Affinity purification of mRNA-protein complexes from *Saccharomyces cerevisiae*

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ABBREVIATIONS

aaRS	aminoacyl-tRNA synthetase
aa-tRNA	aminoacyl-tRNA
A-site	ribosomal acceptor site
BB	boiled beads
CBC	nuclear cap-binding complex
CEN	centromeric DNA
CHX	cycloheximide
CP	coat protein
CTD	carboxy-terminal domain
CYC1	iso-1-cytochrome c
d	distilled
dd	double distilled
dNTP	deoxyribonucleosid triphosphate
dsRBD	double-stranded RNA binding domain
eEF	eukaryotic elongation factor
eIF	eukaryotic initiation factor
EJC	exon-junction complex
ER	endoplasmatic reticulum
eRF	eukaryotic release factor
Exo9	catalytically inactive 9-subunite exosome core
F	phenylalanine
FDR	false discovery rate
FT	flow through
FWB	final wash buffer
G	glycine
gDNA	genomic DNA
H	heavy
hnRNP	heterogenous nuclear ribonucleoprotein
I	input
IgG	immunoglobulin G
IMDH	inosine monophosphate dehydrogenase
K_d	dissociation constant
KH	heterogenous nuclear RNP K homology
L	light
LC-MS/MS	liquid chromatography tandem mass spectrometry
LN_2	liquid nitrogen
Lys0	light $_{12}C_{14}N$ isotope-containing lysine
Lys8	heavy $_{13}C_{15}N$ isotope-containing lysine
Met-$tRNA_i^{Met}$	initiator methionyl tRNA
mRNA	messenger RNA
mRNP	messenger ribonucleoprotein particle
MS	mass spectrometry
MS2L	bacteriophage MS2 stem-loop
n/a	no answer

NAC	nascent polypeptide-associated complex
NMD	nonsense-mediated mRNA decay
NME	N-terminal methionine excision
NPC	nuclear pore complex
nt	nucleotide
Nup	nucleoporin
OD	optical density
ORF	open reading frame
PABP	poly(A)-binding protein
PAP	peroxidase anti-peroxidase soluble complex
P-body	processing body
PBS	phosphate-buffered saline
PCI	phenol-chloroform-isoamyl alcohol
PEG	polyethylene glycol
P_i	inorganic phosphate
PIC	pre-initiation complex
Pol II	RNA polymerase II
poly(A)	poly-adenosyl
PrAx2	four IgG-binding Z domains of *Staphylococcus aureus* protein A
P-site	ribosomal peptidyl transferase site
PTC	premature termination codon
qRT-PCR	quantitative real-time reverse transcription PCR
RAC	ribosome-associated complex
RBD	RNA-binding domain
RBP	RNA-binding protein
RNase	ribonuclease
RNP	ribonucleoprotein particle
RNPB-150	RNP Buffer 150
rpm	revolutions per minute
RRM	RNA-recognition motif
RS domain	domain enriched in arginine and serine residues
RT	room temperature
RT-PCR	reverse transcription PCR
SC	synthetic complete
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SILAC	stable isotope labelling by amino acids in cell culture
SLIC	sequence and ligation-independent cloning
snoRNP	small nucleolar ribonucleoprotein particle
snRNP	small nuclear ribonucleoprotein particle
SR	serine/arginine
ssDNA	single stranded DNA
TC	eIF2-GTP- Met-tRNA$_i^{Met}$ ternary complex
TEV	tobacco etch virus
TREX	transcription and export
TSS	transcriptional start site
U snRNA	uridine-rich small nuclear RNA
uORF	upstream open reading frame
UTR	untranslated region
wt	wild-type
x *g*	relative centrifugal force (rcf)
YEP	yeast extract-peptone
YEPD	yeast extract-peptone-dextrose

SUMMARY

Messenger RNAs (mRNAs) are associated with a dynamic set of RNA-binding proteins as well as additional proteins whose interaction with the mRNA is bridged by protein-protein interactions. These interactions regulate every step of mRNA life cycle from transcription and processing in the nucleus to translation and decay in the cytoplasm. The last decade has seen the development of many new approaches to study mRNA-protein interactions that have greatly extended our knowledge of the mRNA-associated aspects of gene expression regulation. Affinity purification of RNA-binding proteins (RBPs) combined with the identification of co-purifying RNAs by DNA microarray analysis has shown that mRNAs that encode for proteins with related function or sub-cellular location are co-ordinately regulated by specific sets of RBPs (Gerber, Herschlag, and Brown 2004; Hogan et al. 2008a; Gerber et al. 2006; Hieronymus and Silver 2003). These findings suggest that RBPs play a central role in the post-transcriptional regulation of mRNA expression (Hogan et al. 2008a). Proteome-wide screens to identify RNA-protein interactions using high-density protein microarrays suggest the existence of novel RBPs among unexpected classes of proteins such as enzymes (Tsvetanova et al. 2010; Scherrer et al. 2010). Global analysis of mRNA-bound proteome by *in vivo* UV cross-linking combined with polyadenylated RNA purification and quantitative proteomic analysis of the captured proteins has enabled to compile a comprehensive list of RBPs in mammalian cells (Baltz et al. 2012; Castello et al. 2012). Methods also exist for the creation of a transcriptome-wide high-resolution map of RBP-binding sites (reviewed in Ascano et al. 2012). Due to methodological limitations, however, our knowledge of the protein composition of mRNPs assembled on distinct cellular mRNAs is very limited. We have therefore developed a single-step mRNP affinity purification method that is based on the capture of the mRNA component of *in vivo*-assembled mRNPs from the budding yeast *Saccharomyces cerevisiae*. In order to capture the mRNA of interest we make use of the high affinity interaction between the bacteriophage MS2 coat protein and its RNA binding site; integrated after the stop codon, MS2 stem-loops serve as an RNA affinity tag for mRNP capture (Haim et al. 2007). The protein composition of affinity purified mRNPs is analysed by quantitative proteomics. Collectively, our approach should provide an overall picture of the various interactions an mRNP is involved in during its life cycle and also reveal the abundance of specific interactions.

The analysis of the mRNA-bound proteome of two mRNAs encoding for glycolytic enzymes, *PGK1* and *ENO2*, indicates that large mRNP subpopulations are engaged in mRNA translation and 5'→3' exoribonucleolytic decay. The latter process seems to be accelerated for the analysed mRNAs due to the integration of the RNA tag. The analysis of an mRNA-like transcript composed of the RNA tag surrounded by 5' and 3' UTR sequences derived from endogenous genes revealed an mRNP protein composition largely similar to *PGK1* and *ENO2*. This result suggests that the main determinant for recognizing a transcript as an mRNA is not the open reading frame but the 5' and 3' untranslated regions. The three analysed mRNA-bound proteomes contained besides proteins with a well established role in mRNA life cycle also multiple unexpected proteins. Our results point to a possible role for ribosome biogenesis factors, tRNA-modifying enzymes and some metabolic enzymes in mRNA biology and suggest co-translational supramolecular glycolytic enzyme complex formation.

The established mRNP affinity purification method provides a starting point for further analysis of the protein composition of specific *in vivo*-assembled mRNPs in *S. cerevisiae*. Combining the method with UV cross-linking would enable to determine the proteins that directly interact with the mRNA of interest plus the binding sites of these proteins on the mRNA. In order to elucidate the molecular mechanisms that regulate the post-transcriptional fate of mRNA, future studies should aim at identifying the mRNA-associated proteome on a genome-wide scale as well as provide insight into the temporal dynamics of mRNA-protein interactions.

INTRODUCTION

mRNP – the functional form of mRNA

The physiological and developmental stages of a cell are the outcome of spatially and temporally coordinated gene expression. In eukaryotes, gene expression regulation occurs at multiple levels from transcription to post-translational protein modifications. In the centre of many of these control steps is mRNA. In order to be successfully translated, an mRNA has to undergo proper 5' capping, splicing, 3' end processing and export. These steps do not only involve pre-mRNA modifications but also result in the loading of various factors on the transcript. An mRNA together with the associated proteins and non-coding RNAs comprises the messenger ribonucleoprotein particle (mRNP). The composition of an mRNP is highly dynamic. The changing repertoire of mRNA-interacting factors has a profound influence on the fate and function of the mRNA because these factors coordinate and couple the post-transcriptional gene expression events.

The life cycle of an mRNP starts with transcription. As soon as the nascent transcript emerges from RNA polymerase II it is bound by RNA-binding proteins. Some RBPs participate in gene transcription by promoting elongation and preventing RNA-DNA hybrid formation. Other RBPs take part in pre-mRNA processing into 5' capped, spliced and polyadenylated mature transcripts. The interplay between pre-mRNA processing, co-transcriptional mRNA export factor recruitment and nuclear mRNP quality control result in the formation of mature, export competent mRNPs that are subsequently transported through the nuclear pores to the cytoplasm. mRNP export is accompanied by mRNP remodelling which results in the exchange of many mRNP proteins. After export, mRNPs can be directly engaged in translation or they can be localized to distinct cellular regions. mRNP localization is often mediated by specific mRNA-binding adaptor proteins that link mRNPs to motor proteins. Transcript-specific translational regulation, which, among other processes, is also necessary to prevent protein synthesis during mRNA localization, is exerted by RBPs. Finally, mRNAs are degraded by ribonucleases in general cytoplasmic mRNA turnover pathways or in more specialized pathways relying on specific *cis*-acting sequence elements and sequence-specific *trans*-acting factors.

Describing the life cycle of an mRNP, however, as a single linear pathway does not accurately reflect the reality. In fact, mRNPs are a part of a complex post-transcriptional gene

expression regulation system. The importance of post-transcriptional gene expression regulation in mRNP life cycle is emphasized by the highly variable correlation between mRNA and protein expression levels (Ghaemmaghami et al. 2003; Greenbaum et al. 2003). Hence, it is not possible to accurately predict protein abundance based on mRNA expression levels and *vice versa*. An emerging paradigm is the existence of extensive regulatory networks, where mRNAs encoding for proteins that are functionally related or localized in same sub-cellular compartments, are regulated in a coordinated way by distinct RBPs (reviewed in Keene and Tenenbaum 2002; Keene 2007). This concept is supported by evidence provided by several studies (Scherrer et al. 2010; Gerber, Herschlag, and Brown 2004; Hieronymus and Silver 2003; Tsvetanova et al. 2010; Hogan et al. 2008a) and is perhaps best illustrates by the discovery that each of the five members of the Puf family of RBPs associates with a distinct set of functionally related mRNAs in the budding yeast *Saccharomyces cerevisae* (Gerber, Herschlag, and Brown 2004).

The following overview aims at giving an understanding of the life cycle of an mRNP in the context of a complex post-transcriptional regulatory network. In order to provide relevant background information for the experimental part of the thesis, which focuses on the protein composition analysis of affinity purified mRNPs from *S. cerevisiae*, mRNA-protein interactions involved in different steps of the mRNA life cycle will be discussed. Much of the current knowledge about mRNP biogenesis, export and cytoplasmic destiny is based on studies performed in *S. cerevisiae*. Therefore, this overview will focus mostly on this model organism but also refer to relevant findings in higher eukaryotes. In addition, experimental methods to study mRNA-protein complexes will be reviewed.

RNA binding proteins at a glance

RBPs recognize their targets via RNA-binding domains (RBDs). Taken the wide range of functions carried out by RBPs one might assume that an equally large number of protein structures are involved in RNA recognition. However, this does not appear to be the case. A large scale bioinformatics analysis has classified around 40 types of motifs as "non-catalytic" RBDs (Anantharaman, Koonin, and Aravind 2002). Some RBDs, such as the RNA-recognition motif (RRM), are found in hundreds of proteins within a species, whereas other RBDs can be present only in a single proteins (e.g. S6 and L30 ribosomal protein domains) or in proteins with a specific functions (e.g. cap-binding domain) (Anantharaman, Koonin, and Aravind 2002). The better studied RBDs include the above mentioned RRM plus the heterogeneous nuclear RNP K homology (KH) domain, the double-stranded RNA-binding domain (dsRBD), RGG (Arg-Gly-

Gly) box, DEAD/DEAH box, zinc finger (ZnF), Pumilio/FBF (PUF or Pum-HD) domain and the Piwi/Argonaute/Zwiller (PAZ) domain (reviewed in Cléry, Blatter, and Allain 2008; Lunde, Moore, and Varani 2007; Auweter, Oberstrass, and Allain 2006). Rather than containing a single RBD, RBPs usually harbour multiple copies of a certain RBD or several different RBDs. The modular structure of RBPs confers a more specific and higher affinity binding to the cognate substrate and ultimately enables RBPs to recognize a wide variety of targets.

Further functional diversity among RBPs is achieved by combining RBDs with other types of domains which can impart catalytic or protein-binding activities. For instance, both protein kinase R (PKR) and adenosine deminase 2 (ADAR2) contain two dsRBDs in the N-terminus, but differ in their catalytic domains and thus in the cellular functions. PKR harbours a kinase domain (Dar, Dever, and Sicheri 2005; Lemaire et al. 2008; Meurs et al. 1990), whereas ADAR2 contains a deaminase domain catalyzing the conversion of adenosins to inosins (Bass 2002; Macbeth et al. 2005). PKR activation by double-stranded viral RNA leads to translational shut-down, thereby inhibiting viral particle production. ADAR2, on the other hand, can modulate biological processes involving sequence- and structure-specific interactions with the RNA by changing the primary sequence of the RNA (reviewed in Bass 2002).

For some RBPs the ability to form protein-protein interactions can be functionally as important as the ability to bind RNA. A good example here is the serine/arginine (SR)-rich protein family, whose members are involved in various aspects of mRNA metabolism including the regulation of constitutive and alternative splicing (reviewed in Twyffels, Gueydan, and Kruys 2011). SR proteins interact with RNA via one or two conserved RRMs, whereas protein-protein interactions are mediated by a domain enriched in arginine and serine residues (RS domain) (Zuo and Maniatis 1996; Kohtz et al. 1994; Amrein, Hedley, and Maniatis 1994). SR proteins can greatly enhance splicing activity by recruiting spliceosome components to the regulated splice sites and this function is dependent on the protein-binding RS domain (Graveley, Hertel, and Maniatis 1998; Kohtz et al. 1994; Zuo and Maniatis 1996). In other RBPs protein-protein interactions can be mediated also by atypical RRMs or KH domains and dsRBDs (Ramos et al. 2002; Irion et al. 2006; Toba and White 2008). For instance, in some heterogenous nuclear ribonucleoproteins (hnRNPs), which together with SR proteins play an important role in splicing regulation (reviewed in Han, Tang, and Smith 2010), KH domains and RRMs are essential for protein-binding (J. H. Kim et al. 2000).

The versatility of RNA-protein interaction modes among RBPs is further emphasized by a group of RBPs whose mRNA recognition specificity is not dependent on RBDs but on guide RNAs. Identified guide RNA classes that are involved in post-transcriptional gene silencing include microRNAs (reviewed in Bartel 2004), endogenous small interfering RNAs (Tam et al.

2008; Kawamura et al. 2008) and Piwi-interacting RNAs (Aravin et al. 2006; Lau et al. 2006). The first two types of small non-coding RNAs are found in complex with Argonaut proteins and the later with Piwi proteins. Guide RNA function is also provided by small nuclear RNAs during splicing (reviewed in Wahl, Will, and Lührmann 2009) and small nucleolar RNAs during RNA-guided nucleotide modifications (reviewed in Decatur and Fournier 2003).

3 to 11% of all bacterial, archaeal and eukaryotic proteins are involved in RNA metabolism (Anantharaman, Koonin, and Aravind 2002). The list of annotated and predicted RBPs in *S. cerevisiae* comprises over 600 proteins, corresponding to more than 10% of the yeast proteome (Hogan et al. 2008a). Astonishingly, most of these proteins lack known RNA-binding domains, suggesting that many RBPs remain to be elucidated (Hogan et al. 2008a). The existence of RBPs among unexpected classes of proteins, like metabolic enzymes, has been known for about two decades (R. Singh and Green 1993; Kennedy et al. 1992; Nagy and Rigby 1995). The early findings include the discovery that aconitase, the key player in citric acid cycle that converts citrate to isocitrate in mitochondria, also acts as the iron-responsive element-binding protein 1 (IRP-BP 1) in cytosol (Butt et al. 1996; Kennedy et al. 1992). In response to low cellular iron levels IRP-BP 1 binds to the iron-responsive element in its target mRNAs, thereby up- or down-regulating their expression (Kato et al. 2007). More recent data suggests that the ability to bind RNA might be a widespread feature among enzymes (Hogan et al. 2008a; Scherrer et al. 2010; Tsvetanova et al. 2010; Hentze and Preiss 2010). These findings point to the possibility that many metabolic enzymes could have dual functions which could allow them to fine-tune gene expression in response to cell's metabolic state (reviewed in Hentze and Preiss 2010). The question, how proteins without known RBDs bind RNA, remains, in many cases, elusive. One possible explanation, which is exemplified by aconitase, is that evolution has selected for RNA secondary structures capable of protein-binding to establish these RNA-protein interactions.

mRNP life cycle is guided by RNA binding proteins

Transcription elongation and the concomitant pre-mRNA processing events

The guiding role of RBPs in mRNP life cycle is manifested by tight coupling of different steps in mRNP biogenesis. The first important player along this path of interconnected events is RNA polymerase II (Pol II), the enzyme transcribing eukaryotic protein-coding genes. The special feature of Pol II that enables sequential recruitment of mRNA processing factors is the carboxy-terminal domain (CTD) of the largest subunit (reviewed in Meinhart et al. 2005). The CTD

consists of repeats of an amino acid motif with a consensus sequence Tyr-Ser-Pro-Thr-Ser-Pro-Ser. Different stages of the transcription cycle are characterized by specific patterns of CTD post-translational modifications, of which serine phosphorylation has a major impact on transcription and mRNA processing factor recruitment (M. Kim et al. 2009; reviewed in Egloff and Murphy 2008). For instance, the first mRNA processing event – 5' end capping – is dependent on Ser5 phosphorylation that directly recruits the capping machinery to Pol II soon after transcription initiation (Schroeder et al. 2000; Rodriguez et al. 2000; Yue et al. 1997). Capping not only affects mRNA by ensuring mRNA stability (Hsu and Stevens 1993; Walther et al. 1998) and efficient translation (Tarun and Sachs 1996a; Wakiyama, Imataka, and Sonenberg 2000), but also transcription. Several lines of evidence suggest that capping enzymes play a critical role in the transition from abortive early transcription to full elongation (Guiguen et al. 2007; Mandal et al. 2004).

The monomethylated cap structure is co-transcriptionally bound by nuclear cap-binding complex (CBC) (Wong et al. 2007) that likely comprises the first proteins to assemble on a pre-mRNA. The evolutionarily conserved CBC in *S. cerevisia* is composed of Cbc2 (CBP20 in higher eukaryotes), the 20 kDa cap-binding subunit (Colot, Stutz, and Rosbash 1996; Visa et al. 1996), and Cbc1. Cbc1 homolog in higher eukaryotes, CBP80, is the regulatory subunit that enables high affinity binding of CBP20 to cap structure (Mazza et al. 2001; Izaurralde et al. 1994) (Table 1). Studies in yeast have shown that CBC is important for various steps in mRNP biogenesis, further emphasizing the importance of functional coupling in this process. CBC is required for co-transcriptional spliceosome assembly (Görnemann et al. 2005) and can stimulate transcription pre-initiation complex formation on active genes (Lahudkar et al. 2011). In addition, CBC is necessary for proper transcription termination because the complex suppresses the recognition of weak polyadenylation sites (Wong et al. 2007; B Das et al. 2000). CBC has also been shown to participate in rapid mRNA degradation in the nucleus upon mRNA export block (Biswadip Das, Butler, and Sherman 2003; Kuai, Das, and Sherman 2005).

Splicing is another pre-mRNA processing event that is largely co-transcriptional (G. Zhang et al. 1994; J. Singh and Padgett 2009; Lacadie and Rosbash 2005; Görnemann et al. 2005). During splicing intron-containing pre-mRNAs interact with one of the most complex eukaryotic macromolecular machineries – the spliceosome – that catalysis the excision of intronic sequences (reviewed in Will and Lührmann 2011). Spliceosome is assembled from U1, U2, U5 and U4/U6 small nuclear ribonucleoprotein particles (snRNPs) and a multitude of non-snRNP proteins (Fabrizio et al. 2009; Y.-I. G. Chen et al. 2007; Zhou, Licklider, et al. 2002). Each snRNP is composed of one (or two in case or U4/U6) uridine-rich small nuclear RNA (U snRNA), a common set of seven Sm proteins and a varying number of snRNP specific proteins. Proteomic

studies have shown that in total about 90 (Fabrizio et al. 2009) and 170 (reviewed in Jurica and Moore 2003) proteins associate with the yeast and human spliceosome, respectively. Spliceosome composition is highly dynamic. Different stages of splicing are characterized by changes in spliceosome protein composition and extensive remodelling of snRNPs. The large number of spliceosome associated proteins, which in humans comprises two-thirds of the spliceosome mass, indicates the importance of protein-RNA and protein-protein interactions for proper splicing.

Table 1. Selected yeast proteins and their metazoan homologues cited in the thesis. Description refers to the biological function of proteins. Table modified from (Kelly and Corbett 2009) and (Rodríguez-Navarro and Hurt 2011).

S. cerevisiae protein		Metazoan orthologue	Description
Abbreviation	Full name	Abbreviation	
Cbc2/Cbp20	cap-binding protein 20	CBP20	Cap-binding complex with Cbp80
Cbc1/Cbp80	cap-binding protein 80	CBP80	Cap-binding complex with Cbp20
Tho2	THO complex subunit 2	THOC2	THO/TREX component
Hpr1	hyperrecombination protein 1	THOC1	THO/TREX component
Mft1	mitochondrial fusion target protein 1	-	THO/TREX component
Thp2	THO complex subunit THP2	-	THO/TREX component
Sub2	suppressor of BRR1 protein 2	UAP56	RNA helicase, TREX component
Yra1	RNA annealing protein YRA1	Aly/REF	RNA binding protein, TREX component
Mex67	mRNA export factor 67	TAP/NXF1	mRNA export receptor
Mtr2	mRNA transport regulator 2	p15/NXT1	mRNA export receptor
Nab2	nuclear polyadenylated RNA-binding 2	NAB2	RNA binding protein
Npl3	nuclear protein 3	-	RNA binding protein
Pcf11	protein 1 of CF I	PCF11	mRNA 3′ end processing factor
Rna14	mRNA 3′ end processing protein RNA14	CstF77	mRNA 3′ end processing factor
Rna15	mRNA 3′ end processing protein RNA15	CstF64	mRNA 3′ end processing factor
Glc7	serine/threonine-protein phosphatase PP1-2	PP1c	protein phosphatase
Pap1	poly(A) polymerase 1	Pap1	poly(A) tail synthesis
Sac3	nuclear mRNA export protein SAC3	GANP/Xmas-2	TREX-2 component
Thp1	nuclear mRNA export protein THP1	ENST00000246505	TREX-2 component
Sus1	protein SUS1	DC6/ENY2	SAGA and TREX-2 component
Cdc31	cell division control protein 31	CETN3	TREX-2 component
Nup1	nucleoporin NUP1	-	nucleoporin
Nup60	nucleoporin NUP60	Nup153	nucleoporin
Mlp1	myosin-like protein 1	TRP	NPC-associated protein
Mlp2	myosin-like protein 1	TRP	NPC-associated protein
Dbp5/Rat8	DEAD-box protein 5	DDX19	RNA helicase
Gle1	nucleoporin GLE1	hGLE1	nucleoporin
Dis3/Rrp44	chromosome disjunction 3	hDIS3/hDIS3L	exosome component
Rrp6	ribosomal RNA-processing protein 6	hRRP6	exosome component
Trf4/Pap2	topoisomerase 1-related protein 4	hTRF4-1	TRAMP component
Trf5	topoisomerase 1-related protein 5	hTRF4-2	TRAMP component
Air1	Arg methyltransferase-interacting RING-finger 1	ZCCHC7	TRAMP component

Table 1. Continued

S. cerevisiae protein		Metazoan orthologue	Description
Abbreviation	Full name	Abbreviation	
Air2	Arg methyltransferase-interacting RING-finger 2	ZCCHC7	TRAMP component
Mtr4	mRNA-transport regulator 4	hMTR4	TRAMP component
Rat1	ribonucleic acid-trafficking 1	XRN2	5′→3′ exoribonuclease
Rai1	RAT1-interacting protein	DOM3Z	Rat1 co-activator

mRNP export factors are recruited during transcription

Co-transcriptional packaging of Pol II generated transcripts into mRNPs is vital for cells to preserve genome integrity. Naked RNA has a tendency to invade the DNA duplex behind the elongating Pol II and by base pairing with the non-coding DNA strand force the coding strand into single-stranded conformation. Such structures are termed R loops and they are harmful for the cell in several ways (reviewed in Aguilera and García-Muse 2012). R loop formation can impair transcription elongation as this structure is likely to obstruct the next elongating Pol II. R loops can also induce chromosomal DNA rearrangements by blocking replication fork progression (Gómez-González, Felipe-Abrio, and Aguilera 2009; Prado and Aguilera 2005; Gan et al. 2011; Huertas and Aguilera 2003).

A critical role in co-transcriptional mRNA packaging into export competent mRNPs, and thus in preventing R-loop formation (Gómez-González et al. 2011), is played by the evolutionarily conserved transcription and export (TREX) complex (Katja Strässer et al. 2002; Abruzzi, Lacadie, and Rosbash 2004). In *S. cerevisiae* the TREX complex contains the components of the THO complex (Tho2, Hpr1, Mft1, Thp2 and, possibly, Tex1) (Chávez et al. 2000; A. Pena et al. 2012) and two RNA export adapters, Sub2 and Yra1, which are necessary for the recruitment of the mRNA export receptor (Katja Strässer et al. 2002). Chromatin immunoprecipitation experiments have demonstrated that the THO components and the RNA export adapters Sub2 and Yra1 become associated with active chromatin during transcription elongation (A. Pena et al. 2012; Zenklusen et al. 2002). THO recruitment is partly mediated by the C-terminal nucleic acid-binding domain of Tho2 (A. Pena et al. 2012), whereas Sub2 bridges the interaction between THO complex and Yra1 (Zenklusen et al. 2002). A model based on this data suggests that upon association with sites of active transcription THO complex, specifically Hpr1 (Zenklusen et al. 2002), recruits Sub2 to the nascent transcript that further recruits Yra1 (reviewed in Kelly and Corbett 2009). Yra1 can then serve as an adaptor for the general *S. cerevisiae* mRNA export receptor, Mex67/Mtr2 heterodimer (Santos-Rosa et al. 1998; Kadowaki et

al. 1994; Zenklusen et al. 2001; K Strässer and Hurt 2000). As Mex67 and Sub2 share the same binding site on Yra1, Sub2 is likely displaced by Mex67 prior to mRNA export (K Strässer and Hurt 2001). However, evidence is accumulating that mRNA export does not follow a single linear pathway as suggested by the described model. Some of the most notable findings along these lines include the discovery that two shuttling heterogeneous nuclear ribonucleoproteins Npl3 (Wendy Gilbert and Guthrie 2004) and Nab2 (Iglesias et al. 2010) can serve as adaptors for Mex67. The role of Npl3 as an mRNA export adaptor is closely related to 3' end processing and will be therefore discussed in the following chapter.

Nab2 is a polyadenylated RNA-binding protein (J. T. Anderson et al. 1993) with a well-established role in poly(A) tail length control (Viphakone, Voisinet-Hakil, and Minvielle-Sebastia 2008; Hector et al. 2002) and nuclear mRNA export (Fasken, Stewart, and Corbett 2008; Vinciguerra et al. 2005; D. M. Green et al. 2003; D. M. Green et al. 2002; Batisse et al. 2009). The notion that Nab2 can serve as an adaptor for Mex67 is based on the findings that Nab2, Yra1 and Mex67 can form a trimeric complex, and that the lethal phenotype of *⊿yra1* cells can be rescued by Nab2 overexpression (Iglesias et al. 2010). Furthermore, in the same study Yra1 was shown to stimulate the interaction between Mex67 and Nab2, suggesting that Yra1 may not be a *bona fide* mRNA export adaptor but rather a chaperone facilitating Mex67-Nab2 association (Iglesias et al. 2010).

Besides Yra1, Npl3 and Nab2 also the THO complex has been implicated in Mex67 recruitment. Interestingly, RNase treatment affects the association of Sub2 (Abruzzi, Lacadie, and Rosbash 2004; Dieppois, Iglesias, and Stutz 2006) but not Mex67 (Dieppois, Iglesias, and Stutz 2006) with actively transcribed genes and hints that Mex67 recruitment is largely mediated by adaptors associated with the transcription machinery. One such adaptor can be the THO component Hpr1. It has been shown that Mex67 can directly be recruited via its ubiquitin-associated (UBA) domain to Hpr1 and that this recruitment depends on Hpr1 ubiquitination (Gwizdek et al. 2006) (Fig. 1). Hpr1 is ubiquitinated in a transcription-dependent manner leading to Mex67 association with active genes (Gwizdek et al. 2005). Mex67-UBA binding in turn transiently protects Hpr1 from proteasomal degradation (Gwizdek et al. 2006) and can thereby contribute to the coordination of transcription and mRNP assembly.

Not only mRNA export factors are loaded co-transcriptionally to the nascent mRNA, the same holds true for the 3' end processing machinery. A central role in coupling transcription to 3' end formation is played by Pcf11, a conserved subunit of the yeast cleavage factor 1A (CF1A) required for cleavage and polyadenylation steps of 3' end formation (E. J. Steinmetz and Brow 1996; Sadowski et al. 2003) (Fig. 1). Pcf11 can bind both Pol II CTD via phospho-Ser2 (Barillà, Lee, and Proudfoot 2001; Licatalosi et al. 2002; Hollingworth et al. 2006), a phosphorylation

mark coinciding with transcription elongation (Komarnitsky, Cho, and Buratowski 2000; Z. Ni et al. 2004), and RNA (Licatalosi et al. 2002; M. Kim et al. 2004), thereby facilitating 3' end processing machinery co-transcriptional recruitment. However, linking transcription and 3' end formation does not seem to be the only coupling event Pcf11 is participating in. A recent study showed that Yra1 co-transcriptional recruitment was not dependent on Sub2 as anticipated, but on Pcf11 (Johnson, Cubberley, and Bentley 2009). The authors proposed that after the initial Yra1 recruitment by Pcf11, Yra1 is transferred to Sub2. This scenario is supported by the finding that Pcf11 and Sub2 contact with the same region on Yra1, suggesting mutually exclusive binding (Johnson, Cubberley, and Bentley 2009). Yra1 recruitment by 3' end processing machinery could provide an additional mRNP quality control mechanism as export competence, which is acquired through Yra1 loading, depends on proper transcription and 3' end processing.

3' end formation and mRNA export are coupled

All eukaryotic mRNAs, with the exception of replication-dependent histone mRNAs (Dávila López and Samuelsson 2008), carry a homopolymeric tail consisting of adenosyl (poly(A)) residues at their 3' end. These poly(A) tails, which are associated with multiple copies of poly(A)-binding protein (PABP), have a well defined species specific length of 70-80 nt in *S. cerevisiae* and 200-250 nt in mammalian cells (reviewed in Eckmann, Rammelt, and Wahle 2011; Lemay et al. 2010). The two enzymatic activities required in eukaryotes for mRNA 3' end formation are site-specific endonucleolytic cleavage of the pre-mRNA and poly(A) tail addition onto the upstream cleavage product. Despite the seemingly simple biochemistry of the reactions, a megadalton-sized protein machinery is needed both in yeast and mammals for 3' end formation (reviewed in Mandel, Bai, and Tong 2008). The importance of correct 3' end polyadenylation for living organisms is emphasizes by the fact that most 3' end processing factors are encoded by essential genes in *S. cerevisiae* (reviewed in Proudfoot 2011).

The co-transcriptional recruitment of mRNA export factors, jet the nuclear export of only mature mRNPs suggests that cells can efficiently discriminate between mRNPs still on the "assembly line" and export competent mature mRNPs. Evidence has accumulated from studies mainly done on yeast that mRNP export competency is linked to 3' end processing. Similarly to temperature sensitive *mex67-5* cells (Segref et al. 1997), temperature sensitive mutants of the yeast CF1A subunits Rna14, Rna15 and Pcf11 as well as poly(A) polymerase 1 (Pap1) show poly(A) RNA accumulation in the nucleus at restrictive temperature (Hilleren et al. 2001; Hammell et al. 2002; Brodsky and Silver 2000). Conversely, *mex67-5* cells show hyperpolyadenylation of

transcripts at restrictive temperature (T H Jensen, Patricio, et al. 2001; Hammell et al. 2002; Hilleren et al. 2001). This phenotype is also observed in cells defective for 3' end processing (Hammell et al. 2002).

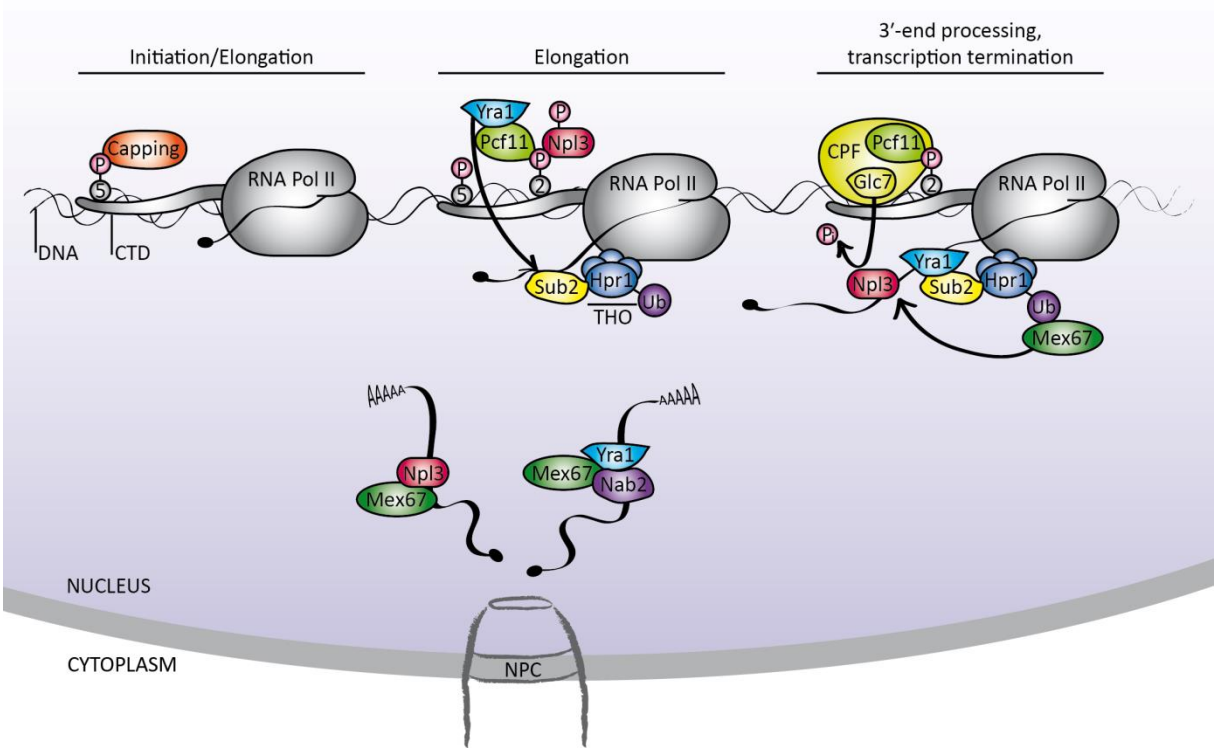


Figure 1. mRNP co-transcriptional assembly. During transcription initiation the C-terminal domain (CTD) of RNA polymerase II (RNA Pol II) becomes phosphorylated at Ser5 in the heptad amino acid repeat composing the CTD. This results in the recruitment of the capping machinery to the CTD and subsequent capping of the nascent transcript, leading to processive transcription elongation. Cap is bound by the cap binding complex that likely represents the first proteins to assemble on the nascent transcript (omitted for simplicity). During transcription elongation CTD is phosphorylated at Ser2, which recruits Pcf11 on the transcription machinery. Pcf11 is a component of cleavage factor 1A (CF1A), and has been shown to be necessary for initial Yra1 association with transcription machinery (Johnson, Cubberley, and Bentley 2009). Yra1 is transferred from Pcf11 to the mRNA export adaptor Sub2, which liberates Pcf11 for interaction with 3' end processing complex. Phospho-Ser2 is necessary also for the recruitment of shuttling mRNA-binding protein Npl3. Among other functions, Npl3 prevents early termination (Bucheli and Buratowski 2005; Bucheli et al. 2007). Npl3 phosphorylation during transcription gradually leads to the loss of its anti-termination activity, leading to cleavage and polyadenylation factor (CPF) association at the 3' end (Dermody et al. 2008). During 3' end formation, CPF component Glc7 dephosphorylates Npl3 which promotes Npl3 interaction with the mRNA export receptor Mex67 and subsequent mRNP export (Wendy Gilbert and Guthrie 2004). The THO complex plays a crucial role in co-transcriptional mRNA export factor recruitment and mature mRNP release form transcription site, as indicated by the impairment of these processes in *tho* mutant yeast strains. The first step in Mex67 recruitment to mRNA is likely mediated by THO component Hpr1. Ubiquitination of Hpr1 during transcription elongation directly recruits Mex67 to the active genes (Gwizdek et al. 2006). Mex67 is loaded together with its adaptors onto the mRNP during 3' end formation. Three proteins – Yra1, Npl3 and Nab2 – have been shown to function as Mex67 adaptors. However, at the moment it is not clear if Mex67 would be loaded onto mRNA in a complex with all three adaptors or with only a subset of them (Kim Guisbert et al. 2005; D. M. Green et al. 2002; Hieronymus and Silver 2003). CTD – C-terminal domain, NPC – nuclear pore complex, RNA Pol II – RNA polymerase II, P – phosphorylation, P_i – dephosphorylation, Ub – ubiquitin. Figure modiefied after (Tutucci and Stutz 2011).

The SR-like protein Npl3 is an abundant shuttling RBP that plays a role in a wide range of processes including mRNA transcription elongation, termination/3' end processing (Wong et al. 2007; Dermody et al. 2008; Bucheli and Buratowski 2005; Bucheli et al. 2007), splicing (Kress, Krogan, and Guthrie 2008), export (Windgassen and Krebber 2003; Singleton et al. 1995; M. S. Lee, Henry, and Silver 1996) and translation (Windgassen et al. 2004). Npl3 directly interacts with Pol II CTD via phosphorylated Ser2 and promotes both transcription (Dermody et al. 2008) and co-transcriptional splicing factor recruitment (Kress, Krogan, and Guthrie 2008) (Fig. 1). During transcription elongation Npl3 also prevents early termination (Bucheli et al. 2007; Bucheli and Buratowski 2005) but this activity is gradually lost due to Npl3 phosphorylation, which leads to the recruitment of cleavage and polyadenylation factor (CPF) at the 3' end (Dermody et al. 2008). Npl3 dephosphorylation by CPF component Glc7 elegantly links 3' end processing to mRNA export receptor Mex67 loading (Wendy Gilbert and Guthrie 2004). Namely, Npl3 dephosphorylation by Glc7 enables Npl3 to act as an mRNA export adapter protein by promoting its direct binding to Mex67 and mature mRNP nuclear export. In the cytoplasm Npl3 is rephosphorylated, which leads to its release form the mRNP and nuclear re-import (W Gilbert, Siebel, and Guthrie 2001).

In addition to Npl3, Sub2 and the THO complex also have a clear role in 3' end processing and mRNP release form the transcription site. In *tho/sub2* mutants the 3' end of the *HSP104* locus is trapped in a dense chromatin fraction that besides the gene's 3' region also contains RNA, Pol II, pre-mRNA 3' end processing machinery and nuclear pore complex (NPC) components (Rougemaille et al. 2008). In the same study 3' regions of nearly 400 yeast genes were found to be associated with dense chromatin in *tho* mutants. These results suggest that THO and Sub2 are required to dissociate the 3' end processing machinery and to release the export competent mRNP form the transcription site. Remarkably, transcription site release seems to take place in close proximity to NPC that could further facilitate mRNP export (Rougemaille et al. 2008).

mRNP export form nucleus

Mature mRNPs are exported through the nuclear pore complexes to the cytoplasm. The yeast NPC contains about 30 different proteins termed nucleoporins (Nups). The central NPC transport channel is filled and surrounded with Nups containing domains rich in phenylalanine (F) and glycine (G) (FG-Nups), which create a physical barrier for macromolecules larger than about 40 kDa (reviewed in Terry and Wente 2009). This barrier is overcome by cargo export

receptors by binding to FG-repeats. At the nuclear side of the NPC eight filaments, connected by a ring at the end, reach into the nucleoplasm and form a structure termed the nuclear basket (Kiseleva et al. 2004; Ris and Malecki 1993). The filaments reaching into the cytoplasm are not connected to each other and are therefore highly mobile (Kiseleva et al. 2004).

mRNP export starts at the nuclear basket where export competent mRNPs can concentrate using multiple mechanisms (Fig. 2). In yeast, TREX-2 complex, which is composed of Sac3, Thp1, Sus1 and Cdc31(Rodríguez-Navarro et al. 2004; T. Fischer et al. 2002), has been shown to mediate the repositioning of actively transcribed *GAL* genes from the nuclear interior to the NPC (Rodríguez-Navarro et al. 2004; Cabal et al. 2006). Subsequent production of mRNA in close vicinity to NPC is likely to increase mRNP export efficiency. TREX-2 mediates the repositioning of active genes to the nuclear pores through binding to both the nuclear basket and to the SAGA transcriptional co-activator complex (Brownell et al. 1996; Grant et al. 1997). TREX-2 component Sac3 tethers TREX-2 to the nuclear basked through interactions with nucleoporins Nup1 and Nup60 (T. Fischer et al. 2002). Sus1, which is a functional component of both the SAGA and TREX-2 complexes, binds to Sac3, thereby anchoring active genes to nuclear pores (Cabal et al. 2006; Rodríguez-Navarro et al. 2004; Jani et al. 2009). However, TREX-2 does not seem to comprise the only link between sites of active transcription and nuclear pores. Nuclear basket-associated proteins Mlp1 and Mlp2, which, similarly to Sac3, bind this structure via Nup60 (Feuerbach et al. 2002), have been shown to physically associate with SAGA components on transcriptionally active *GAL* genes (Luthra et al. 2007). In addition to physically linking sites of active transcription to nuclear pore, Mlp1 can also facilitate the docking of export competent mRNPs at the nuclear basket. Namely, the protein has been shown to interact with Nab2 (Fasken, Stewart, and Corbett 2008). The existence of a complex web of protein-protein interactions at the nuclear basket is further emphasized by the finding that Mex67 can interact with Sac3 both *in vivo* and *in vitro* (T. Fischer et al. 2002).

The phenomenon of actively transcribed gene docking at NPC is thus far well documented only for *S. cerevisiae*. However, the evolutionary conservation of SAGA (Brand et al. 1999; Martinez et al. 2001), TREX-2 (Jani et al. 2012) and the NPC including the Mlp proteins (Mendjan et al. 2006; Strambio-de-Castillia, Blobel, and Rout 1999) suggests that the mechanism could exist also in higher eukaryotes. Indeed, SAGA has been shown to function in anchoring of a subset of active transcription sites to the nuclear pores in *Drosophila melanogaster* (Kurshakova et al. 2007).

In vivo imaging of single endogenous mRNPs in mammalian cells has revealed that mRNA export contains three basic steps: docking, transport and release (Grünwald and Singer 2010). Surprisingly, transport through the NPC central channel is a very rapid process and is completed

in less than 20 ms. Most of the about 200 ms needed for export, the mRNP spends equally between the docking and the release phase (Schmitt et al. 1999). mRNA export directionality is likely achieved by mRNP reorganization at the cytoplasmic face of the NPC. One protein implicated in this processed is the conserved RNA helicase Dbp5 (also known as Rat8) (Schmitt et al. 1999; Snay-Hodge et al. 1998; Tseng et al. 1998) (Fig. 2). Dbp5 is a shuttling protein (Estruch and Cole 2003; Zhao et al. 2002; Hodge et al. 1999) that at steady state locates at the cytoplasmic filaments of the NPC (Weirich et al. 2004; Hodge et al. 1999; Schmitt et al. 1999). The low intrinsic RNA-dependent ATPase activity of Dbp5 (Tseng et al. 1998) is greatly stimulated at the cytoplasmic filaments upon interaction with Gle1. Inositol hexakisphosphate binding to Gle1 has been shown to enhance Gle1-mediated stimulation of Dbp5 ATPase activity (Weirich et al. 2006; Alcázar-Román et al. 2006). Even though there is no clear consensus about how Dbp5 activity results in mRNP remodelling (reviewed in Linder 2008), the protein has been shown to facilitate the removal of export factors such as Mex67 (Lund and Guthrie 2005) and Nab2 (Tran et al. 2007).

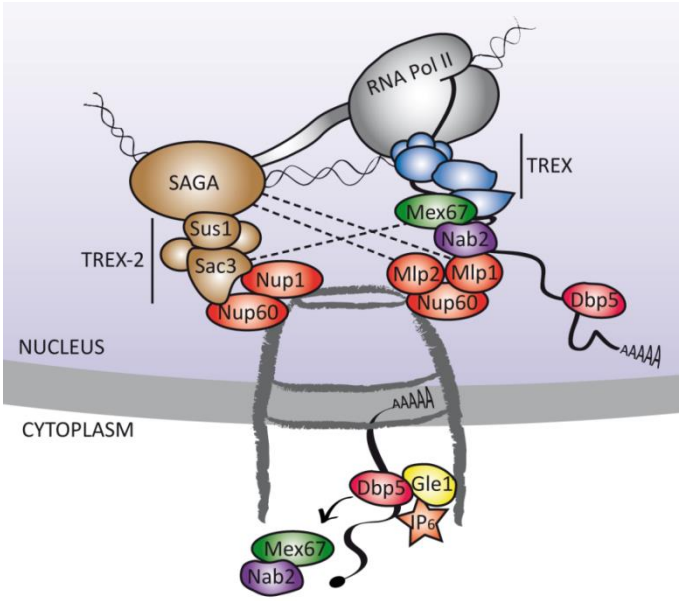


Figure 2. Anchoring of active genes to nuclear pore complex (NPC) and mRNP remodelling upon export. Active genes can be docked at the NPC by a four subunit protein complex termed TREX-2 or by Mlp proteins. TREX-2 bridges the interaction between nucleoporins (Nups) of the nuclear basket and the SAGA transcriptional co-activator complex (T. Fischer et al. 2002; Jani et al. 2009). Another link between SAGA and NPC is provided by NPC-associated proteins Mlp1 and Mlp2 (Luthra et al. 2007) (represented by dotted lines). Export competent mRNP docking at the NPC can be mediated by Nab2, which has been shown to interact with Mlp1 (Fasken, Stewart, and Corbett 2008), and by Sac3, which can bind Mex67 (T. Fischer et al. 2002) (represented by dotted line). mRNA export leads to mRNP remodelling. An important player in this process, the RNA helicase Dbp5, is loaded onto the mRNP in the nucleus. Dbp5 ATPase activity is greatly stimulated at the cytoplasmic face of the NPC by Gle1 bound to inositol hexakisphosphate (IP6) (Weirich et al. 2006; Alcázar-Román et al. 2006). Dbp5 activation results in mRNP remodelling, leading to the dissociation of mRNA export factors such as Mex67 and Nab2 (Lund and Guthrie 2005; Tran et al. 2007). Figure modified after (Iglesias et al. 2010) and (Köhler and Hurt 2007).

Nuclear mRNP quality control

Formation of a mature, export-competent mRNP is a multistep process where each mRNP maturation reaction is inherently error-prone. Functional coupling of the different mRNP biogenesis steps helps cells to monitor the overall accuracy of the process. The crosstalk between proteins involved in mRNP maturation and quality control leads to the destruction, nuclear arrest or transcriptional downregulation of aberrant transcripts.

mRNA degradation is the best studied nuclear mRNP quality control mechanism (Fig. 3). The first ribonucleolytic activity identified in this cellular compartment belongs to the multi-subunit exosome complex (P. Mitchell et al. 1997; Bousquet-Antonelli, Presutti, and Tollervey 2000). The eukaryotic nuclear exosome is composed of 9 core subunits forming a barrel-like structure, and two enzymatically active subunits. 3'→5' exonuclease activity of the exosome in *S. cerevisiae* is provided by Dis3 (also known as Rrp44) (Allmang et al. 1999; Dziembowski et al. 2007) and Rrp6 (Allmang et al. 1999; Liu, Greimann, and Lima 2006). Dis3 also displays endonucleolytic activity (Schaeffer et al. 2009; Schneider et al. 2009; Lebreton et al. 2008). mRNA degradation by exosome is stimulated by the TRAMP (Trf4/5-Air1/2-Mtr4) polyadenylation complex, which marks aberrant transcripts for degradation by adding a short poly(A) tail that facilitates exosome recruitment (LaCava et al. 2005; Wyers et al. 2005). Functional exosome is required for the rapid degradation of unspliced pre-mRNAs (Bousquet-Antonelli, Presutti, and Tollervey 2000), mRNAs with defective poly(A) tails (Milligan et al. 2005; Burkard and Butler 2000; Libri et al. 2002) and nucleus-restricted mRNAs upon mRNA export block (Biswadip Das, Butler, and Sherman 2003). In addition, catalytically active exosome is also involved in transcript retention at the site of synthesis observed in mRNA export deficient yeast strains (Assenholt et al. 2008). Interestingly, this phenotype is not dependent on the exosome co-factor TRAMP, indicating a functional difference between the two complexes (Rougemaille et al. 2007).

The other main ribonucleolytic activity in the cell nucleus is provided by 5'→3' exonuclease Rat1. Together with its co-factor Rai1, Rat1 is implicated in transcription termination of RNA Pol II (West, Gromak, and Proudfoot 2004; M. Kim et al. 2004) as well as RNA Pol I (Kawauchi et al. 2008; El Hage et al. 2008). According to the "torpedo" model of transcription termination, Rat1 attacks the 5' end formed after pre-mRNA cleavage by the 3' end processing machinery and degrades the RNA produced by Pol II downstream of the polyadenylation site, causing Pol II to terminate (Connelly and Manley 1988; Luo, Johnson, and Bentley 2006). In nuclear RNA quality control Rat1 is needed for the 5'→3' exonucleolytic degradation of unsuccessfully capped mRNAs (Jiao et al. 2010; Jimeno-González et al. 2010). The substrate for Rat1-mediated mRNA decay, 5'-monophosphorylated RNA, is generated by Rat1 co-factor Rai1

by removing the unmethylated cap or by hydrolyzing the 5' end triphosphate of an uncapped RNA (Xiang et al. 2009; Jiao et al. 2010).

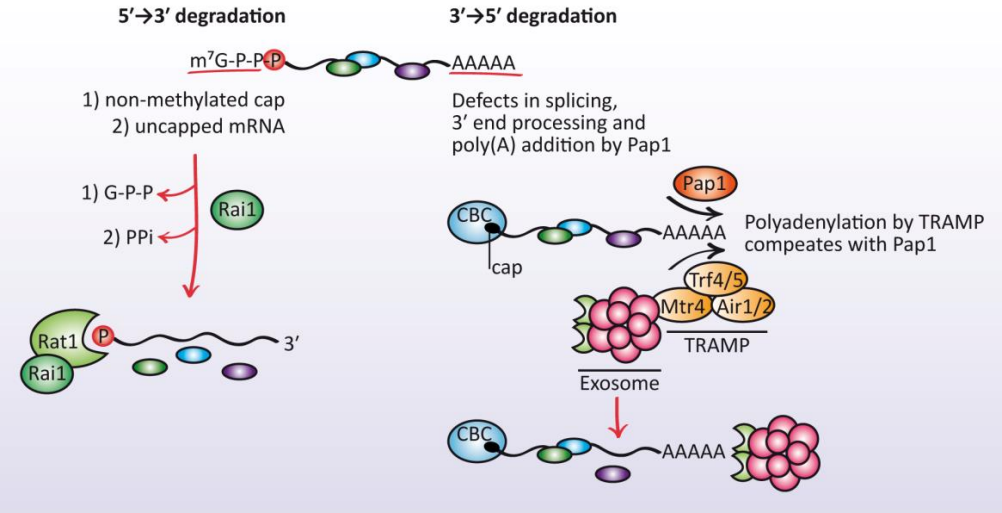


Figure 3. mRNA degradation during nuclear mRNA quality control. Defects in pre-mRNA processing to 5′ capped, spliced and polyadenylated mature transcripts or in mRNA export result in nuclear retention, transcriptional downregulation or degradation of these transcripts. Nuclear mRNA degradation involves 5′→3′ and 3′→5′ exonucleolytic, as well as endonucleolytic cleavage. The 5′→3′ degradation pathway is responsible for the removal of mRNAs with aberrant 5′ ends. Rai1 removes the non-methylated cap structure or hydrolyzes the 5′ triphosphate of an uncapped RNA to leave 5′-monophosphorylated RNA, which can then act as a substrate for Rat1 5′→3′ exoribonuclease. The 3′→5′ degradation is involved in the surveillance of proper mRNA 3′ end formation. Inefficient plyadenylatation by poly(A) polymerase 1 (Pap1) due to defective splicing or 3′ end processing results in the addition of a short poly(A) tail by the non-canonical poly(A) polymerase of the TRAMP complex. TRAMP then recruits the nuclear exosome, leading to rapid mRNA degradation form the 3′ end. The exosome contains nine conserved core subunits plus two 3′→5′ exonucleases, Dis3 and Rrp6. Dis3 also harbours endonucleolytic activity. CBC – cap binding complex. Figure modified after (Tutucci and Stutz 2011).

Nuclear mRNA quality control does not reduce the level of aberrant transcripts only by mRNA degradation. Non-optimal mRNP formation can have a direct negative effect on transcriptional rates. Evidence for that has come both from the analysis of defective splicing and mRNA export. In the mammalian system promoter-proximal 5' splice site mutation has been shown to strongly reduce the steady state levels of the mRNA in an mRNA decay-independent fashion (Damgaard et al. 2008; Furger et al. 2002). As U1 snRNA can interact both with the 5' splice site (Massimo Caputi et al. 2004; Kammler et al. 2001) and TFIIH (Kwek et al. 2002), it has been suggested to stimulate transcription initiation by enhancing pre-initiation complex assembly (Damgaard et al. 2008). Transcriptional downregulation upon promoter proximal 5' splice site mutation could therefore be an outcome of this disrupted communication. Similarly to the mammalian system, the removal of a promoter proximal intron in yeast reduces transcription levels of the gene (Furger et al. 2002). How does a cells profit form transcriptional downregulation of aberrant mRNAs? The answer could lie in the observation that artificially

reduced transcription rates in *tho/sub2* mutants can suppress several associated phenotypes (T H Jensen, Boulay, et al. 2001; Torben Heick Jensen et al. 2004). This finding suggests that under challenging conditions mRNP assembly efficiency can be increased by reducing the production level of aberrant mRNAs.

mRNP cytoplasmic destiny

Translation is accompanied by mRNP remodelling

In rapidly growing *S. cerevisiea* cells most newly exported mRNPs will be immediately engaged in translation in the cytoplasm (Arava et al. 2003). Translation is accompanied by major changes in mRNP composition, which involves the dissociation of several nuclear-acquired proteins as well as the recruitment of the elongation-competent 80S ribosome. Translation-accompanied changes in mRNP composition not only enable bulk protein synthesis but also ensure the quality of gene expression through translation-dependent mRNA surveillance pathways (reviewed in Maquat, Tarn, and Isken 2010; Isken and Maquat 2007). Nuclear-acquired proteins that travel with the mRNA to the cytoplasm include the nuclear cap-binding complex, PABP and, in case of mammalian pre-mRNAs subjected to splicing, the exon-junction complex (EJC) deposited ~20-24 nucleotides (nt) upstream of exon-exon junctions. The newly exported mRNPs seem to acquire a set of proteins characteristic for mRNAs involved in steady-state translation predominantly by the end of the first or the so-called "pioneer" round of translation (Gehring et al. 2009; Sato and Maquat 2009; Hosoda, Lejeune, and Maquat 2006; Ishigaki et al. 2001; S.-Y. Chiu et al. 2004). In mammalian cells, where translation-dependent mRNP remodelling has been extensively studied, these changes include the replacement of CBP80-CBP20 heterodimer with eukaryotic translation initiation factor 4E at the 5' cap, the exchange of nuclear poly(A) binding protein PABPN1 by cytoplasmic PABPC1 and the removal of EJCs (Sato and Maquat 2009; Gehring et al. 2009; Dostie and Dreyfuss 2002; Lejeune et al. 2002).

mRNP interactions in cap-dependent translation

Besides the 79 proteins that are loaded onto the mRNA as part of the yeast 80S ribosome (reviewed in D. N. Wilson and Cate 2012), mRNA translation involves numerous accessory factors that participate in translation initiation, elongation, termination and ribosome recycling and interact with the mRNA either directly or indirectly through protein-protein interactions with

other components of the translation machinery (reviewed in R. J. Jackson, Hellen, and Pestova 2010; Hinnebusch 2011; Dever and Green 2012). The largest number of accessory factors, at least 10, participate in translation initiation (reviewed in R. J. Jackson, Hellen, and Pestova 2010). Eukaryotic initiation factors (eIFs) help to separate the ribosomal subunits after translation termination, prepare the mRNA and the small (40S) ribosomal subunit for binding with each other and participate in locating the start codon and in subsequent large (60S) subunit joining with 40S, after which translation elongation can proceed (reviewed in R. J. Jackson, Hellen, and Pestova 2010; Hinnebusch 2011).

The "end product" of translation initiation step is an elongation-competent 80S ribosome, which is defined by base-pairing between the mRNA's start codon and the anticodon loop of the initiator methionyl tRNA (Met-tRNA$_i^{Met}$) occupying the ribosomal peptidyl (P) site. The molecular events enabling elongation-competent 80S formation start with post-termination ribosome separation into free 40S and 60S subunits. After translation termination, the 80S ribosome remains bound to at least three factors: mRNA, P-site deacylated tRNA, and eukaryotic release factor (eRF) 1 and, possibly, eRF3 (Pisarev, Hellen, and Pestova 2007) (Fig. 4). At a low (1 mM) free Mg^{2+} concentration, which enables greater flexibility of the ribosomal subunits (Shenvi et al. 2005), eIF3, eIF1 and eIF1A are sufficient to mediate ribosome recycling into free 40S and 60S subunits (Pisarev, Hellen, and Pestova 2007; Pisarev et al. 2010). *In vivo*, however, efficient ribosome recycling likely needs an additional factor, ABCE1, which is an essential (Z.-Q. Chen et al. 2006; Dong et al. 2004) and highly conserved protein of the ATP-binding cassette (ABC) transporter superfamily (reviewed in Dean and Annilo 2005). Importantly, ABCE1 can mediate the separation of post-termination ribosomes into free 60S and mRNA- and tRNA-bound 40S subunits in a wide range of Mg^{2+} concentrations (Pisarev et al. 2010). The subsequent release of mRNA and deacylated tRNA from the 40S subunit is promoted by eIF3, eIF1 and eIF1A (Pisarev, Hellen, and Pestova 2007), which are recruited to the 40S subunit during ribosome recycling (reviewed in R. J. Jackson, Hellen, and Pestova 2010). All three eIFs remain associated with the released 40S subunit and participate in the following steps of translation initiation.

The binding of eIF1 and eIF1A triggers a conformational change in 40S subunit that opens the mRNA binding channel – a change that is proposed to convert the "closed", scanning-incompetent 40S structure into an "open", scanning-competent 43S pre-initiation complex (PIC) (Passmore et al. 2007). 43S pre-initiation complex contains besides the 40S subunit and the eIFs 3, 1 and 1A also the eIF2-GTP- Met-tRNA$_i^{Met}$ ternary complex (TC), which delivers the initiator tRNA to the ribosomal P-site (Shin et al. 2011), and eIF5 (reviewed in R. J. Jackson, Hellen, and Pestova 2010; Hinnebusch 2011). Biochemical data indicate that eIF1- and eIF1A-induced conformational change enhances the rate of eIF2-GTP- Met-tRNA$_i^{Met}$ ternary complex binding to

40S, producing a 43S PIC capable of directly docking the mRNA into the mRNA binding channel during translation initiation (Passmore et al. 2007). It should be noted that in addition to the step-by-step association of eIFs with the 40S subunit, there is evidence for an alternative pathway for 43S PIC formation where eIFs 1, 3, 5, and the TC associate into a large multifactor complex (MFC) prior to binding to 40S subunit (K Asano et al. 2000; Sokabe, Fraser, and Hershey 2012; Dennis, Person, and Browning 2009).

The 43S PIC is loaded onto the mRNA at the 5' cap-proximal region and subsequently scans downstream the 5' untranslated region (UTR) to locate the initiation codon (reviewed in R. J. Jackson, Hellen, and Pestova 2010; Hinnebusch 2011). 43S PIC is able to attach to an scan along an unstructured 5' UTR (Tatyana V Pestova and Kolupaeva 2002; S. F. Mitchell et al. 2010). However, attachment to an even weakly structured 5' UTR depends on eIF4F and eIF4B or eIF4H that cooperate to unwind the 5' cap-proximal region for 43S PIC loading and assist 43S PIC in scanning (Tatyana V Pestova and Kolupaeva 2002; S. F. Mitchell et al. 2010; Rogers et al. 2001; Marintchev et al. 2009). eIF4F is composed of three proteins: (1) eIF4E, the cap-binding protein; (2) eIF4A, a DEAD-box RNA helicase; and (3) eIF4G, a large modular protein acting as a scaffold for the assembly of highly stable eIF4F at the mRNA's 5' end (reviewed in Prévôt, Darlix, and Ohlmann 2003; Hinnebusch 2011). eIF4G directly interacts with both eIF4E and eIF4A, thereby directing the RNA helicase to the cap-proximal region (J. D. Gross et al. 2003; Volpon et al. 2006; S. F. Mitchell et al. 2010; P. Schütz et al. 2008). Furthermore, the ATPase activity of eIF4A is stimulated upon binding to eIF4G (P. Schütz et al. 2008). The affinity of eIF4E for the cap structure is also enhances by eIF4G-eIF4E interaction (J. D. Gross et al. 2003).

Besides interacting with mRNA's 5' end, eIF4G also contacts the 3' poly(A) tail via PABP, thereby physically linking the mRNA termini (E.-H. Park et al. 2011; Svitkin et al. 2009; Tarun et al. 1997; Craig et al. 1998; Le et al. 1997; AMRANI et al. 2008). The formation of a "closed-loop" structure is not absolutely required for translation *in vivo* as indicated by genetic analysis in *S. cerevisiae* (E.-H. Park et al. 2011; Tarun et al. 1997). However, eIF4G-PABP interaction is thought to promote 43S PIC attachment, and thus translation initiation, because it contributes to the stability of mRNA binding by eIF4F (reviewed in Hinnebusch 2011). In addition to participating in a network of interactions that stabilize eIF4F binding, eIF4G can promote translation initiation by directly recruiting 43S PIC to the mRNA. Namely, in mammalian cells eIF4G interacts with the 43S PIC component eIF3 (LeFebvre et al. 2006; Morino et al. 2000; Lamphear et al. 1995; Korneeva et al. 2000). In yeast, the interaction between the aforementioned proteins is bridged by eIF5 or eIF1, which simultaneously interact with both eIF4G and eIF3 (H. He et al. 2003; Katsura Asano et al. 2001; S. F. Mitchell et al. 2010).

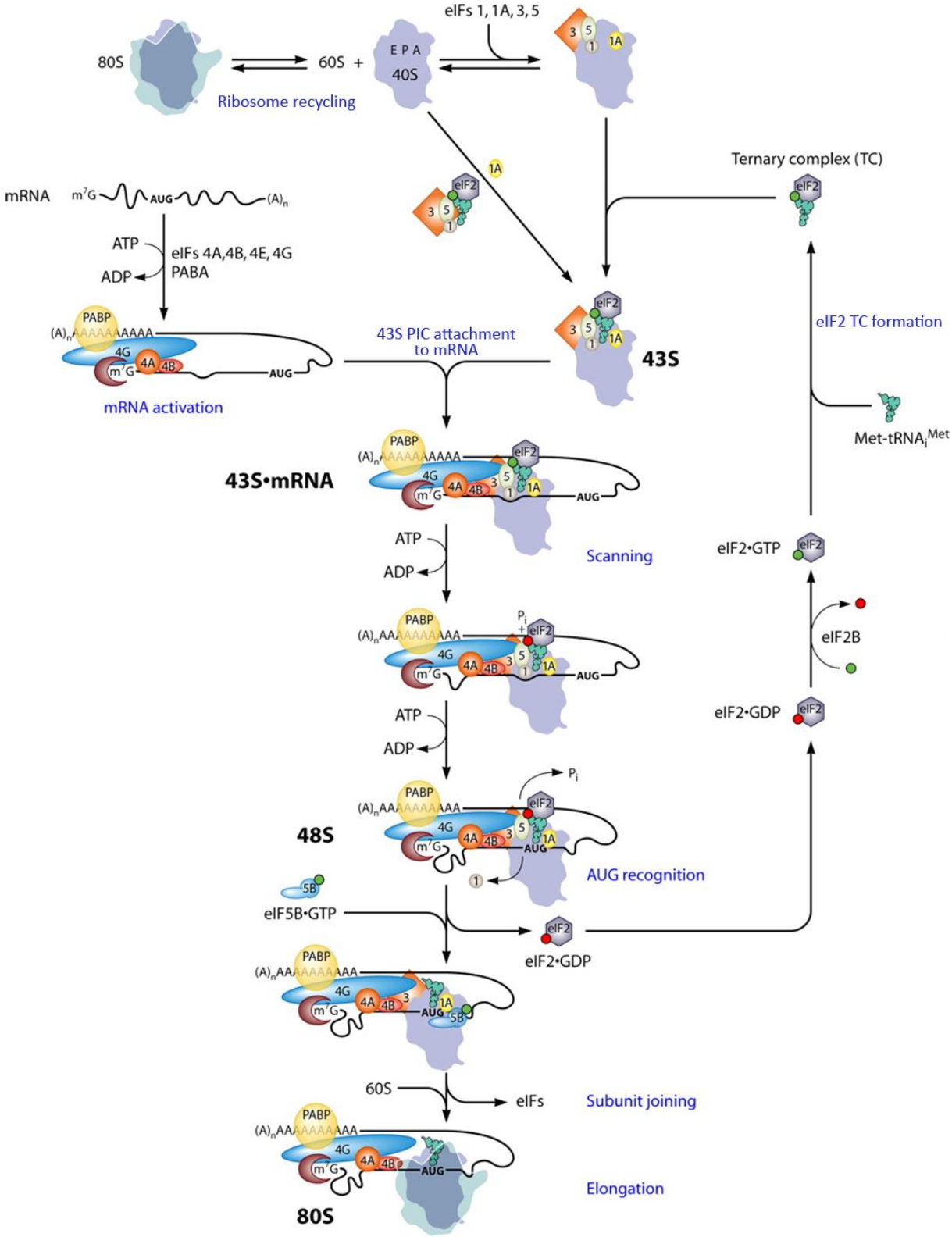


Figure 4. Model of eukaryotic translation initiation by ribosomal scanning. Translation initiation is a multitep process (single steps in blue type) that starts with the separation of post-termination ribosomal complexes into free 40S and 60S ribosomal subunits (shapes depicting ribosomal subunits correspond to crystal structures of bacterial 70S and 30S ribosomal species). Association of a subset of eukaryotic translation initiation factors (eIFs, dipicted as numbered shapes) and the eIF2-GTP- Met-tRNA$_i^{Met}$ ternary complex (TC) with 40S subunit results in the formation of 43S pre-initiation complex (PIC). eIFs and TC may be recruited to 40S subunit in a sequential manner or as a pre-formed multifactor complex. Association of eIF4F (eIF4E/eIF4G/eIF4A) with m^{7}G cap and poly(A)-binding protein (PABP) with poly(A) tail activates mRNA for translation initiation – the DEAD-

box RNA helicase eIF4A with eIF4B unwinds the mRNA to generate a single-stranded region at mRNA's 5' end, which is bound by 43S PIC. 43S PIC scans the 5' UTR until initiation codon recognition and 48S PIC formation, which commits the ribosome to initiate at the selected start codon and is paralleled by P_i release from GDP•P_i-bound TC and the dissociation of eIF1. Subsequent 60S subunit joining and the release of eIFs is catalysed by the GTPase eIF5B. eIF2-GDP is recycled to eIF2-GTP by eIF2B, the guanine nucleotide exchange factor for eIF2. For more details, see text. A – aminoacyl-tRNA binding site, P – peptidyl-tRNA binding site, E – deacylated-tRNA binding site (exit), GTP – green ball, GDP – red ball, P_i – inorganic phosphate. Figure modified from (Hinnebusch 2011).

Once loaded at the 5' cap-proximal region, 43S PIC scans the mRNA in search of an initiation codon, which is commonly the first AUG triplet (reviewed in Hinnebusch 2011). During scanning the "open" conformation of 43S PIC is stabilized by eIF1 and eIF1A, thereby enabling 43S PIC to thread along the mRNA (Passmore et al. 2007). Perfect base-pairing between the initiation codon and the anticodon of Met-$tRNA_i^{Met}$ leads to a conformational change in 40S subunit, which likely closes the mRNA binding channel to prevents further scanning, thereby forming 48S PIC (Passmore et al. 2007; Maag et al. 2005; Unbehaun et al. 2004). Subsequent dissociation of eIF1 from the pre-initiation complex allows the release of inorganic phosphate (P_i) from eIF2-GDP•P_i, which drives GTP hydrolysis by eIF2 to completion and commits the ribosome to initiate at the selected start codon (Unbehaun et al. 2004; Maag et al. 2005; Algire, Maag, and Lorsch 2005). eIF2-bound GTP hydrolysis is stimulated by eIF5, however, during scanning the reaction seems to be reversible because the presence of eIF1 on 43S PIC precludes the release of P_i (Algire et al. 2002; Algire, Maag, and Lorsch 2005). eIF1 thus ensures the fidelity of translation initiation by allowing irreversible eIF2-bound GTP hydrolysis only upon the establishment of codon-anticodon base-pairing (Algire, Maag, and Lorsch 2005).

60S subunit joining is accompanied by eIF release from the small ribosomal subunit and is catalyzed by the ribosome-dependent GTPase eIF5B (T V Pestova et al. 2000; Fringer et al. 2007). Efficient subunit joining depends on the interaction between eIF5B and the C-terminus of eIF1A. This interaction accelerates the rate of subunit joining and, after 60S recruitment, enhances the GTP hydrolysis activity of eIF5B (Acker et al. 2006; Acker et al. 2009; Fringer et al. 2007). GTP hydrolysis is required for the rapid release of eIF5B and eIF1A, thereby producing and elongation-competent 80S ribosome (Acker et al. 2009; J. H. Lee et al. 2002; Shin et al. 2002). eIF2-GDP dissociates from a small fraction of 48S complexes upon P_i release and its release is further promoted by eIF5B binding to 48S PIC (Pisarev et al. 2006). However, complete dissociation of eIF2-GDP from the small ribosomal subunits is paralleled by 60S subunit joining (Pisarev et al. 2006).

In contrast to translation initiation, which is paralleled by the recruitment of a large number of initiation factors, translation elongation in most eukaryotes requires the association of only two factors with the ribosome. The GTP-bound eukaryotic elongation factor (eEF) 1A

delivers the aminoacyl-tRNA (aa-tRNA) to the ribosomal acceptor (A) site (reviewed in Rodnina and Wintermeyer 2001). Correct codon-anticodon base-pairing triggers GTP hydrolysis by eEF1A followed by the dissociation of eEF1A-GDP from the ribosome, which enables the aa-tRNA to fully accommodate in the A-site. Subsequent peptide bond formation leaves a deacylated tRNA in the P-site and the newly formed peptidyl-tRNA in the A-site. Before the next round of translation elongation can proceed the ribosome needs to move by one codon on the mRNA, thereby placing the deacylated tRNA and the peptidyl-tRNA in ribosomal E- and P-sites, respectively, and the next codon in the A-site. Translocation is catalyzed by eEF2, which hydrolyzes GTP to enable ribosome movement (Taylor et al. 2007; VanLoock et al. 2000). A third elongation factor, eEF3, exists exclusively in fungi, where it mediates the release of deacylated tRNA from the E-site and the binding of eEF1A-GTP-aa-tRNA ternary complex to the A-site (Triana-Alonso, Chakraburtty, and Nierhaus 1995; Andersen et al. 2006).

Translation is terminated after the entry of one of the three stop codons into the ribosomal A-site (reviewed in Kapp and Lorsch 2004). In eukaryots, this process is governed by two release factors, eRF1 and eRF3. eRF1 recognizes the three stop codons and catalyzes the hydrolysis of peptidyl-tRNA, whereas eRF3 strongly stimulates peptide release by eRF1 through GTP hydrolysis. According to the current model eRF1 and eRF3 are recruited to the ribosomal A-site in a ternary complex with GTP (Alkalaeva et al. 2006). Importantly, the interaction between eRF1 and eRF3 increases the affinity of eRF3 for GTP (Hauryliuk et al. 2006; Pisareva et al. 2006). eRF3's GTPase activity is triggered upon binding to the ribosome and is thought to result in a conformational change in eRF1 that activates peptidyl-tRNA hydrolysis by eRF1 (Frolova et al. 1996; Alkalaeva et al. 2006). Interestingly, in *S. cerevisiae* mRNA export factors Dbp5 and Gle1 together with inositol hexakisphosphate have been implicated in translation termination (Bolger et al. 2008; T. Gross et al. 2007). Both Dpb5 and Gle1 physically and genetically interact with release factors and have been proposed to participate in mRNP remodelling prior to termination (Bolger et al. 2008; T. Gross et al. 2007). However, the exact molecular mechanism how these factors promote efficient translation termination remains to be determined.

Cytoplasmic mRNA decay

Cytoplasmic mRNA degradation machinery serves two major functions: (1) it maintains normal mRNA decay rates and thereby regulates the abundance of functional proteins; (2) it performs mRNA quality control by eliminating aberrant mRNAs that otherwise could give rise to toxic

proteins (reviewed in Houseley and Tollervey 2009). The five hydrolytic activities participating in cytoplasmic mRNA decay mediate decapping at the 5' end, 5'→3' exonucleolytic decay, deadenylation of the 3' poly(A) tail, 3'→5' exonucleolytic decay and endonucleolytic cleavage within the transcript (reviewed in Schoenberg 2011; C.-Y. A. Chen and Shyu 2011; Ling, Qamra, and Song 2011). The accessibility of an mRNA to these activities is determined by the structure of the mRNP, i.e. the complement of mRNA associated RBPs and small non-coding RNAs. These mRNA-associated factors can regulate mRNA decay rates directly by promoting or hindering the recruitment of mRNA decay machinery, and indirectly by influencing the translational status and/or subcellular localization of the mRNA. Depending on the substrate and cellular conditions, mRNA decay can be initiated by deadenylation, decapping or endonucleolytic cleavage followed by exonucleolytic digestion (reviewed in Garneau, Wilusz, and Wilusz 2007).

In order to give relevant background information for the experimental part of the thesis, this chapter will focus on deadenylation dependent mRNA decay and on mRNA quality control mechanism termed nonsense-mediated mRNA decay.

Deadenylation dependent mRNA decay

Deadenylation

The initial and often rate-limiting step in most eukaryotic cytoplasmic mRNA degradation pathways is deadenylation (Muhlrad, Decker, and Parker 1994; M Tucker et al. 2001). The bulk poly(A)-specific 3' exoribonuclease activity is provided by the evolutionarily conserved Pan2-Pan3 and Ccr4-Not complexes in *S. cerevisiae*. In Pan2-Pan3 heterodimer both subunits are required for enzymatic activity even though the catalytic site is harboured by Pan2 (Boeck et al. 1996; C E Brown et al. 1996). The deadenylase activity in the multisubunit Ccr4-Not complex resides in Ccr4 (Morgan Tucker et al. 2002; Goldstrohm et al. 2007) and, at least in metazoans, in Caf1 (also known as Pop2) (Moser et al. 1997; Temme et al. 2010; Cooke, Prigge, and Wickens 2010; Viswanathan et al. 2004; Wagner, Clement, and Lykke-Andersen 2007). Studies in yeast have shown that Pan2-Pan3 complex is recruited to mRNA via the interaction between Pan3 and the major poly(A)-binding protein Pab1 (David A Mangus, Smith, et al. 2004), which leads to the activation of Pan2 exonuclease activity (Alan B. Sachs and Deardorff 1992; Lowell, Rudner, and Sachs 1992; David A Mangus, Evans, et al. 2004). Pan2-Pan3 is suggested to trim the initially synthesized ~90 nt long poly(A) tails to mRNA-specific lengths of ~55-70 nt (Christine E. Brown and Sachs 1998). The loss of Pan2-Pan3 activity has a modest effect on mRNA deadenylation and decay as only a slight increase in the average poly(A) tail length of total steady-state mRNA was observed in *pan2* and *pan3* deletion strains (C E Brown et al. 1996). In contrast,

combining *pan2* deletion with *ccr4* deletion led to the loss of detectable mRNA deadenylation activity (M Tucker et al. 2001), indicating that Ccr4-Not complex is the predominant cytoplasmic poly(A) nuclease (Morgan Tucker et al. 2002; M Tucker et al. 2001; J. Chen, Chiang, and Denis 2002). Unlike Pan2-Pan3, Ccr4-Not complex does not directly bind to Pab1 (Yao et al. 2007) and is instead selectively recruited to mRNA through the interaction with other RBPs (reviewed in Doidge et al. 2012).

Decapping

Deadenylated mRNAs can undergo further decay along two different routes. The unprotected 3' end can be attacked by the cytoplasmic exosome, which degrades the mRNA body in 3'→5' direction (reviewed in S. Lykke-Andersen et al. 2011). Alternatively, the mRNA can be decapped and degraded by the 5'→3' exoribonuclease Xrn1 (reviewed in Jones, Zabolotskaya, and Newbury 2012). In *S. cerevisiae*, the bulk mRNA seems to be degraded in the latter pathway (C. J. Decker and Parker 1993; Hsu and Stevens 1993). This is suggested by the findings that inactivation of decapping or 5'→3' exonucleolytic decay leads to the accumulation of deadenylated full-length transcripts (Muhlrad, Decker, and Parker 1994; Beelman et al. 1996; Muhlrad, Decker, and Parker 1995; Dunckley and Parker 1999). Such decay intermediates can be degraded, albeit slowly, in 3'→5' direction, indicating that in yeast the two decay pathways are, at least to some extent, redundant (Muhlrad, Decker, and Parker 1994; Muhlrad, Decker, and Parker 1995).

In wild-type (wt) yeast cells the poly(A) tail is shortened to an oligo(A) length of ~12 nt before the mRNA can enter the decapping pathway (C. J. Decker and Parker 1993). The packing density of Pab1 on poly(A) tract is approximately one molecule per 25 A residues (Baer and Kornberg 1980; A. B. Sachs, Davis, and Kornberg 1987). The shortening of poly(A) tail below this length is likely to disrupt the communication between the 5' cap and the 3' poly(A) tail due to the loss of Pab1 (Tarun and Sachs 1996b; Tarun et al. 1997; Wells et al. 1998) and consequently lead to a decrease in translation initiation efficiency (Munroe and Jacobson 1990; Gallie 1991). Inefficient translation initiation enables the decapping machinery to gain access to the mRNA (Beelman and Parker 1994; D C Schwartz and Parker 2000; LaGrandeur and Parker 1999) as indicated by the findings that a stable secondary structure in the 5' UTR (Muhlrad, Decker, and Parker 1995), a poor AUG context (LaGrandeur and Parker 1999) or mutations in the translation initiation factors increase *in vivo* mRNA decapping rates (David C. Schwartz and Parker 1999).

The catalytic core of the evolutionarily conserved decapping complex is Dcp2 (Steiger et al. 2003; Deshmukh et al. 2008; van Dijk et al. 2002; Z. Wang et al. 2002), which in *S. cerevisiae* forms a holoenzyme with Dcp1 (Beelman et al. 1996; Dunckley and Parker 1999; Steiger et al.

2003). Kinetic studies have revealed that Dcp1 enhances the catalytic step (Deshmukh et al. 2008; Floor et al. 2010). Unexpectedly, the overexpressed human decapping enzymes DCP1A and DCP2 do not form a detectable complex *in vivo* (Fenger-Grøn et al. 2005) and the bacterially produced DCP1A is not capable to stimulate the activity of DCP2 *in vitro* (Jens Lykke-Andersen 2002; van Dijk et al. 2002). This discrepancy from the results obtained in yeast was explained by the finding that the metazoan-specific protein Hedls (also known as Ge-1 and Edc4) promotes complex formation between Dcp2 and Dcp1 in human and in *A. thaliana* and that this interaction enhances the catalytic activity of Dcp2 (Fenger-Grøn et al. 2005; J. Xu et al. 2006; Chang et al. 2014).

Regulation of mRNA decapping. Decapping is an irreversible process that leads to the rapid degradation of the mRNA body and therefore this activity needs to be tightly regulated in cells. The mRNA specific decapping rates seem to be determined by two properties of individual mRNAs. Firstly, mRNA structural features that reduce translation initiation efficiency also increase deadenylation and decapping rates. As mentioned above, such features include a poor AUG context or a stable secondary structure in the 5' UTR (Muhlrad, Decker, and Parker 1995; LaGrandeur and Parker 1999). Secondly, some mRNAs contain binding sites for regulatory proteins that can either stimulate or inhibit decapping (Olivas and Parker 2000; Mauchi, Ohtake, and Irie 2010).

Considering the inverse correlation between translation initiation and decapping efficiency, translation initiation factors can be viewed as general decapping inhibitors. Indeed, the major cytoplasmic cap-binding protein eIF4E inhibits decapping *in vitro* due to its ability to bind the cap structure (D C Schwartz and Parker 2000; Ramirez et al. 2002) and mutations in the subunits of eIF4F or eIF3 complexes increase the rate of decapping (David C. Schwartz and Parker 1999; D C Schwartz and Parker 2000). Likewise, the poly(A) tail has a negative effect on decapping, which is partly mediated through Pab1 (C. J. Decker and Parker 1993; Caponigro and Parker 1995). General decapping activators, on the other hand, enhance decapping of both stable and unstable mRNAs. Such proteins can act by interfering with translation, by promoting the catalytic activity of Dcp2, or by directly binding to the mRNA and providing a scaffold for the assembly of the decapping machinery. In *S. cerevisiae* the core set of decappin activators includes Pat1, Dhh1, Scd6, Edc3 and Lsm1-7, which are all conserved proteins (Sundaresan Tharun et al. 2000; Bonnerot, Boeck, and Lapeyre 2000; Bouveret et al. 2000; J. M. Coller et al. 2001; N. Fischer and Weis 2002; Decourty et al. 2008).

Several decapping activators like the DEAD-box RNA helicase Dhh1, Pat1 and Scd6 promote decapping indirectly through mRNA translational repression. Overexpression of these

proteins inhibits growth of yeast cells. Further analysis of cells overexpressing either Dhh1 or Pat1 revealed reduced rates of translation (J. Coller and Parker 2005; Nissan et al. 2010). Consistent with a role in translational repression, all three proteins repress translation *in vitro* by inhibiting the formation of a stable 48S PIC (J. Coller and Parker 2005; Nissan et al. 2010). In case of Scd6, further *in vitro* analysis showed that the protein inhibits translation by directly binding to eIF4G, which likely blocks the recruitment of 43S PIC to the mRNA (Rajyaguru, She, and Parker 2012). Interestingly, *in vivo* experiments indicate that Dhh1 is able to repress translation also after the initiation step. Namely, Dhh1 was found to repress translation at a step subsequent to initiation by slowing ribosome movement (Sweet, Kovalak, and Coller 2012). A reduction in ribosome transit rate is a potent activator of mRNA turnover as suggested by the finding that rare codons, which restrict ribosome elongation, in the open reading frame (ORF) of a reporter mRNA stimulated mRNA decapping in a Dhh1-dependent manner (Sweet, Kovalak, and Coller 2012).

Pat1 is an exceptional protein among decapping activators because it can stimulate mRNA decapping both through indirect and direct mechanisms. Besides repressing translation, Pat1 act as a scaffold for the assembly of the decapping machinery and enhances the catalytic activity of Dcp2 (S Tharun and Parker 2001; Sundaresan Tharun et al. 2000; Bonnerot, Boeck, and Lapeyre 2000; Pilkington and Parker 2008; Nissan et al. 2010). Recombinant Pat1 directly interacts with Dhh1, Scd6, Lsm1-7 complex, Xrn1, Dcp1 and Dcp2 (Nissan et al. 2010) and has strong two-hybrid interactions with Edc3 (Pilkington and Parker 2008). The multitude of interactions with the decapping machinery suggests that Pat1 is a key protein in promoting decapping. Indeed, except for *dcp1* and *dcp2* deletion strains, which lack mRNA decapping activity, deletion of *pat1* results in the strongest defect in decapping as compared to any other known mutation (S Tharun and Parker 1999; Dunckley and Parker 1999; Sundaresan Tharun et al. 2000; Bouveret et al. 2000; J. M. Coller et al. 2001; Kshirsagar and Parker 2004; Decourty et al. 2008).

Similarly to Pat1, Edc1, Edc2 and Edc3 directly bind Dcp1-Dcp2 decapping complex and stimulate its activity (D. Schwartz, Decker, and Parker 2003; Carolyn J Decker, Teixeira, and Parker 2007; Tritschler et al. 2007; Harigaya et al. 2010; Nissan et al. 2010; Borja et al. 2011). Unlike Pat1, however, the loss of these proteins does not cause defects in mRNA decay (Dunckley, Tucker, and Parker 2001; Kshirsagar and Parker 2004). Instead, Edc1, Edc2 and Edc3 become necessary for mRNA decapping when the function of Dcp1 or Dcp2 is partially compromised due to mutations (Dunckley, Tucker, and Parker 2001; Kshirsagar and Parker 2004). Besides interacting with the decapping enzymes, Edc3 shows two-hybrid interactions with Pat1, Dhh1 and Lsm1-7 complex and has therefore been proposed to function as a scaffold for

the decapping mRNP assembly (Pilkington and Parker 2008; Fromont-Racine et al. 2000; Carolyn J Decker, Teixeira, and Parker 2007).

Co-immunoprecipitation experiments combined with RNase treatment, which enables to determine the RNA-dependence of the interactions, have helped to shed light on the dynamics of decapping mRNP assembly. Pat1, for instance, associates in an RNase-sensitive manner with eIF4E, eIF4G and Pab1, suggesting that Pat1 can bind to the mRNA when it still has a functional poly(A) tail (S Tharun and Parker 2001). Lsm1-7 complex, on the other hand, has been found to preferentially interact with deadenylaed mRNAs (S Tharun and Parker 2001). Consistent with the loss of the poly(A) tail, eIF4E, eIF4G and Pab1 do not co-immunoprecipitate with Lsm proteins (S Tharun and Parker 2001). Instead, Lsm1-7 complex associates in an RNA-dependent manner with Dcp1-Dcp2 (Sundaresan Tharun et al. 2000). These results imply that Pat1 and Lsm1-7 bind to mRNAs destined for decay at different times. However, once both Pat1 and Lsm1-7 are present on the mRNA they seem to form a stable complex as suggested by the finding that Pat1 co-immunopreciptiates with Lsm proteins in an RNase-insensitive manner (Sundaresan Tharun et al. 2000; S Tharun and Parker 2001). According to the current model the interaction of Pat1 with Lsm1-7 complex results in the formation of a binding site for Dcp2 in the C-terminal domain of Pat1 and subsequent activation of decapping (reviewed in Parker 2012).

The binding efficiency of Lsm1-7 has been proposed to be the key factor in determining if an mRNA will be degraded in 5'→3' or in 3'→5' pathway (reviewed in Sundaresan Tharun 2009). Remarkably, Lsm1-7 shows a strong binding preference for oligoadenylated mRNAs over polyadenylated mRNAs (S Tharun and Parker 2001; Chowdhury, Mukhopadhyay, and Tharun 2007). Mutations in *LSM1* gene that abolish the preferential binding of the Lsm1-7 complex to oligoadenylated mRNA impair mRNA decay (Sundaresan Tharun et al. 2005). These observations suggest that the Lsm1-7 complex can act as a sensor of the poly(A) tail length and that this ability is essential for efficient mRNA decay *in vivo*. Consistent with Lsm1-7 binding preference for oligo(A) tail, unadenylated mRNAs that are generated *in vivo* by ribozyme cleavage are not good substrates for Lsm1-7 mediated 5'→3' decay (Chowdhury and Tharun 2008). Instead, such mRNAs are efficiently degraded by the exosome in the 3'→5' decay pathway (Chowdhury and Tharun 2008; Meaux and Van Hoof 2006). It is therefore conceivable, that mRNAs that escape binding by the Lsm1-7 complex at an oligo(A) tail stage are fully deadenylated and subsequently degraded by the exosome (Sundaresan Tharun 2009).

The relationship between mRNA decapping and P-bodies. mRNA decay intermediates together with proteins involved in decapping and, to a lesser extent, deadenylation can be found

in cytoplasmic aggregates known as processing bodies (P-bodies) (Sheth and Parker 2003; Teixeira and Parker 2007). Therefore, P-bodies have been proposed to be the actual sites of mRNA decapping (reviewed in Parker and Sheth 2007; Franks and Lykke-Andersen 2008). The extent of P-body assembly correlates with the cellular concentration of non-translating mRNPs (reviewed in Franks and Lykke-Andersen 2008). For instance, P-bodies increase in number and size when translation initiation is inhibited by cellular stress or mutations in translation initiation factors (Teixeira et al. 2005). Conversely, trapping the mRNA in polyribosomes by blocking translation elongation leads to the disappearance of visible P-bodies (Teixeira et al. 2005; Sheth and Parker 2003). The assembly of decapping mRNPs into macroscopically visible P-bodies, however, is not necessary for efficient mRNA decay, as the deletion of proteins or protein domains involved in individual mRNP aggregation into P-bodies does not substantially reduce mRNA turnover rates (Carolyn J Decker, Teixeira, and Parker 2007; Reijns et al. 2008). The functional significance of P-body formation remains therefore elusive, although the evolutionary conservation of the mechanism argues for an adaptive advantage. Blocking mRNA decapping or 5'→3' degradation results in an increase in P-bodies, suggesting that aggregation of individual mRNPs into P-bodies might facilitate mRNA decay under conditions where mRNA decay factors are limited (Sheth and Parker 2003; Andrei et al. 2005; Cougot, Babajko, and Séraphin 2004; Teixeira and Parker 2007). Sequestration of decapping mRNPs into P-bodies might also help to avoid aberrant mRNA decapping by physically separating non-translating mRNPs from translating mRNPs (reviwed in Franks and Lykke-Andersen 2008).

3'→5' mRNA decay

As mentioned above, deadenylated mRNAs can also be degraded in 3'→5' direction by the cytoplasmic exosome. The catalytically inactive 9-subunite exosome core (Exo9), which is identical between the nuclear and cytoplasmic versions of the exosome (Hernandez et al. 2006), is associated with one enzymatically active protein in the cytoplasm – Dis3 (Dziembowski et al. 2007; Allmang et al. 1999). Biochemical and structural studies suggest that RNA substrates reach the exoribonucleolytic site of Dis3 after having been threaded through the central channel of Exo9 (Bonneau et al. 2009; Malet et al. 2010). The length of the RNA binding path in Exo9 central channel is 31-33 nt (Bonneau et al. 2009). Consequently, only RNAs with a 3' single stranded region longer than 31-33 nt are efficient degraded by the cytoplasmic exosome (Lorentzen et al. 2008; Liu, Greimann, and Lima 2006; Bonneau et al. 2009). Dis3 also contains an endoribonucleolytic site, which, unlike the exoribonucleolytic site, is accessible from solvent

and does not depend on substrate threading through the Exo9 central channel (Bonneau et al. 2009).

The cytoplasmic exosome functions together with Ski proteins as indicated by the findings that in yeast strains where 5'→3' mRNA decay is blocked in *cis*, the loss of any of the four Ski proteins leads to the stabilization of mRNA 3' end fragments (J. S. Anderson and Parker 1998; A van Hoof et al. 2000). The evolutionarily conserved Ski2, Ski3 and Ski8 proteins (Orban and Izaurralde 2005) form a complex (J. T. Brown, Bai, and Johnson 2000; L. Wang, Lewis, and Johnson 2005) that in *S. cerevisiae* is recruited to the exosome via Ski7, which is a yeast-specific protein (Orban and Izaurralde 2005) that directly binds to the exosome (Araki et al. 2001; Ambro van Hoof et al. 2002). Ski2 belongs to the DExH family of RNA helicases and could therefore mediate ATP-dependent RNA unwinding and mRNP remodelling before the substrate is delivered to the exosome (Halbach, Rode, and Conti 2012). This notion is supported by recent biochemical data suggests that Ski complex forms a continuous RNA channel to the exosome, thereby coupling the RNA helicase and the exoribonuclease (Halbach et al. 2013). The RNA helicase activity seems to play an important role in exosome-mediated mRNA decay since also the TRAMP complex, which regulates the activity of the nuclear exosome (Jia et al. 2012; Jia et al. 2011), contains a Ski2-related RNA helicase Mtr4 (Halbach, Rode, and Conti 2012).

Cytoplasmic mRNA quality control

Cytoplasmic mRNA quality control mechanisms target mRNAs with defects in translation. The three types of translational defects that trigger mRNA decay include: (1) aberrant translation termination due to a premature translation termination codon in the protein coding region; (2) translation into 3' poly(A) tail due to the absence of a stop codon; and (3) stalled translation elongation due to a barrier for ribosome progression. Each of the defects activates a specific mRNA decay pathway that have been reviewed in (Isken and Maquat 2007; Parker 2012). A common theme among these decay pathways seems to be how aberrant mRNAs are distinguished from normal transcripts. Namely, mRNA translational defects lead to the recruitment of adaptor proteins that interact with the translation machinery and direct the mRNA into a decay pathway (reviewed in Doma and Parker 2007).

Nonsense-mediated mRNA decay

The best studied translation-dependent mRNA quality control mechanism is nonsense-mediated mRNA decay (NMD), which targets mRNAs with nonsense codons in the protein coding region

in organisms ranging from yeast to human (reviewed in Kervestin and Jacobson 2012). Such nonsense codons are referred to as premature termination codons (PTCs) and they can arise in any gene due to gene mutations and errors in mRNA transcription and splicing (Massimo Caputi, Kendzior, and Beemon 2002; Sayani et al. 2008; Mort et al. 2008). If translated, PTC-containing mRNAs can produce truncated proteins with dominant negative or gain-of-function potential, as illustrated by truncated β-globine, which causes a dominant negative form of β-thalassemia in humans (Thein et al. 1990; Hall and Thein 1994). Therefore, the purpose of NMD seems to be to protect cells from the potentially toxic effects of mistakes that routinely occur during gene expression (reviewed in Isken and Maquat 2007). It is important to note, however, that in case of human genetic disease efficient NMD can also lead to haploinsufficiency because truncated proteins, which otherwise would retain sufficient wt function, are not produced (reviewed in J T Mendell and Dietz 2001; Peixeiro, Silva, and Romao 2011).

NMD targets and the mechanism of their recognition. Nonsense mutations are likely to arise only at a low frequency during gene expression (Korona, LeCompte, and Pursell 2011; M. J. Thomas, Platas, and Hawley 1998; Lynch 2010). Therefore, the presence of NMD pathway in all eukaryotes examined to date suggests that mRNAs, which contain PTCs introduced by mutations, are not the only targets of NMD (Baserga and Benz 1988; Brogna 1999; Isshiki et al. 2001; Leeds et al. 1991; Cali and Anderson 1998). Indeed, genome wide studies in yeast, worm, fruitfly, plant and human have revealed that NMD regulates the expression of many physiological non-mutated transcripts (Lelivelt and Culbertson 1999; Feng He et al. 2003; Joshua T Mendell et al. 2004; Rehwinkel et al. 2005; Wittmann, Hol, and Jack 2006; Ramani et al. 2009; Kurihara et al. 2009; Chan et al. 2007). The physiological NMD substrates in yeast include unspliced pre-mRNAs that contain nonsense codons in their introns (F He et al. 1993; Sayani et al. 2008), transcripts with upstream open reading frames (uORFs) (Gaba, Jacobson, and Sachs 2005; Guan et al. 2006) or alternative AUG initiation codons beyond the initiator AUG that are out of frame with the main ORF (Welch and Jacobson 1999), and transcripts that induce elongating ribosomes to shift the reading frame by one base in 5' or 3' direction and consequently direct the ribosomes to PTCs (Belew, Advani, and Dinman 2010). In higher eukaryotes alternative splicing contributes to the production of NMD substrates (reviewed in L. Huang and Wilkinson 2012; McGlincy and Smith 2008). Most PTC-containing alternative mRNA isoforms targeted by NMD are likely the result of splicing errors as suggested by their relatively low abundance and by the lack of tissue-specific expression pattern (Pan et al. 2006). However, in case of a subset of PTC-containing alternative mRNA isoforms the NMD pathway is exploited to exert post-transcriptional gene expression regulation (reviewed in L. Huang and Wilkinson 2012; McGlincy and Smith 2008). A

remarkable example here is the SR family of splicing regulators, where the expression of all the family members is regulated by alternative splicing-coupled NMD (Lareau et al. 2007; J. Z. Ni et al. 2007; Saltzman et al. 2008).

How do cells distinguish between a PTC and a normal translation termination codon? Experiments using mRNAs with artificially extended 3' UTRs have helped to shed light on this question. Namely, in organisms ranging from yeast to human a normal translation termination codon can be recognized as premature when positioned too far upstream of the poly(A) tail (Muhlrad and Parker 1999; Amrani et al. 2004; Behm-Ansmant et al. 2007; Longman et al. 2007; Kertesz et al. 2006; Eberle et al. 2008; Bühler et al. 2006; G. Singh, Rebbapragada, and Lykke-Andersen 2008). Conversely, PTC-containing mRNAs can be stabilized by positioning the poly(A) tail closer to the PTC either by deleting the coding region downstream of the PTC or by folding back the 3' UTR (Peltz, Brown, and Jacobson 1993; Hagan et al. 1995; Eberle et al. 2008). These observations indicate that the local mRNP structure downstream of the site of translation termination is a critical determinant for PTC recognition. But how does the 3' UTR regulate PTC recognition? Studies in yeast, fruitfly, plant and human cells have shown that NMD reporter transcripts can be stabilized by localizing poly(A)-binding protein close to the PTC, thereby mimicking a normal 3' UTR (Amrani et al. 2004; Behm-Ansmant et al. 2007; Kerényi et al. 2008; Eberle et al. 2008; G. Singh, Rebbapragada, and Lykke-Andersen 2008). The inhibitory effect of PABP on NMD could be mediated by PABP's role in normal translation termination. Both Pab1 in yeast and PABPC1 in mammals have been shown to directly interact with eRF3 (Hoshino et al. 1999; Cosson et al. 2002). Furthermore, Pab1 overexpression promotes translation termination, whereas depletion of PABPC1 increases nonsense codon readthrough (Cosson et al. 2002; Ivanov et al. 2008). Disrupted communication between PABP and eRF3 may therefore signal that the translation termination event is premature and lead to NMD activation (reviewed in Kervestin and Jacobson 2012). This notion is also supported by the finding that human *β-globin* mRNA effectively evades NMD if the PTC is located not more than 23 codons downstream of the initiator AUG (Inácio et al. 2004; Silva et al. 2006). NMD resistance of such transcripts was shown to depend on PABPC1 and eRF3 interaction, which likely facilitates normal translation termination at an AUG-proximal PTC (Peixeiro, Silva, and Romao 2011). However, NMD activation cannot solely be a consequence of a missing interaction between eRF3 and PABP as indicated by the findings that, at least in yeast cells, neither Pab1, mRNA poly(A) tail nor the Pab1-interacting domain of eRF3 are necessary for PTC-containing mRNA recognition and destabilization by NMD (Meaux, van Hoof, and Baker 2008; Kervestin et al. 2012).

Even though the exact molecular mechanism involved in PTC recognition remains unknown, it has been convincingly shown that translation termination at a PTC is mechanistically

different form translation termination at a normal termination codon (Amrani et al. 2004; Peixeiro et al. 2011). The latter proceeds without ribosome pausing at the termination codon as primer extension inhibition assay, which identifies the position of single ribosomes on mRNA, failed to yield toeprint signals at normal yeast and mammalian termination codons (Amrani et al. 2004; Peixeiro et al. 2011). In contrast, PTC-containing transcripts yielded toeprint signals corresponding to ribosomes stalled with nonsense codons occupying the ribosomal A-sites. Collectively, these data indicate that translation termination at a PTC is aberrant. It has been suggested that the inefficient release of eRF3 from a terminating ribosome, possibly due to the absence of PABP, could be the aberrant step in translation termination at a PTC, which leads to the activation of NMD pathway (reviewd in Kervestin and Jacobson 2012).

NMD factors and consequences of their activation. In all eukaryotes studied to date NMD substrates are identified and eliminated through the recruitment of up-frameshift (Upf) proteins, Upf1 (also known as Nam7), Upf2 (also known as Nmd2) and Upf3 (Hodgkin et al. 1989; Leeds et al. 1991; F He and Jacobson 1995; F He, Brown, and Jacobson 1997; Perlick et al. 1996; Cui et al. 1995; J Lykke-Andersen, Shu, and Steitz 2000; Serin et al. 2001; Gatfield et al. 2003). Single deletion of any of the *UPF* genes in yeast inhibits NMD to the same extent as Δ*upf1-3*, indicating that each of the Upf proteins is essential for NMD activation (F He, Brown, and Jacobson 1997). Besides Upf1-3, additional proteins participate in NMD activation in metazoans (reviewed in Isken and Maquat 2008; Isken and Maquat 2007; Kervestin and Jacobson 2012).

The key effector of the NMD pathway appears to be Upf1, which is thought to link aberrant translation termination at a PTC to NMD activation. Specifically, in the absence of Upf1 aberrant toeprints corresponding to ribosomes stalled at PTCs fail to accumulate, suggesting that Upf1 regulates the extent to which a ribosome remains associated with a PTC (Amrani et al. 2004). A role of Upf1 in translation termination is also supported by the finding that Upf1 co-immunoprecipitates with eRF3 and eRF1 (K Czaplinski et al. 1998; Ivanov et al. 2008; Kashima et al. 2006; W. Wang et al. 2001). Likewise, Upf2 and Upf3 co-immunoprecipitate with eRF3 but not with eRF1, indicating sequential assembly of the NMD machinery (W. Wang et al. 2001).

Upf1 has an amino terminal cysteine-histidine-rich zinc-knuckle domain (CH domain) connected by a flexible linker to helicase motifs common to superfamily 1 nucleic acid helicases (Weng, Czaplinski, and Peltz 1996; Bhattacharya et al. 2000). Biochemical and structural analysis indicates that in the absence of Upf2, the CH domain enhances the extent of RNA binding by the catalytic ATP-dependent RNA helicase domain, thereby inhibiting the ATPase activity of Upf1 (Chamieh et al. 2008; Chakrabarti et al. 2011). These observations support a model for Upf1 activation during NMD where the formation of the "surveillance complex", i.e. a complex

where the interaction of Upf1 and Upf3 is bridged by Upf2 (F He, Brown, and Jacobson 1997; Chamieh et al. 2008; Serin et al. 2001), reduces the extent of RNA binding by the Upf1 helicase domain and triggers its ATPase activity (Chakrabarti et al. 2011). However, it should be noted that data obtained by yeast two-hybrid and genetic analysis supports a contradicting model where Upf1 binding to its target mRNAs is stabilized, but not destabilizes, upon interaction with Upf2 (Feng He, Ganesan, and Jacobson 2013). Despite no clear consensus about the mechanism of Upf2-mediated activation of Upf1 ATP-dependent RNA helicase activity, the latter activity is necessary for mRNP disassembly. Namely, it has been found that in HeLa cells that harbour UPF1 mutants, which fail to bind or hydrolyze ATP, partially degraded NMD target mRNAs accumulate in P-bodies in complex with NMD factors (Franks, Singh, and Lykke-Andersen 2010). Upf1 has also been shown to play an important role in ribosome recycling at PTCs, thereby enabling subsequent rounds of translation (Ghosh et al. 2010). However, it remains to be determined if Upf1 ATPase activity is required for this process.

How exactly is Upf1 recruited to NMD target mRNAs and how Upf1 recruitment is linked to NMD activation? These questions have primarily been studied in the mammalian system (reviewed in Popp and Maquat 2014; Schweingruber et al. 2013). On newly synthesised mRNAs UPF1 has been found to transiently or weakly interacts with nuclear cap-binding complex component CBP80 (Hwang et al. 2010). In the presence of a PTC, UPF1-CBP80 interaction promotes UPF1 binding to eRF1-eRF3 complex at the terminating ribosome (Hwang et al. 2010). UPF1 binding to an NMD target is further enhanced by the presence of an EJC sufficiently downstream of the PTC (Kurosaki and Maquat 2013). The role of EJC in promoting UPF1 recruitment is likely related to EJC serving as a binding platform for UPF2 and UPF3, thereby facilitating UPF1-UPF2-UPF3 interaction (reviewed in Schweingruber et al. 2013; Popp and Maquat 2014), which leads to the activation of UPF1 helicase activity (Chakrabarti et al. 2011) required for the destruction of NMD targets (Franks, Singh, and Lykke-Andersen 2010). Importantly, PTC-free mRNAs can be bound by UPF1, albeit less efficiently than their PTC-containing counterparts (Hwang et al. 2010; Kurosaki and Maquat 2013). The critical determinant of whether an mRNA is subjected to NMD therefore cannot be the binding of UPF1 to an mRNA but rather seems to be the association of UPF1-UPF2-UPF3 proteins as part of the decay-inducing complex (reviewed in Schweingruber et al. 2013; Popp and Maquat 2014), which contains the ribosome, the UPF2- and UPF3-associated EJC and several proteins including UPF1, eRF1 and eRF3 (Yamashita et al. 2009). However, it should be noted that EJCs are removed by the translating ribosome likely during the pioneer round of translation (Gehring et al. 2009; Dostie and Dreyfuss 2002; Lejeune et al. 2002) but PTC-containing transcripts can be subjected to NMD also during subsequent rounds of translation, as indicated by the recent

finding that eIF4-bound mammalian mRNAs are not immune to NMD (Rufener and Mühlemann 2013; Durand and Lykke-Andersen 2013). These findings raise the question how UPF1-UPF2-UPF3 interaction occurs in the absence of an EJC further downstream of a PTC? In the absence of EJCs, UPF2 and UPF3 have been proposed to bind to ribosome-bound UPF1 (reviewed in Stalder and Mühlemann 2008), which is "found" possibly by diffusion (reviewed in Schweingruber et al. 2013).

Whereas Upf1-mediated NMD activation depends on translation, Upf1 binding to mRNA seems to be translation-independent. Global analysis of UPF1-mRNA interactions by UV cross-linking and immunoprecipitation combined with high-throughput sequencing has revealed that UPF1 associates with mRNAs prior to translation and is displaced by translating ribosomes from ORF sequences (Zünd et al. 2013; J. A. Hurt, Robertson, and Burge 2013).

NMD activation results in the rapid degradation of PTC-containing mRNAs that in *S. cerevisiae* and also in mammalian cells can be initiated from both the 5' and 3' end of the mRNA (reviewed in Isken and Maquat 2007). In *D. melanogaster*, the first step in the degradation of nonsense mRNAs is generally an endonucleolytic cleavage event in the vicinity of the PTC (Gatfield et al. 2003). PTC-proximal endonucleolytic cleavage has also been reported for mammalian cells (Eberle et al. 2009). Remarkably, in *S. cerevisiae* decapping of NMD substrates does not depend on prior deadenylation, which is a notable difference from the general deadenylation-dependent mRNA decay pathway (Muhlrad and Parker 1994; Cao and Parker 2003). Besides destabilization of PTC-containing mRNAs, NMD activation has also been suggested to lead to Upf1-dependent proteasom-mediated destruction of truncated proteins (Kuroha, Tatematsu, and Inada 2009). Even though the prevalence of NMD-coupled protein decay has yet to be determined (reviewed in Parker 2012), it is tempting to speculate that this mechanism has an important role in protecting cells from potentially toxic proteins derived from nonsense mRNAs (Kuroha, Tatematsu, and Inada 2009).

RNA-based RNP affinity purification

Isolation of ribonucleoprotein particles (RNPs) has provided valuable knowledge about RNA-protein interactions, which, in turn, has led to a better understanding of post-transcriptional gene expression regulation. Multiple methods have been developed to isolate RNPs. However, two general approaches exist – RNP capture via the protein or via the RNA component of the complex. With a focus on the technical aspects, the following chapter will give an overview

about RNP affinity purification methods that relay on the capture or the RNA component of the RNP.

RNA-based RNP affinity purification methods can be divided into two categories according to how RNPs are formed. For methods in the first category RNA-protein complex assembly takes place *in vivo* inside living cells. Methods in the second category are based on *in vitro* RNP formation that is performed after cell lysis. A common theme among all RNA-based RNP affinity purification methods is that the RNA of interest has to be "tagged" for purification. This tag can either be a chemical modification of the RNA or an RNA secondary structure element that binds with high affinity to a specific ligand. In both cases the introduced modification serves the purpose of capturing the bait RNA on the affinity matrix. The strategy used to tag the bait RNA depends on the nature of the bait. *In vitro* transcribed RNA or synthesized RNA oligonucleotides can be chemically modified. Naturally occurring or artificially selected RNA secondary structure elements can be incorporated into the RNA of interest during *in vivo* or *in vitro* transcription.

RNP affinity purification using chemically modified bait RNA

Many studies have made use of the high affinity biotin-streptavidin or biotin-avidin interaction (N. M. Green 1990; Sano and Cantor 1995) to capture ribonucleoprotein complexes. Biotinylated nucleotide analogs can be incorporated into the bait RNA during *in vitro* transcription. Typically, the labelled RNA is incubated with cell extract to allow *in vitro* RNP formation and the formed complexes are captured onto streptavidin-coated matrix. In the early studies this strategy was used to isolate spliceosome components from nuclear extracts of mammalian cells (Bindereif and Green 1987; Grabowski and Sharp 1986) and iron-responsive element-binding protein form human liver cytosolic extract (Rouault et al. 1989). One possible drawback of biotin labelling is that it can lead to structural changes of the bait RNA, which could affect RNP assembly (Walker et al. 2008). More recent studies have therefore utilized direct covalent coupling of the unlabelled bait RNA to the affinity matrix (Sela-Brown et al. 2000; Copeland et al. 2000; Allerson et al. 2003; M Caputi et al. 1999).

Despite the technical improvements in chemically modified RNA use for RNP affinity purification, the dependence on *in vitro* RNP assembly is a considerable drawback of the method because such complexes might not contain the whole complement of interaction partners present *in vivo*. However, in some cases the method can also be adopted for the affinity purification of *in vivo* assembled RNPs. Namely, antisense 2'-O-methyl RNA oligonucleotides complementary to

single-stranded regions in the RNA of interest have been successfully used to purify U2 and U4/U6 snRNPs as well as telomerase from nuclear extracts (Wenz et al. 2001; Lingner and Cech 1996; Blencowe et al. 1989). However, antisense RNA oligonucleotides have not found wide use in RNP affinity purification experiments. Single-stranded regions tend to destabilize RNP architecture and therefore it might not always be possible to find suitable regions in the bait RNA for affinity oligonucleotide annealing.

Artificially selected RNA affinity tags

The studies of RNP composition and function have greatly profited from the development of *in vitro* selection technology SELEX (systematic evolution of ligands by exponential enrichment), which has enabled to identify RNA and DNA sequences termed aptamers that bind with high affinity to specific ligands (reviewed in D. S. Wilson and Szostak 1999). Four different RNA aptamers have been commonly used to study RNA-protein interactions. All these aptamers are around 40 nt long stably folding RNA sequences that bind to their cognate ligand with an affinity in the micromolar to nanomolar range.

The first RNA aptamer to be adopted for RNA-protein interaction studies binds to the aminoglycosid antibiotic streptomycin. The binding is Mg^{2+} dependent and has a dissociation constant (K_d) of around 1 μM (Wallace and Schroeder 1998). The streptomycin binding RNA aptamer, or the so-called StreptoTag (Bachler, Schroeder, and von Ahsen 1999) and its improved version STagT (Dangerfield et al. 2006), have thus far been used in *in vitro* studies. In a typical experiment the *in vitro* transcribed hybrid RNA containing an RNA motif of interest and the streptomycin aptamer, is added to the cell lysate to allow RNA-protein complex formation and then loaded onto streptomycin-coupled sepharose column. After washing, the bound complexes are specifically eluted under native conditions with high concentration of free streptomycin. This approach has been used to isolate 48S PICs form rabbit reticulocyte lysate, thereby significantly facilitating the preparation of pure 48S PIC for downstream applications (Locker, Easton, and Lukavsky 2006). In another study glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was found to be an enhancer of group II intron splicing in *S. cerevisiae* (Böck-Taferner and Wank 2004). However, the identified RNA-protein interaction does not likely take place *in vivo*, since GAPDH is a cytosolic protein but the studied intron is located in yeast mitochondria. As eukaryotic GAPDH is more similar to the eubacterial homolog than to the archaeal homolog (Martin et al. 1993), the authors speculate that this result could hint to glycolytic pathway acquisition from the mitochondrial genome. Even though type II intron-GAPDH interaction

might have a biological meaning, this finding also draws the attention to the problem of possible falls-positive interaction identification due to *in vitro* RNA-protein complex assembly. In addition, the same study revealed that large hybrid RNAs could not be efficiently bound to the streptomycin matrix (Böck-Taferner and Wank 2004; Windbichler and Schroeder 2006). This is likely caused by folding problems of large *in vitro* transcribed RNA molecules and thus limits the size of hybrid RNAs, which can still be efficiently bound by streptomycin column, to about 600 nt.

Another RNA aptamer that under physiological conditions binds with a high affinity (K_d 5 nM) to an aminoglycoside antibiotic is the tobramycin-binding J6f1 RNA (Hamasaki et al. 1998). This aptamer fused to the 3' end of a pre-mRNA (hybrid RNA total size 267 nt) was successfully used to isolate human prespliceosomes, resulting in the identification of more than 70 prespliceosome-associated proteins by mass spectrometry (Hartmuth et al. 2002).

SELEX using the gel filtration matrix Sephadex G-100 as a target ligand resulted in the identification of D8 RNA aptamer (Srisawat, Goldstein, and Engelke 2001), which has been used to purify *in vivo* assembled ribonuclease P form *S. cerevisiae*. The main advantage of D8 aptamer is that its affinity matrix Sephadex is relatively cheap and can directly be used for tagged RNA purification. However, the affinity of the aptamer is not very high and therefore bound RNA will be gradually lost form the matrix during washing steps (Walker et al. 2008).

From the four RNA aptamers used for RNA-protein interaction studies the streptavidin-binding S1 aptamer has received the widest use. S1 aptamer binds to streptavidin with a K_d of about 70 nM (Srisawat and Engelke 2001). The elution of S1 aptamer form the affinity matrix can be performed under native conditions in the presence of d-biotin. The unusually high-affinity binding of biotin to streptavidin (K_d about 10^{-14} M) is essentially irreversible (N. M. Green 1990), thus enabling efficient elution and preventing rebinding of the eluted RNA to the matrix. The main disadvantage of the system is that the cellular biotin moieties have to be blocked before the lysate can be used for affinity purification. However, this can easily be done by pre-incubating the cell extract with egg white avidin, which binds biotin with a similar affinity as streptavidin.

S1 aptamer has been used in various experimental setups to study both *in vitro* (Butter et al. 2009; Leonov et al. 2003) and *in vivo* (Y. Li and Altman 2002; Vasudevan and Steitz 2007; Srisawat and Engelke 2001) assembled RNA-protein complexes. To highlight a few studies, Butter et al. developed a screening method of RNA-protein interactions using *in vitro* transcribed RNA motives as bait (Butter et al. 2009). In this approach the S1-tagged RNA is first coupled to paramagnetic beads and then incubated with metabolically labelled mammalian cell extract. Metabolic labelling is performed by stable isotope labelling by amino acids in cell culture (SILAC), which enables to perform high-resolution, quantitative mass spectrometry (MS) to

analyze RNA-protein complex composition. Due to the high specificity of RNA elution by biotin, it was possible to perform gel-free, single-run MS analysis, thus accelerating sample throughput. The method proved to be highly reliable, i.e. both previously reported RNA-protein interactions could be identified and new interactions could be proved in follow-up experiments.

It was recently reported that the S1 aptamer-mediated RNP pull-down efficiency could be increased 10-fold by adding a tRNA scaffold between the S1 aptamer sequence and the RNA motif of interest (Iioka et al. 2011). Interestingly, the attempt to increase the original S1 aptamer binding efficiency to the matrix by creating a hybrid RNA with six consecutive S1 repeats resulted in the opposite outcome – reduced RNA tethering efficiency as compared to hybrid RNA with a single S1 aptamer. The authors reasoned that this effect could be caused by one aptamer repeat interfering with another one, which might lead to misfolding of the RNA and subsequent affinity loss to streptavidin. The described tests were performed with matrix-tethered *in vitro* transcribed RNA because *in vivo* assembled RNA-protein complexes containing the RNA motif of interest fused to either one S1 aptamer, six S1 aptamers or S1 aptamer-tRNA scaffold could not be efficiently captured from human cell lysate. This is in contrast to some previous reports where, for instance, *in vivo* assembled RNase P could be purified via the S1 aptamer (Y. Li and Altman 2002). This discrepancy underlines the need to optimize RNA-based RNP affinity purification conditions for each RNP of interest.

Naturally occurring RNA secondary structure elements as RNA affinity tags

Several powerful tools for studying RNP structure and function have been developed based on RNA-protein interactions found in bacteriophages. One such interaction, which, among other applications, has been successfully adopted for RNP affinity purification, occurs between a stem-loop structure in the bacteriophage MS2 single stranded RNA genome and its coat protein (MS2CP). The 13.7 kDa MS2CP binds to the 19 nt long stem-loop as a dimer by contacting the nucleotides in the loop region and a bulged adenosine in the stem (C. Z. Ni et al. 1995; Valegård et al. 1994). The drawback of the wild type version of the coat protein for many experimental setups is its property to aggregate into capsid-like structures (Beckett, Wu, and Uhlenbeck 1988; Beckett and Uhlenbeck 1988). Structural studies of the coat protein have revealed a region important for the interaction of MS2CP dimers in the capsid. Several mutations in this region can prevent bulk capsid formation, even though multimers higher than a dimer can still form (Peabody and Ely 1992; LeCuyer, Behlen, and Uhlenbeck 1995). Mutational analysis of the RNA stem-loop in the bacteriophage R17, which is closely related to MS2, has identified a loop

sequence with increased affinity to the coat protein. The binding of the wt loop sequence AUUA to the coat protein has a K_d of 1-3 nM, whereas the mutated sequence AUCA has a K_d of 0.2-0.6 nM (Carey et al. 1983; Lowary and Uhlenbeck 1987; Romaniuk et al. 1987; H. N. Wu, Kastelic, and Uhlenbeck 1988). Due to the higher affinity, the mutated loop sequence is now generally used to tether the MS2 or R17 coat protein (R17CP) to the stem-loop.

The first report describing an affinity purification method based on the interaction of R17CP and its cognate stem-loop binding site was published by Bardwell and Wickens (Bardwell and Wickens 1990). The authors demonstrated that *in vitro* transcribed RNA containing either one or two R17 stem-loops could be captured with equal efficiencies by R17CP immobilized onto solid support. However, in the presence of additional sequences two loops were necessary to enable efficient capture. The applicability of the MS2CP/MS2-loop (MS2L) system for *in vitro* or *in vivo* assembled RNP affinity purification has since been demonstrated by many groups. For instance, the method has been adopted for the purification of spliceosomal complexes under native conditions (R. Das, Zhou, and Reed 2000; Zhou, Sim, et al. 2002; Deckert et al. 2006; Bessonov et al. 2008). In this approach, the MS2CP is fused to maltose-binding protein (MBP) and amylose beads, which bind the MBP-moiety of the fusion protein, are used for affinity selection. Captured complexes are eluted from the matrix under mild conditions with an excess of maltose. In the first attempt to affinity purify *in vivo* formed RNPs from prokaryotes, MS2CP-MBP was employed to capture MS2L-tagged small non-coding RNAs (sRNAs) from *Salmonella*. sRNAs tagged with two MS2Ls were either expressed from plasmid or from the genomic locus and RNPs were immobilized onto MS2CP-MBP-coupled amylose column (Said et al. 2009). It was shown that the isolated MS2L-tagged sRNAs co-purified with the common sRNA-binding protein Hfq, suggesting that sRNAs were recovered in their native form.

In the studies described in the previous paragraph only relatively short RNA sequences had been used for RNP affinity purification via the MS2L tag. Slobodin and Gerst have demonstrated that the MS2CP/MS2L system is also applicable for the purification of RNPs containing full-length mRNAs form *S. cerevisiae* (Slobodin and Gerst 2010). With the aim to identify new RBPs taking part in mRNA trafficking, they developed a method termed RNA-binding protein purification and identification (RaPID). In this method, the mRNA of interest, which is expressed form its genomic locus, is captured via 12 tandem copies of MS2Ls inserted behind the coding region. In addition, the yeast strain contains a plasmid encoding for the tag-binding protein under the control of a galactose inducible promoter. The tag-binding protein is comprised of three functional unites: (1) the MS2CP that binds to the MS2Ls; (2) GFP that helps to monitor *in vivo* formation of mRNPs and; (3) straptavidin binding protein that enables to capture the mRNPs on streptavidin-conjugated matrix. The bound mRNPs are eluted under

native conditions by competition with free biotin. The analysis of the affinity-eluates by reverse transcription PCR (RT-PCR) and immunobloting confirmed previously known RNA-protein interactions. In addition, Sec27, a subunit of the COPI vesicle coating complex, was identified as a possible interaction partner of *OXA1* mRNA by MS analysis of a specifically enriched band on silver stained SDS-PAGE.

An alternative approach to the MS2CP/MS2L system has been developed base on the interaction between bacteriophage lambda N-antiterminator protein and boxB stem-loop (Kevin Czaplinski et al. 2005). The N-protein forms a 1:1 complex with boxB by interacting with nucleotides both in the stem and the loop region (Van Gilst et al. 1997; Cilley and Williamson 1997). The K_d of the complex determined by flouorometry is about 1 nM (Van Gilst et al. 1997). The first 22 N-terminal amino acids of the N-protein have been shown to bind to the boxB stem-loop with a similar affinity and specificity as the full-length protein (Tan and Frankel 1995). This short peptide can be fused to the protein of interest, which can then be recruited onto the target RNA containing the 19 nt boxB stem-loop (Baron-Benhamou et al. 2004).

The versatility of possible experimental approaches to purify RNP complexes is emphasized by the development of RNA Affinity in Tandem (RAT) method (Hogg and Collins 2007). This method uses an RNA tag composed of two different stem-loops. In the first purification step RNPs are selected based on the interaction between *Pseudomonas aeruginosa* phage 7 coat protein (PP7CP) and its 25-nt binding site. The second step is provided by the binding of J6f1 RNA aptamer to tobramycin. PP7CP is structurally similar to MS2CP (Chao et al. 2008) and both coat proteins bind to their cognate stem-loop with a comparable affinity (K_d about 1 nM) (Francis Lim and Peabody 2002; Van Gilst et al. 1997). However, due to the tolerance to a broader range of salt concentration and pH than MS2CP-MS2L interaction, PP7CP and its binding site might represent a more robust tool for RNP affinity purification (F Lim, Downey, and Peabody 2001; Francis Lim and Peabody 2002; Hogg and Collins 2007). Indeed, optimization of the RAT tag revealed that RNP yield was higher if PP7CP/PP7-loop (PP7L) system was used as compared to MS2CP/MS2L system (Hogg and Collins 2007). Another innovative approach by Hogg and Collins besides a two-step purification strategy based on a double RNA tag, is the use of tobacco etch virus (TEV) protease to selectively elute RNPs after the first affinity purification step. Namely, the PP7CP contains a TEV protease cleavage site between the coat protein and protein A tag. The latter tag enables to capture RNPs onto IgG-coupled affinity matrix during the first purification step. The method was used in mammalian cell culture system to purify endogenous RNPs assembled on non-coding RNAs. Combined with mass spectrometry, hnRNP K was identified as a component of 7SK ncRNA-containing RNPs.

The RNP affinity purification method established by Tsai et al. adds two novel approaches to the toolbox of *in vivo*-assembled RNP affinity purification strategies – affinity purification under denaturing conditions and identification of affinity purified proteins by SILAC-based quantitative mass spectrometry (Tsai et al. 2011). In order to capture RNPs under denaturing conditions, RNA-protein interactions were cross-linked by UV light prior to affinity purification and the high affinity biotin-straptavidin interaction was used to isolate RNPs. Specifically, the MS2CP was fused to HTBT tag that contains an *in vivo* biotinylation site, which allowed the capture of RNPs onto streptavidin-coated superparamagnetic beads (X. Wang et al. 2007). This strategy enabled Tsai et al. to quantitatively identify 36 proteins binding to internal ribosomal entry site (IRES) of lymphoid enhancer factor-1 mRNA and to determin the RNP proteins common to the IRES-containing and control mRNA (Tsai et al. 2011).

MATERIALS and METHODS

Materials

General buffers and media were prepared as described in (Jellbauer 2009).

Chemicals

Standard chemicals were used as listed in (Fundakowski 2012).

Special chemicals and reagents

AppliChem	Pepstatin A Triethylamine
Bio-Rad	Protein Assay Dye Reagent Concentrate
Carl Roth	Roti®-Aqua-Phenol/Chloroform/Isoamyl alcohol for RNA extraction
EMD Millipore	Pellet Paint® Co-Precipitant
Life Technologies	Ambion® Linear Acrylamide Applied Biosystems® TRI Reagent® Solution
Roche Applied Science	Blocking Reagent Complete Protease Inhibitor Cocktail Tablets CSPD, ready-to-use
Sigma-Aldrich	1-Bromo-3-chloropropane Antifoam B Emulsion IgG from rabbit serum, reagent grade Molecular BioProducts RNase AWAY®
Thermo Scientific	Pierce ECL Western Blotting Substrate

Consumables

5 Prime	Phase Lock Gel™ Heavy 2 ml
BGB Analytik	GL Microfiber 25 mm Syringe Filter (pore size 3.1 µm and 1.2 µm)
Biotium	GelRed™ Nucleic Acid Gel Stain
GE Healthcare Life Sciences	Amersham Hybond™-P PVDF Transfer Membrane Whatman™ GF6 Glass Fibre Filter (Ø 10 cm, pore size 1-3 µm)
Life Technologies	Applied Biosystems® MicroAmp® Fast Optical 96-Well Reaction Pate Dynabeads® M-270 Epoxy Invitrogen™ NuPAGE® Novex 4-12% Bis-Tris Gel (1.0 mm)

	SYBR® Safe DNA Gel Stain
Roche Applied Science	Nylon Membrane, positively charged
Sarstedt	Filtropur S Sterile Syringe Filter (pore size 0.2 µm)
Thermo Scientific	Fermentas GeneRuler DNA Ladder Mix Fermentas PageRuler Prestained Protein Ladder Fermentas PageRuler Unstained Protein Ladder

Equipment

Alpha Innotec	FluorChem® FC2 Imaging System
Analytik Jena AG	FlexCycler
Bachofer	Vacuum Concentrator
Bio-Rad	Mini-PROTEAN® Tetra Cell Mini Trans-Blot® Electrophoretic Transfer Cell PowerPac™ Basic and HC High-Current Power Supplyis Trans-Blot® SD Semi-Dry Electrophoretic Transfer Cell
EMD Millipore	SNAP i.d.™ Protein Detection System
Eppendorf	Centrifuge 5415 R Centrifuge 5702 Centrifuge 5810 R Thermomixer comfort 1.5 ml
Fujifilm	LAS-3000 Imager
GE Healthcare Life Sciences	Amersham Hybridization oven/shaker Amersham Typhoon™ Variable Mode Imager DynaMag™-15 Magnet Ultrospec 10 Cell Density Meter
IKA	Vibrax® VXR basic
Infors HT	Minitron
LI-COR Biosciences	Odyssey® Infrared Imaging System
Life Technologies	Applied Biosystems® StepOnePlus™ Real-Time PCR System XCell SureLock® Mini-Cell
Retsch	Mixer Mill MM400
Schleicher & Schuell BioScience	GV 100/0 Vacuum Filter Holder
Thermo Scientific	GENESYS 10 Bio UV-Vis Spectrophotometer NanoDrop® 1000 Spectrophotometer Sorvall® RC-6 PLUS

Enzymes

Agilent Technologies	Herculase II Fusion DNA Polymerase
Amsbio	Zymolyase® 20T

Axon	Taq DNA Polymerase
Life Technologies	Ambion® RNase Cocktail
New England Biolabs	conventional restriction enzymes RecA
Promega	GoTaq® DNA Polymerase RQ1 RNase-Free DNase
Roboklon	TEV Protease
Thermo Scientific	Fermentas Calf Intestine Alkaline Phosphatase (CIP) Fermentas conventional restriction enzymes Fermentas FastDigest restriction enzymes Fermentas Proteinase K Fermentas RevertAid Premium Reverse Transcriptase Fermentas RiboLock RNase Inhibitor Fermentas T4 DNA Ligase Fermentas T4 DNA Polymerase

Commercial kits

Agilent Technologies	Prime-It II Random Primer Labelling Kit
Qiagen	QIAquick Gel Extraction Kit QIAquick PCR Purification Kit QIAprep Spin Miniprep Kit
Life Technologies	Applied Biosystems® High Capacity cDNA Reverse Transcription Kit Fast SYBR® Green Master Mix Invitrogen™ Colloidal Blue Staining Kit Invitrogen™ MEGAshortscript™ T7 Kit Invitrogen™ SilverQuestTM Silver Staining Kit

Antibodies

Primary antibodies			**Corresponding secondary antibodies**		
Name	Dilution[a]	Supplier	Name	Dilution[a]	Supplier
Peroxidase Anti-Peroxidase (PAP)	1:5000	Sigma-Aldrich			
Anti-She2 (clone 1C3-11)	1:150	AG Jansen	Peroxidase-conjugated Rabbit Anti-Rat IgG (H+L)	1:2000	Jackson ImmunoResearch
Anti-c-myc (clone 9E10)	1:1000	Roche Applied Science	Peroxidase-conjugated Sheep Anti-Mouse IgG (H+L)	1:3000	Jackson ImmunoResearch
Anti-Pgk1 (clone 22C5D8)	1:3500	Invitrogen™ Life Technologies	Peroxidase-conjugated Sheep Anti-Mouse IgG (H+L)	1:4000	Jackson ImmunoResearch
			IRDye 680-conjugatedanti Goat Anti-Mouse IgG (H+L)	1:3500	LI-COR Biosciences

[a] Dilution corresponds to antibody dilution used for western blot analysis.

Oligonucleotides

Oligonucleotides for epitope tagging, gene knockout and checking of transformants were designed according to published protocols (Janke et al. 2004; Knop et al. 1999; Haim et al. 2007). Oligonucleotides for fusion PCR were designed according to Shevchuk et al. (Shevchuk et al. 2004) and for sequence- and ligation-independent cloning (SLIC) according to Li and Elledge (M. Z. Li and Elledge 2007). Oligonucleotides for template DNA amplification for *in vitro* transcription with T7 RNA polymerase were designed following the guidelines of manufacturer's manual of MEGAshortscript T7 Kit (Life Technologies).

Oligonucleotides for epitope tag integration and gene knockout

RJO	Name	5'- 3' sequence
3560	PGK1_m-TAG_F	GGAATTGCCAGGTGTTGCTTTCTTATCCGAAAAGAAATAAcgctgcaggtcgacaaccc
3561	PGK1_m-TAG_R	GGGAAAGAGAAAAGAAAAAAATTGATCTATCGATTTCAATTCAATTCAATgcataggccactagtggatc
3590	MEX67_S1_F	AAGAGTAAAATAAATCGTTAAAAATTCTGCATCGCTAATAGCAGCAAAAAAAATGcgtacgctgcaggtcgac
3591	MEX67_S2_R	CTGTATATTTTTTGTGATACTGTGCGGCTGAAACAGGGAACAATATCATTAatcgatgaattcgagctcg
3848	ENO2_m-TAG_F	CTACGCCGGTGAAAACTTCCACCACGGTGACAAGTTGTAAcgctgcaggtcgacaaccc
3849	ENO2_m-TAG_R	CTATGATGAAAAAATAAGCAGAAAAGACTAATAATTCTTAGTTAAAAGCACTgcataggccactagtggatc
3934	NAM7_S3_F	GAGAAGAACAAAAGCATGAATTGTCAAAAGACTTCAGCAATTTGGGAATAcgtacgctgcaggtcgac
3935	NAM7_S2_R	GTATCACAAGCCAAGTTTAACATTTTATTTTAACAGGGTTCACCGAATTAatcgatgaattcgagctcg

Oligonucleotides for verification of proper epitope tag integration or gene knockout

RJO	Name	5'- 3' sequence
3562	PGK1_Det_F	GGCTTTGTTAGACGAAGTTGTC
3563	HIS3_Det_R	GACTGTCAAGGAGGGTATTCTG
3564	PGK1_Det_R	CCCGAACATAGAAATATCGAATGGG
3589	MEX67_-235_F	CATGCCCACTTGCCTTTCGTAG
3850	ENO2_Det_F	CATTGCTGACTTGGTTGTCGG
3851	ENO2_Det_R	CCAGTGCATTATGCAATAGACAGC
3936	NAM7_Det_F	GTACCAGGAGGAGGCTTCTC
3937	NAM7_Det_R	TGCAAATTGCGAGTCTATCTCG

Oligonucleotides for RT-PCR

RJO	Name	5'- 3' sequence
2205	Ash1-RTPCR-E1-for	CTTTATCTAAGAGACCGGAGCGC
2206	Ash1-RTPCR-E1-rev	CTTGGACGACCTAGTCGATTCC
3491	PGK1_mid_F	GGTTTTGGAAAACACTGAAATCGG
303	PGK1-rev	TAAGAAAGCAACACCTGGCA
3509	SOD1_+240_F	ACATGTCGGTGACATGGGTAACG
3510	SOD1_+424_R	ACCACAGGCTGGTCTTGGAC
3515	SRL1_+413_F	AGGTCAAGTCCTTTGAACAGGCT
3516	SRL1_+570_R	CCATTGTACGTTACCTGGAGAGGT

Oligonucleotides for qRT-PCR

RJO	Name	5'- 3' sequence
2920	Act1_qPCR_1_for	TCAGAGCCCCAGAAGCTTTG
2921	Act1_qPCR_1_rev	TTGGTCAATACCGGCAGATTC
2916	Eno2_qPCR_2_for	GGTTGTCGGTTTGAGAACTGG
2917	Eno2_qPCR_2_rev	TTCGATTCTCAACAATTGGTTCA
4132	PGK1-RT_F	GAACGGTCCACCAGGTGTT
4133	PGK1-RT_R	GACGGTGTTACCAGCAGCAG
4135	TPI1_F	TGGTACCGGTTTGGCTGCT
4136	TPI1_R	ATTCGCTGGCAGCCTTGTC
4139	18S_F	TCAACACGGGGAAACTCACC
4148	18S_qRT_R	CTAAGAACGGCCATGCACCA
4141	26S_F	GCTTGTGGCAGTCAAGCGT
4149	26S_qRT_R	ACAATCCAACGCTTACCGAA

Oligonucleotides for cloning

RJO	Name	5'- 3' sequence
3487	pLOX_5'MS2L_F	GTTTAAACGAGCTCTCGAGAACC
3494	MS2CP_F	GGTCGCTGAATGGATCAGCTC
3683	pUC/M13 Forward	CCCAGTCACGACGTTGTAAAACG
3684	pUC/M13 Reverse	AGCGGATAACAATTTCACACAGG
3747	MET25_F	cccctcgaggtcgacggtatcgataagcttAGCTCCGGATGCAAGGG
3750	TEV-PrA_R	ggtggcggccgctctagaactagtggatccGGCCGCAAATTAAAGCCTTCG
3802	2_PrA5un_3un_R	CTTCATCGTGTTGCGCGGAATTCGCGTCTAC
3803	3_PrA3un_5un_F	GTAGACGCGAATTCCGCGCAACACGATGAAGCCGTG
4059	SLIC_pRS4_PGK1gen_F	ccctcgaggtcgacggtatcgataagcttTGCAAGTACCACTGAGCAGG
4061	SLIC_PGK1prom_MS2L_R	CGACCTGCAGCGgctagcTGTTTTATATTTGTTGTAAAAAGTAG ATAATTAC

4062	SLIC_MS2L_PGK1prom_F	CAACAAATATAAAACAgctagcCGCTGCAGGTCGACAACCC
4063	SLIC_MS2L_CYC1_R	gtgacataactaattacatgGCATGCGCATAGGCCACTAGTGGATC
4064	SLIC_CYC1_MS2L_F	GCCTATGCGCATGCcatgtaattagttatgtcacgc
4065	SLIC_CYC1_pRS4_R	ggcggccgctctagaactagtggatccaaagccttcgagcgtccc

Oligonucleotides for generation of hybridization probes

RJO	Name	5'- 3' sequence
176	sASH1nco	CCAATAGAACCATGGAGCGC
217	ASH1 1892 REV	GAAGATGCCGCGGCGTG
302	PGK1-forw.	CTTCAAAGTTGTCTGTCCAAG
303	PGK1-rev.	TAAGAAAGCAACACCTGGCA
3491	PGK1_mid_F	GGTTTTGGAAAACACTGAAATCGG
3939	PGK1_T7p_R	taatacgactcactatagggGGCATCAGCAGAGAAAGCATC
4109	m-TAG_F	CGCTGCAGGTCGACAACCC
3938	MS2L_T7p_R	taatacgactcactatagggGCAGACATGGGTGATCCTCATG
4139	18S_F	TCAACACGGGGAAACTCACC
4140	18S_T7_1302_R	taatacgactcactatagggCGTTCGTTATCGCAATTAAGCAG
4141	26S_F	GCTTGTGGCAGTCAAGCGT
4120	26S_T7_R	taatacgactcactatagggCTCACGACGGTCTAAACCC

Plasmids

RJP	Name	Origin
88	YEplac181-ASH1	pC3319 in (Long et al. 1997)
407	pSH47	(Prein, Natter, and Kohlwein 2000)
1116	pUN100-LEU2-mex67-5	(Segref et al. 1997)
1117	pUN100-LEU2-MEX67	(Segref et al. 1997)
1433	p414 Gal1 Ash1	Susanne Lange, Gene Center, Munich
1573	p414 GALS 6MS2 PGK1	Susanne Lange, Gene Center, Munich
1712	ploxP-HIS5-6xMS2L	this study
1751	pRS316-MET25-MS2CP-TEV-PrAx2	this study
1783	pRS416-PGK1prom-6MS2L-CYC1	this study
1814	YCplac22-MET25-MS2CP-PrAx2	this study

RJP 1712, which is a PCR template plasmid for the amplification of 6MS2L-containig m-TAG cassette, was constructed from plasmid RJP 1485 (pLOXHIS5MS2L) (Haim et al. 2007) by the replacement of 12MS2L with 6MS2L. RJP 1485 was digested with *Eco*RV and the 3850 bp

fragment comprising the vector backbone was blunt-end ligated with 380 bp BamHI-BglII fragment cut out from plasmid RJP 232 (YEP lac112-LZ-MS2-ASH1) (Bertrand et al. 1998).

RJP 1751 (CEN6, URA3), which, under the control of MET25 promoter, expresses MS2CP fused to four IgG-binding Z domains of protein A (PrAx2), was constructed using fusion PCR to create the insert (Shevchuk et al. 2004) and SLIC (M. Z. Li and Elledge 2007) to assemble the insert and the vector backbone. The insert comprising the sequences of MET25 promoter, MS2CP, TEV protease cleavage site, PrAx2 and iso-1-cytochrome c (CYC1) transcription terminator was created as follows. First, fragment 1 (MET25-MS2CP-TEV-PrA, 1383 bp) and fragment 2 (PrA-CYC1, 772 bp) were amplified form plasmid RJP 1748 (pRS316-MET25-MS2CP-TEV-PrA) with primers RJO 3684/RJO 3802 and RJO 3803/RJO 3802, respectively. Due to a 31 nt homology region between the 3'-end of fragment 1 and 5'-end of fragment 2, the fragments could be fused in a PCR step containing polymerase and dNTPs but lacking primers (Shevchuk et al. 2004). The PCR fusion product was used for insert amplification (1948 bp) with nested primers RJO 3747/RJO 3750, which contained at 5'-ends a stretch of 30 nt homologous to vector backbone. Plasmid RJP 148 (pRS316) (Sikorski and Hieter 1989) opened with *Hind*III-*Bam*HI (4857 bp) was annealed with the insert following the protocol for SLIC sub-cloning using T4 DNA polymerase treated inserts with RecA (M. Z. Li and Elledge 2007).

RJP 1783 (CEN6, URA3) encodes for an RNA that contains loxP-6MS2L sequence as it is present in the endogenously expressed mRNA tagged with 6MS2L (Haim et al. 2007). The insert (1676 bp) was created by a two step fusion PCR form three fragments: (1) *PGK1* promoter containing 947 nt upstream of *PGK1* start codon was amplified form yeast genomic DNA (gDNA) (RJY 3731) with primers RJO 4059/ RJO 4061; (2) loxP-6MS2L was amplified form yeast gDNA (RJY 3731) with primers RJO 4062/RJO 4063; and (3) CYC1 transcription terminator was amplified form plasmid RJP 111 (p413-GAL1) (Mumberg, Muller, and Funk 1994) with primers RJO 4064/4065. The first round of fusion PCR resulted in fragment 1-2 (amplified with RJO 4059/4063) and fragment 2-3 (amplified with RJO 4062/4065) fusion. The resulting PCR products were fused in a second round of PCR and amplified with primers RJO 4059/4065. Plasmid RJP 291 (pRS416) (Sikorski and Hieter 1989) opened with *Hind*III-*Bam*HI (4868 bp) was annealed with the insert following the protocol for SLIC sub-cloning using T4 DNA polymerase treated inserts with RecA (M. Z. Li and Elledge 2007).

RJP 1814 (CEN4, TRP1) expresses MS2CP-TEV-PrAx2 fusion protein under the control of MET25 promoter. The insert (1882 bp) comprising the sequence of MET25 promoter and the

fusion protein was cut out with *Hind*III-*Bam*HI from plasmid RJP 1751 and inserted into HindIII-BamHI site of RJP 138 (YCplac22) (Gietz and Akio 1988).

All the plasmids created in this study were verified by sequencing.

E. coli strains

Strain	Essential genotype	Origin
TOP10	F- *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) φ80*lac*ZΔM15 Δ*lac*X74 *nup*G *rec*A1 *ara*D139 Δ(*ara-leu*)7697 *gal*E15 *gal*K16 *rps*L(Str^R) *end*A1 λ$^-$	Invitrogen

S. cerevisiae strains

All yeast strains that were generated for this work are based on either haploid (RJY 359) or diploid (RJY 925) W303 wildtype cells.

RJY	Essential genotype	Origin
135	*MATalpha, ade2-1, trp1-1, can1-100, leu2-3, 112, his 3-11,15, ura3, ash1::URA3*	(Nasmyth et al. 1990)
359	*MATalpha, ade2-1, trp1-1, can1-100, leu2-3, 112, his3-11,15, ura3, GAL, psi+*	n/a
3166	*MATa, ade2-1, can1-100, his3-11,15, leu2-3,112, trp1-1, ura3, GAL, psi+, loxP-ProtA-TEV-CBP-SHE2*	Stephan Jellbauer, Gene Center, Munich
3550	*MATalpha, ade2-1, trp1-1, can1-100, leu2-3,112, his3-11,15, ura3, GAL, psi+, PGK1-loxP-HIS5-loxP-6MS2L*	this study
3558	MATa, ade2-1, trp1-1, can1-100, leu2-3,112, his3-11,15, ura3, GAL, psi+, lys1::kanMX6	Katja Sträßer, Gene Center, Munich
3639	*MATa/MATalpha, ade2-1, trp1-1, can1-100, leu2-3,112, his3-11,15, ura3, GAL, psi+, LYS1/lys1::kanMX6, PGK1/PGK1-loxP-HIS5-loxP-6MS2L,* pSH47(RJP 407)	this study
3641	*MATa/MATalpha, ade2-1, trp1-1, can1-100, leu2-3,112, his3-11,15, ura3, GAL, psi+, LYS1/lys1::kanMX6, PGK1/PGK1-loxP-HIS5-loxP-6MS2L, MEX67/mex67::natNT2,* pUN-*LEU2-mex67-5* (RJP 1116), pSH47(RJP 407)	this study
3644	*MATalpha, ade2-1, trp1-1, can1-100, leu2-3,112, his3-11,15, ura3, GAL, psi+ lys1::kanMX6, mex67::natNT2, PGK1-loxP-6MS2L,* pUN100-*LEU2-mex67-5* (RJP 1116)	this study
3645	*MATalpha, ade2-1, trp1-1, can1-100, leu2-3,112, his3-11,15, ura3, GAL, psi+ lys1::kanMX6, mex67::natNT2, PGK1,* pUN100-*LEU2-mex67-5* (RJP 1116)	this study
3682	*MATalpha, ade2-1, trp1-1, can1-100, leu2-3,112, his3-11,15, ura3, GAL, psi+, lys1::kanMX6, mex67::natNT2, PGK1-loxP-6MS2L,* pUN100-*LEU2-MEX67* (RJP 1117), pRS316-*MET25-TEV-PrAx2* (RJP 1751)	this study
3683	*MATa, ade2-1, trp1-1, can1-100, leu2-3,112, his3-11,15, ura3, GAL, psi+, lys1::kanMX6, mex67::natNT2, PGK1,* pUN100-*LEU2-MEX67* (RJP 1117), pRS316-*MET25-TEV-PrAx2* (RJP 1751)	this study

3715	*MATa, ade2-1, trp1-1, can1-100, leu2-3,112, his3-11,15, ura3, GAL, psi+, lys1::kanMX6, mex67::natNT2, ENO2-loxP-6MS2L,* pUN100-*LEU2-MEX67* (RJP 1117), pRS316-*MET25-MS2CP-TEV-PrAx2* (RJP1751)	this study
3731	*MATalpha, ade2-1, trp1-1, can1-100, leu2-3,112, his3-11,15, ura3, GAL, psi+ lys1::kanMX6, mex67::natNT2, PGK1-loxP-6MS2L, NAM7-3myc::His3MX6,* pUN100-*LEU2-mex67-5*	this study
3739	*MATalpha, ade2-1, trp1-1, can1-100, leu2-3,112, his3-11,15, ura3, GAL, psi+, lys1::kanMX6, mex67::natNT2, PGK1-6MS2L, NAM7-3myc::His3MX6,* pUN100-*LEU2-mex67-5* (RJP 1116), pRS316-*MET25-MS2CP-TEV-PrAx2* (RJP 1751)	this study
3740	*MATalpha, ade2-1, trp1-1, can1-100, leu2-3,112, his3-11,15, ura3, GAL, psi+, lys1::kanMX6, mex67::natNT2, PGK1, NAM7-3myc::His3MX6,* pUN100-*LEU2-mex67-5* (RJP 1116), pRS316-*MET25-MS2CP-TEV-PrAx2* (RJP 1751)	this study
3827	*MATalpha, ade2-1, trp1-1, can1-100, leu2-3,112, his3-11,15, ura3, GAL, psi+, lys1::kanMX6, mex67::natNT2, PGK1-loxP-6MS2L, NAM7-3myc::His3MX6,* pUN100-*LEU2-MEX67* (RJP 1117), pRS316-*MET25-MS2CP-TEV-PrAx2* (RJP 1751)	this study
3828	*MATa, ade2-1, trp1-1, can1-100, leu2-3,112, his3-11,15, ura3, GAL, psi+, lys1::kanMX6, mex67::natNT2, PGK1, NAM7-3myc::His3MX6,* pUN100-*LEU2-MEX67* (RJP 1117), pRS316-*MET25-MS2CP-TEV-PrAx2* (RJP 1751)	this study
3989	*MATa, ade2-1, trp1-1, can1-100, leu2-3,112, his3-11,15, ura3, GAL, psi+, lys1::kanMX6, mex67::natNT2, PGK1, NAM7-3myc::His3MX6,* pUN100-*LEU2-MEX67* (RJP 1117), pRS416-*PGK1prom-6MS2L-CYC1* (RJP 1783), YCplac22-*MET25-MS2CP-TEV-PrAx2* (RJP 1814)	this study

Methods

Standard methods in molecular biology and *Escherichia coli*-specific techniques were performed as described in (Jellbauer 2009).

Working with *S. cerevisiae*

Optical density of yeast culture

Yeast culture optical density (OD) was determined using Ultrospec 10 Cell Density Meter (GE Healthcare Life Sciences) at 600 nm. One OD unit at 600 nm (1 OD_{600}) corresponds to 2.7 x 10^7 cells.

Transformation of yeast cells

Plasmid DNA transformation was carried out, with minor modifications, according to the protocol published by Chen et al. (D. C. Chen, Yang, and Kuo 1992). Specifically, instead of

incubating the cells in DNA/One-Step Buffer mix at 45°C, the incubation was performed at 30°C followed by incubation at 42°C for 10 or 5 min. 5 min incubation time was used for strains carrying the temperature sensitive *mex67-5* allele.

PCR products were introduced by high-efficiency transformation according to a protocol adapted from Hami-Vilmovsky and Gerst (Haim-Vilmovsky and Gerst 2009). Cells were harvested at OD_{600} 0.6-0.8 by centrifugation at 1000 x *g* for 3 min at room temperature (RT; same settings were used also for subsequent centrifugation steps for cell suspension in 15 or 50 ml tubes), washed with 0.5 culture volume of TE and thereafter with 0.1 culture volume of 0.1 M lithium acetate (LiAc). Cells were resuspended in 1 ml 0.1 M LiAc, transferred into 1.5 ml tube and pelleted by centrifugation at 16 000 x *g* for 15 sec. Subsequently, cells were resuspended in 0.1 M LiAc to have around 3 OD_{600} units of cells per 50 µl cell suspension. In parallel to cell washing, PCR tubes containing 8 µl of PCR product (3-1 µg DNA) and 525 µl of polyethylene glycol (PEG)/LiAc/single stranded DNA (ssDNA) mix were prepared. A negative control was prepared by omitting the PCR product. 50 µl of cell suspension was added to each tube, mixed by brief vortexing and incubated for 30 min at 30°C. Cells were then exposed to heat shock at 42°C for 10 or for 5 min in case of temperature sensitive strains. After heat shock 600 µl yeast extract-peptone (YEP) was added to the tubes and cells were pelleted by centrifugation at 1000 x *g* for 3 min. Washing was repeated once with 1 ml YEP. Finally, cells we resuspended in 1 ml YEP and 200 µl of cell suspension corresponding approximately to 0.6 OD_{600} units of cells was plated onto an appropriate selection plate. Colonies were allowed to form for 2-3 days at 26°C. If cloNAT (nourseothricin) or G418 (geneticin) was used for selection, cells were allowed to recover after the heat shock for 4-16 h in yeast extract-peptone-dextrose (YEPD) before plating.

One-Step Buffer	**PEG/LiAc/ssDNA mix per one transformation**
0.2 M LiAc	50 µg ssDNA (stock 2 µg/µl)
40% (w/v) PEG 3350	400 µl 45% (w/v) PEG 4000
100 mM DTT	100 µl 95 mM LiOAc (in TE)
Filter sterilized and stored at -20°C	Prepared fresh before use. ssDNA added directly before use to avoid reannealing of ssDNA

Summary of mRNP affinity purification- and SILAC-compatible yeast strain creation

PGK1 was tagged with 6MS2L using a PCR-based genomic tagging strategy termed m-TAG (Haim et al. 2007). Genomic deletion of *LYS1* was achieved by mating two haploid strains of opposite mating types: RJY 3558 carrying *⊿lys1* and RJY 3550 carrying *PGK1-loxP-Sphis5*$^{+}$*-loxP-6MS2L*. In addition to *LYS1* deletion, strain RJY 3558 contained plasmid RJP 407 with URA3 selection marker. Mating of stains was performed as described in (Jellbauer 2009) and diploid cells were selected on synthetic complete (SC) plates lacking uracil and histidine. Yeast colony

PCR was performed to confirm the diploid status of selected colonies (Huxley, Green, and Dunham 1990). The resulting diploid strain RJY 3639 was next used for the genomic deletion of *mex67* for experimental purposes not discussed in this thesis. After successful *mex67* deletion the resulting strain RJY 3641 was sporulated and tetrads were dissected. One tetrad was identified with two haploid spores with desired genotypes: (1) *⊿lys1, ⊿mex67, PGK1* (RJY 3645); and (2) *⊿lys1, ⊿mex67, PGK1-loxP-6MS2L* (RJY 3644). These strains were used as background strains to create all other strains for mRNP affinity purification experiments.

mRNP affinity purification

Coupling of Dynabeads® M-270 Epoxy with rabbit IgG

300 mg of Dynabeads® M-270 Epoxy paramagnetic beads (Life Technologies) were coupled with 50 mg of rabbit IgG (Sigma-Aldrich) as described in (Oeffinger, Wei, and Rout), except that rabbit IgG was reconstituted in 150 mM NaCl instead of double distilled (dd) H_2O.

Culturing cells for mRNP affinity purification optimization experiments

Yeast strains were grown in SC medium containing 6.7 g/L yeast nitrogen base, 69 mg/L adenine sulfate, amino acids (Table 2) and 2% glucose. In the morning, 10 ml of medium was inoculated with a small amount of cells form freshly streaked plate and allowed to grow over day at 26°C with shaking at 120 rpm (growth conditions here and hereinafter). In the evening, cells were diluted to OD_{600} 0.05 in 200 ml medium and grown overnight. The next morning, 1900 ml medium was inoculated to OD_{600} 0.2 and the culture was allowed to reach mid-log phase (OD_{600} 0.8) before harvesting.

Table 2. Final concentration of amino acids in SC medium lacking uracil (SC -ura).

Amino acid	mg/L
L-arginine monohydrochloride	42
L-histidine hydrochloride monohydrate	42
L-isoleucine	42
L-leucine	84
L-lysine	50
L-methionine	84
L-phenylalanine	63
L-serine	42
L-threonine	42
L-tryptophan	63
L-tyrosine	69
L-valine	189

Metabolic labelling by SILAC for mass spectrometry-based quantitative proteomics

SILAC-compatible yeast strains were first tested for SILAC amino acid incorporation rate. For this, cells were allowed to divide ~7 times in 4 ml of appropriate SC medium containing the "heavy", i.e. the stable $_{13}C_{15}N$ isotopic form of the amino acid lysine (Lys8). Yeast cells were disrupted in MS Cell Breakage Buffer by vigorous shaking with acid washed glass beads (Ø 0.2-0.3 mm) for 10 min at 4°C, stopping the shaking after each 2 min to cool down the lysate on ice for 2 min. Protein concentration of the clarified lysate was determined using Protein Assay Dye Reagent (Bio-Rad) according to manufacturer's manual and the lysate was stored at -20°C. Lys8 incorporation test was performed by the Proteome Center Tübingen using 50 µg of lysate. The strains used in this study had Lys8 incorporation rates > 95%.

For mRNP purification combined with quantitative analysis of MS2L-tagged mRNA co-isolating proteins, two strains were grown in parallel in SC -ura medium (see previous chapter and table 2 for medium composition) supplemented either with "light" $_{12}C_{14}N$ isotope-containing lysine (Lys0) or Lys8. In order to precisely determine the number of cell divisions in Lys8-containing medium, both strains were first grown overnight in 2 ml medium supplemented with Lys0. The next morning the strains were inoculated to OD_{600} 0.2 in 4 ml of SILAC medium, which for one strain was supplemented with Lys0 and for the other strain with Lys8, and were allowed to grow for about eight hours. Then cells were diluted to OD_{600} 0.1 in 80 ml of SILAC medium and were grown overnight. The following morning, the two cultures were diluted to OD_{600} 0.2 in 1200 ml SILAC medium. In order to prevent both strains from reaching OD_{600} 0.8 at the same time, dilutions were performed with 1 h gap in between. Cells should be allowed to double in SILAC medium for at least five times for full incorporation of the heavy amino acid (Gruhler and Kratchmarova 2008). The described growth strategy enabled cells to double at least 8 times in SILAC medium and the determined Lys8 incorporation rates were > 95%.

MS Cell Breakage Buffer
6 M urea
2 M thiourea
10 mM Tris HCl, pH 8.0
1x Protease Inhibitor Cocktail (Roche Applied Science)
Prepared fresh

Harvesting of large scale yeast culture according to *Öffinger* et al.

Yeast cell harvesting was modified from (Oeffinger, Wei, and Rout). Briefly, 2 L culture was divided into three 1 L centrifugation tubes and spun down at 4000 x *g* for 10 min at 4°C (Sorvall® RC-6 PLUS, Thermo Scientific). Supernatant was decanted and centrifugation tubes were placed on ice. Pellets were resuspended in 25 ml ice cold ddH_2O per pellet. Cell suspension

was divided into two 50 ml pre-cooled conical tubes and spun down at 2600 x *g* for 5 min at 4°C (5810 R, Eppendorf). After decanting the supernatant, cells were resuspended in 25 ml ice cold ddH_2O per pellet. Cell suspension in two 50 ml tubes was pooled into one and cells were spun down at 2600 x *g* for 5 min at 4°C. After centrifugation supernatant was decanted, cells were resuspended in ice cold Resuspension Buffer equal to the volume of the cell pellet and spun down at 2600 x *g* for 10 min at 4°C. Supernatant was removed by aspiration and the pellet was spun down once more at 2600 x *g* for 10 min at 4°C. The remaining of the supernatant was removed by aspiration and yeast cells were frozen following the protocol by Öffinger et al. (Oeffinger, Wei, and Rout).

Resuspension Buffer	Before use Resuspension Buffer was supplemented with:
1.2% (w/v) PVP-30	1x Protease Inhibitor Cocktail
20 mM Hepes (pH 7.4)	1:100 Solution P (stock: 0.4 mg/ml Pepstatin A, 18 mg/ml PMSF in absolute EtOH)
Stored at 4°C	1:1000 1M DTT

Harvesting of large scale yeast culture according to *Inada* et al.

Yeast growth medium was removed by vacuum filtration (GV 100/0, Schleicher & Schuell BioScience) using 2 pieces of GF6 glass fibre filters (Whatman™, GE Healthcare Life Sciences) pre-wetted in distilled (d) H_2O. The maximal volume of mid-log phase culture that could be filtered before clogging of the system was 2 L. After filtration, the filter with the captured cells was placed into a 600 ml wide-mouth glass beaker and the cells were removed by rinsing with 25 ml of Cell Wash Buffer pre-warmed to 30°C (Inada et al. 2002). Cell suspension was transferred into a 50 ml conical tube and the filter was washed with an additional 20 ml of Cell Wash Buffe in order to collect remaining cells. Cell suspension was pelleted at 2600 x *g* for 4 min at 24°C and supernatant was removed by decanting. In order to remove the rest of the supernatant, the 50 ml tube was left standing upside down on a towel paper for 10 sec before proceeding with freezing the cells (Oeffinger, Wei, and Rout).

Cell Wash Buffer
20 mM Hepes (pH 7.4)
2 mM $MgAc_2$
100 mM KAc
2% (w/v) glucose
1x amino acids
1x Protease Inhib. Cocktail
Prepared fresh before use

10x Amino Acids				
0.2 g	adenine sulfate		1 g	L- isoleucine
0.2 g	uracil		1 g	L- phenylalanine
0.2 g	L- tyrosine		1 g	L- glutamic acid
1 g	L- tryptophan		1 g	L- aspartic acid
1 g	L-histidine hydrochloride monohydrate		3 g	L- valine
1 g	L- arginine monohydrochloride		4 g	L- threonine
1 g	L- leucine		8 g	L- serine

Volume brought to 1 L with ddH_2O, filter sterilized, stored at 4°C

Disruption of yeast cells by cryolysis

Frozen cells were disrupted under cryo conditions by grinding in mixer mill (MM400, Retch). The technical details of grinding are listed in table 3. Before transferring the frozen cells into grinding jars, the jars, as well as the steel grinding balls, were cooled down in liquid nitrogen (LN_2). Care was taken to remove all LN_2 before closing the screw cap of the grinding jar. Cells were ground for 6 cycles, each cycle lasting 3 min. After every cycle (including the last one) the grinding jars were cooled down in LN_2 for about 2 min until LN_2 had stops boiling. During cooling down, the jars were not entirely immersed in LN_2 in order to prevent LN_2 from seeping into the jars. After grinding, the grindate was transferred into 50 ml conical tubes previously cooled down in LN_2 and stored at -80°C.

Table 3. Grinding specification. Sample volume refers to the volume of frozen yeast cells.

Initial culture vol.	Sample vol.	Grinding jar vol.	Ball charge	Grinding cycles (Hz)
2 L	≥5 ml	50 ml	1 x Ø 25 mm	20, 2 x 14, 3 x 12
1.2 L	2-3 ml	10 ml	1 x Ø 12 mm	20, 5 x 16

mRNP affinity purification protocol

The method of mRNP isolation via the mRNA component of the complex was developed based on a method for immunoaffinity purification of protein complexes (Oeffinger et al. 2007). The experiments were performed under essentially RNase-free conditions. Only RNase-free solutions and water (HPLC-purified or DEPC-treated H_2O) were used. In order to prevent RNase contamination through pipettes, filter tips and serological plastic pipettes were used. In addition, the work area and pipettes were regularly cleaned with RNase AWAY® (Molecular BioProducts Sigma-Aldrich).

Yeast cell grindate amount used for mRNP isolation depended on the downstream application (Table 4). Before starting with the experiment, grindate was weighed out into a 15 ml or 50 ml conical tube pre-cooled in LN_2, and stored in LN_2 or at -80°C until needed. Due to the loss of some material during lysate preparation 1.3 times more grindate was weighed out than eventually needed for the experiment (in case of grindate amounts ≤ 300 mg 1.5-2 times more grindate was weighed out). Once ready to proceed with the experiment, the tubes were immersed in ice and the grindate was allowed to thaw until it resembled thick ice cream (15 to 30 min depending on grindate amount). In parallel, IgG-coupled Dynabeads were washed four times with ice cold RNP Buffer 150 (RNPB-150). Per gram of grindate 100 µl IgG-coupled Dynabeads (concentration 150 mg/ml) were washed and per 100 µl beads 2 ml of RNPB-150 was used for

washing. Depending on the bead volume, washing was performed in 1.5 ml safe seal tube (safe seal tubes were used throughout the experiment) or in 15 ml conical tube. For washing, the beads were resuspended by pipetting or by short vortex, captured on magnet and the supernatant was removed by pipetting (1.5 ml tube) or by aspiration (15 ml tube). If bead washing had been performed in a 15 ml tube, the beads were transferred into a 1.5 ml tube after the fourth washing steps by rinsing the 15 ml tube twice with 1 ml RNPB-150. After washing, 100-200 µl RNPB-150 was added to the beads and the beads were stored on ice until needed.

Table 4. Grindate amount used for mRNP affinity purification.

Grindate amount	Downstream application
300-400 mg	Analysis of bead-captured RNA by northern blot and qRT-PCR[a]
200 mg	Analysis of bead-captured RNA co-isolating proteins on silver stained SDS-PAGE[b]
100 mg	Immunodetection of bead-captured RNA co-isolating proteins[b]
1.15 g	Quantitative MS analysis of bead-captured RNA co-isolating proteins[b]

[a] Isolated material was enough to perform several analyses.
[b] Isolated material was enough to perform one analysis.

Thawed grindate was resuspended in ice cold RNPB-150 using 9 ml of buffer per one gram of grindate. After adding the buffer, the tube was vortexed for ≤ 30 sec. If after 30 sec of vortexing the lysate still contained a frozen clump of cells, the tube was inverted until the clump disappeared and the lysate was immediately spun down at 940 x *g* for 3 min at 4°C. During the centrifugation step preparations were made for subsequent lysate filtration: (1) per lysate two 50 ml wide-mouthed 100 ml glass beakers were placed on ice; (2) the piston of a fresh 10-20 ml syringe was removed; (3) a 25 mm glass microfiber syringe filter with a pour size of 3.1 µm (BGB Analytik) was attached to the syringe and placed into the glass beaker on ice. Immediately after centrifugation the supernatant was poured into the prepared syringe avoiding the transfer of cell debris and the lysate was filtered avoiding foaming. The filtration step was repeated using a syringe filter with pore size of 1.2 µm (BGB Analytik). The appropriate volume of clarified lysate (mg grindate intended to use for the experiment times 9) was transferred into a 15 ml conical tube (or 1.5 ml tube) and washed IgG-coupled beads were added. In case of working with several lysates in parallel, care was taken to use an equal volume of each lysate for the experiment and to divide the IgG-coupled beads equally between the samples. mRNA-protein complexes were captured by rotating the samples at minimal rpm (Reax 2, Heidolph) for 30 min at 4°C. A 100 µl aliquot of input material was stored at -20°C for western blot analysis of MS2CP-PrAx2 capture and cell grinding efficiency. A second aliquot of 150 µl was snap frozen in LN_2 and stored at -20°C for total RNA extraction. During lysate-bead incubation the tube containing the cell debris, which had been stored on ice while preparing the lysate, was spun down at 3020 x *g* for 5 min at

4°C, the supernatant was removed by aspiration and the pellet was resuspended in ice cold ddH_2O equal to the volume of RNPB-150 used for resuspending the grindate. A 100 µl aliquot was removed and stored at -20°C for western blot analysis of cell grinding efficiency.

After 30 min of binding, the tubes were placed on magnet and the supernatant was allowed to clear. 100 µl of cleared supernatant (flow through) was transferred into a 1.5 ml tube and stored at -20°C for western blot analysis of MS2CP-PrAx2 capture efficiency. A second aliquot of 150 µl was snap frozen in LN_2 and stored at -20°C for total RNA extraction. The supernatant was removed from beads by pipetting (1.5 ml tube) or by aspiration (15 ml tube). 1 ml of ice cold RNPB-150 was added to the tube for washing and the beads were resuspended by gentle pipetting in order to prevent disruption of captured complexes. During the first washing step beads were transferred from 15 ml tube into 1.5 ml tube. Washing with RNPB-150 was repeated once, followed by two washing steps with 1 ml of ice cold Final Wash Buffer (FWB). After this, either bead-captured RNA was extracted or mRNP proteins were release by RNase treatment.

RNP Buffer 150
20 mM Hepes (pH 7.4)
110 mM KAc
150 mM NaCl
0.5% (v/v) Triton X-100
0.1% (v/v) Tween-20
0.02% (v/v) Antifoam B (Sigma-Aldrich)
1:100 Solution P (added directly before use)
Prepared fresh before use

Final Wash Buffer
20 mM Hepes (pH 7.4)
1 mM $MgCl_2$
40 mM NaCl
Prepared fresh before use

Bead-captured RNA extraction

IgG-coupled beads with the bound mRNPs were resuspended in FWB (2 times the initial bead volume) supplemented with 0.1% (v/v) SDS and 200 µg/ml Proteinase K (Fermentas Thermo Scientific) and incubated on a water bath at 30°C for 30 min. During the incubation, beads were mixed every 10 min by tapping the 1.5 ml tube with a finger. After Proteinase K treatment, equal volume of TRI Reagent (Applied Biosystems® Life Technologies) was added to the beads and mixed by vortexing for 30 sec. The sample was incubated at RT for 5 min, after which 1/10 TRI Reagent volume of 1-bromo-3-chloropropane (Sigma-Aldrich) was added. The sample was mixed by vortexing and incubated at RT for additional 10-15 min. The sample wash shortly spun down in order to collect liquid form the lid and placed on magnet to capture the beads. The organic and aqueous phase was mixed by pipetting and transferred onto a pre-spun Phase Lock Gel Heavy 2 ml (PGL, 5 Prime) tube. The PGL tube was centrifuged at 16 000 x *g* for 5 min at RT (5415 R, Eppendorf) and the aqueous RNA-containing phase was transferred into a fresh 1.5 ml tube. An

equal volume of chloroform (Sigma-Aldrich) was added to the tube, mixed by pipetting and incubated at RT for 2 min. The sample was loaded onto a pre-spun PGL tube and the centrifugation was repeated as above. The aqueous phase was transferred into a new 1.5 ml tube. The following solutions were added to the RNA sample: 1/10 RNA sample volume 3 M sodium acetate (pH 5.2), 0.5 µl Pellet Paint Co-Precipitant (EMD Millipore), 2.5 RNA sample volume absolute EtOH and 10 µg/ml Linear Acrylamide (Ambion® Life Technologies, amount calculated according to the final volume of RNA sample plus EtOH). The sample was mixed by vortexing and RNA was precipitated at -20°C overnight. The next day RNA was collected by centrifugation at 16 000 x *g* for 15 min at 4°C. After removing the supernatant by pipetting, 500 µl of 75% EtOH was added into the tube in order to remove salt from RNA pellet and the sample was centrifuged at 16 000 x *g* for 5 min at 4°C. After removing most of the 75% EtOH, the sample was briefly spun down and the remaining of the liquid was removed by pipetting. The sample was allowed to air-dry for 2 min at RT, after which the pellet was dissolved in 12 µl HPLC-H_2O per 400 mg initial grindate.

mRNP protein release by RNase treatment

For RNase treatment beads were resuspended in 1.2 times the initial bead volume of FWB. Subsequently, 1/24 of FWB volume of RNase Cocktail (Ambion® Life Technologies) was added and the sample was mixed at 24°C for 30 min on Thermomixer comfort (Eppendorf) using the following settings: 10 sec at 1400 rpm, 1 min break. After RNase treatment the sample was shortly spun down and the beads were captured on magnet. RNase eluate containing the mRNP proteins was transferred into a fresh 1.5 ml tube. The beads were washed with one initial bead volume of HPLC-H_2O by pipetting, the water was pooled with the eluate and the sample was vacuum dried (Vacuum Concentrator, Bachofer). Vacuum drying took about 1.5 h for 250 µl sample. The dry eluate samples, as well as the magnetic beads, were stored at -20°C for further analysis.

RNase treatment was performed with a modification for SILAC-labelled grindates. Specifically, before RNase treatment the washed beads from two parallel affinity purifications were mixed. For this, the beads in one tube were resuspended in 200 µl FWB and added to the beads in the other tube. The empty tube was rinsed with an additional 200 µl of FWB in order to collect all the beads into one tube. FWB was removed from the pooled beads and RNase treatment was performed as described above.

Total cellular RNA extraction

An equal volume of phenol-chloroform-isoamyl alcohol (PCI, Roti-Aqua-PCI for RNA extraction, Carl Roth) was added to the frozen input (I) and flow through (FT) samples and vortexed until the samples had thawed. In order to allow mRNA-protein complexes to dissociate, samples were incubated at RT for 5 min and then the organic and RNA-containing aqueous phase were separated by centrifugation at 16 000 x *g* for 5 min at RT. The upper aqueous phase was transferred into new 1.5 ml tubes, 2.5 volumes of cold absolute EtOH was added, mixed and RNA was precipitated for 10 min at RT. The samples were centrifugation at 16 000 x *g* for 10 min at 4°C to collect the RNA. RNA pellets were washed with 1 ml 75% cold EtOH, centrifuged at 16 000 x *g* for 5 min at 4°C and the air-dried pellets were dissolved in 50 µl HPLC-H_2O.

A small amount of grindate (~ 50 µl) was transferred into a pre-cooled 1.5 ml tube. 400 µl PCI and 600 µl Cross RNA Buffer I were added to the tube and vortexed for mixing. The sample was incubated at RT for 5 min and RNA extraction was continued as described for I and FT samples.

Cross RNA Buffer I
0.3 M NaCl
10 mM Tris-HCl (pH 7.5)
1 mM EDTA (pH 8.0)
0.2% (w/v) SDS
Stored at 4°C
Before use the bottle was swirled to equally distribute precipitated SDS

RNA analysis

Northern blot analysis

Denaturing agarose gel electrophoresis and capillary transfer of RNA onto positively charged nylon membrane was performed as described in (Jellbauer 2009).

Hybridization probe synthesis

Radiolabelled probe synthesis. α-[^{32}P]-dCTP labeled DNA probes (Table 5) were synthesized using Prime-lt II Random Primer Labeling Kit (Agilent Technologies) according to the manufacturer's manual. 25 ng PCR product was used per labelling reaction and the reaction products were purified from unincorporated nucleotides with probe cleanup spin columns.

Table 5. Radiolabeled DNA probes for northern blot analysis.

DNA probe	Template DNA for labelling by random priming		DNA probe length (nt)
	PCR template	PCR primers	
ASH1	RJP 88	RJO 176 RJO 217	1020
PGK1	RJP 1573	RJO 302 RJO 303	1226

DIG-labelled probe synthesis. Antisense RNA probes were synthesized by *in vitro* transcription in the presence of digoxigenine-11-UTP (Roche Applied Science) using MEGAshortscript Kit (Life Technologies) according to the manufacturer's manual (Table 6). 100 nM PCR product containing a T7 RNA polymerase promoter site was used as template DNA for *in vitro* transcription.

Table 6. Antisense RNA probes for northern blot analysis.

RNA probe	Template DNA for *in vitro* transcription		*In vitro* transcription	
	PCR template	PCR primers	DIG-UTP:UTP	Antisense RNA probe length (nt)
PGK1-ORF	RJP 1573	RJO 3491 RJO 3939	1:2.5	154
MS2L	gDNA, RJY 3731	RJO 4109 RJO 3938	1:2	120
18S	gDNA, RJY 3731	RJO 4139 RJO 4140	1:2.5	138
25S	gDNA, RJY 3731	RJO 4141 RJO 4120	1:2.5	148

Northern blot hybridization

Hybridization of DNA probes. DNA probe hybridization and signal detection were performed as described in (Jellbauer 2009).

Hybridization of antisense RNA probes. Hybridization was performed with minor modifications as described in Engler-Blum et al. (Engler-Blum et al. 1993). Briefly, the membrane was prehybridized at 68°C with gentle rolling in hybridization oven/shaker (Amersham GE Healthcare Life Sciences) for a minimum of 2 h in 20 ml/100 cm^2 Prehybridization Solution. 100 ng denatured DIG-labelled RNA probe was added to the hybridization tube per ml of Prehybridization Solution and incubated overnight. After hybridization, the membrane was washed 3 x 20 min at 65°C with 50 ml of preheated Wash Buffer I. Subsequently, the membrane was transferred from the hybridization tube into a box and incubated with gentle rocking for 5 min at RT in Wash Buffer II. Before antibody incubation the membrane was blocked in 1 ml/cm^2 Blocking Solution for 1 h, after which Anti-Digoxigenin-alkaline phosphatise, Fab fragments (Roche Applied Science) was added at 1:5000 dilution. After 1 h of antibody incubation the membrane was washed 2 x 15 min in 100 ml Wash Buffer II. The membrane was

equilibrated in Detection Buffer for 3 min and ready-to-use CSPD chemiluminescent substrate (Roche Applied Science) was applied on the membrane according to manufacturer's instructions. Chemiluminescence was detected using LAS-3000 image acquisition system (Fujifilm) equipped with a cooled digital CCD camera.

Prehybridization Solution
250 mM Na-phosphate buffer (pH 7.2)
10% (w/v) SDS
0.5% (w/v) Blocking Reagent (Roche)
Prepared fresh by adding Blocking Reagent (stock 5% (w/v))

Wash Buffer I
20 mM Na-phosphate buffer (pH 7.2)
1 mM EDTA
1% (w/v) SDS
Stored at RT

Wash Buffer II
0.1 M maleic acid (pH 8.0)
3 M NaCl
0.3% (v/v) Tween-20
Stored at RT

Blocking Solution
Wash Buffer II
0.5% (w/v) Blocking Reagent
Prepared fresh by adding
Blocking Reagent (stock 5% (w/v))

Detection Buffer
0.1 M Tris-HCl (pH 9.5)
0.1 M NaCl
50 mM $MgCl_2$
Stored at RT

Real-time quantitative reverse transcription PCR (qRT-PCR)

DNase treatment and cDNA synthesis

Input and Flow Through RNA samples. In order to digest genomic DNA, total cellular RNA was treated with RQ1 RNase-Free DNase (Promega) prior to cDNA synthesis. 9 µl reaction containing 1 µg RNA, 1 U RQ1 DNase, 1 µl 10x RQ1 DNase Reaction Buffer and HPLC-H_2O was pipette into a 200 µl thin wall PCR tube and incubated for 30 min at 37°C. All incubation steps during DNase treatment and cDNA synthesis were performed in a PCR cycler with a heated lid. Reaction was terminated by adding 1 µl RQ1 DNase Stop Solution (sample total volume 10 µl) and subsequently DNase was denatured by incubating the reaction for 10 min at 65°C. RNA was reverse-transcribed using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems® Life Technologies). cDNA synthesis was performed in a total volume of 12 µl containing 600 ng of DNase treated RNA, 1.2 µl 10x RT Buffer, 1.2 µl 10x RT Random Primers, 0.6 µl 25 mM dNTPs, 0.6 µl MultiScribe Reverse Transcriptase and HPLC-H_2O. Per each RNA sample an 8 µl control reaction containing 400 ng of DNase treated RNA and all cDNA synthesis components except reverse transcriptase was prepared. cDNA synthesis was performed using the following settings: 10 min 25°, 120 min 37°C, 15 min 85°C.

Bead-captured RNA samples. DNase treatment and cDNA synthesis were performed using the same reagents and incubation conditions as described for I and FT samples. 2 µl bead-

captured RNA was treated with 0.2 U of DNase in a total volume of 9 µl. 6 µl of the DNase-treated RNA was used for cDNA synthesis and 4 µl for a control reaction without reverse transcriptase.

qRT-PCR

qRT-PCR was performed using the StepOnePlus Real-Time PCR System (Applied Biosystems® Life Technologies). Each reaction contained 5 µl Fast SYBR Green PCR Master Mix (Applied Biosystems® Life Technologies), 4 µl appropriately diluted cDNA and 1 µl of primer mix (500 nM each). Primers were designed using FastPCR software (PrimerDigital, http:// primerdigital.com/fastpcr.html). The thermocycling profile included an initial denaturation for 20 sec at 95°C, followed by 40 cycles of amplification, which comprised denaturation at 95°C for 3 sec and annealing/elongation at 60°C for 30 sec. A single fluorescence measurement was performed at the end of the elongation step of every amplification cycle. After PCR amplification melting curve analysis of amplification products was performed to test for the formation of primer-dimers and non-specific PCR products. For this, PCR amplification products were denatured at 95°C for 15 sec and allowed to reanneal at 60°C for 1 min. Subsequently, the temperature was increased in 0.3°C increments to 95°C (step-and-hold fluorescence measurement). Only those primer pairs (Table 7) were used for further analysis that did not generate any primer-dimers during the 40 PCR amplification cycles. Reactions were run in duplicate or in triplicate and included a no template control (cDNA replaced by ddH_2O).

Table 7. qRT-PCR target genes. Input refers to the RNA extracted from clarified lysate before the mRNP isolation step. Bead captured-RNA refers to the RNA extracted from IgG-coupled beads after mRNP isolation. Amplification efficiency (E) estimates for each target were calculated using a 5 fold serial dilution curve with five data points.

Target gene	Primers	cDNA dilution		E
		Input	Bead-captured RNA	
PGK1	RJO 4132 RJO 4133	20 or 100	100	1.926
TPI1	RJO 4135 RJO 4136	20 or 100	100	1.896
ACT1	RJO 2920 RJO 2921	20 or 100	100	1.944
ENO2	RJO 2916 RJO 2917	20 or 100	100	1.935
25S	RJO 4141 RJO 4149	1000	100	1.935
18S	RJO 4139 RJO 4148	1000	100	1.986

qRT-PCR data analysis

qRT-PCR amplification efficiency. In order to determine qRT-PCR amplification efficiency (E) for each target, 5 fold serial dilutions were prepared from a cDNA sample corresponding to input RNA of strain RJY 3739. cDNA dilutions ranging from 10-fold to 6250-fold were run in triplicate and StepOne Software version 2.2 was used to generate the standard curve for each target. Using the slope of the linear regression line, the software calculates E of one cycle in the exponential phase according to the equation: $E=5^{(-1/slope)}$. Only those primer pairs were used for further analysis that showed an E value between 0.9-1 (Table 7).

Relative quantification of qRT-PCR results. Relative quantification of qRT-PCR results was performed by comparative C_T method (also known as the $2^{-\Delta\Delta C_T}$) combined with kinetic qRT-PCR efficiency correction (Pfaffl 2001). In this model the relative expression ratio (R) of a target gene is calculated based on its E, and threshold cycle (C_T) difference (Δ) of one unknown sample (e.g. drug treated sample) versus one control (e.g. untreated sample), and expressed in comparison to a reference gene (Pfaffl 2001) (Equation 1).

Equation 1 $R = (E_{target})^{\Delta C_{T\ target}\ (control\ -\ sample)} \div (E_{ref})^{\Delta C_{T\ ref}\ (control\ -\ sample)}$

In order to give an overview about how equation 1 was applied in this study, the steps of calculating *PGK1-6MS2L* relative enrichment after *PGK1*-mRNP affinity purification are listed. In this calculation *PGK1-6MS2L* and *PGK1* are defined as "target" mRNAs and *TPI1*, *ACT1* and *ENO2* as "reference" mRNAs. For *PGK1-6MS2L* mRNA (and its respective reference mRNAs) "control" is the C_T value of input sample and "sample" is the C_T value of bead-captured RNA sample (both C_T values correspond to the strain harbouring *PGK1-6MS2L*). Conversly, for *PGK1* mRNA (and its respective reference mRNAs) "control" is the C_T value of input sample and "sample" is the C_T value of bead-captured RNA sample (both C_T values correspond to strain harbouring *PGK1*). Relative quantification of all the qRT-PCR experiments in this study was done analogous to the below described case.

1) Calculation of ΔC_T (normalization)
   a) C_T values of strain harbouring *PGK1-6MS2L*
      Target $\Delta C_{T\ PGK1\text{-}6MS2L}$ ($C_{T\ input\ PGK1\text{-}6MS2L}$ - $C_{T\ bead\text{-}captured\ PGK1\text{-}6MS2I}$)
      Reference $\Delta C_{T\ TPI1/ACT1/ENO2}$ ($C_{T\ input\ TPI1/ACT1/ENO2}$ - $C_{T\ bead\text{-}captured\ TPI1/ACT1/ENO2}$)
   b) C_T values of strain harbouring *PGK1*
      Target $\Delta C_{T\ PGK1}$ ($C_{T\ input\ PGK1}$ - $C_{T\ bead\text{-}captured\ PGK1}$)
      Reference $\Delta C_{T\ TPI1/ACT1/ENO2}$ ($C_{T\ input\ TPI1/ACT1/ENO2}$ - $C_{T\ bead\text{-}captured\ TPI1/ACT1/ENO2}$)

2) Calculation of $E^{\Delta CT}$ from target and reference ΔC_T values

3) Calculation of relative expression ratio R
   a) $E^{\Delta CT\ PGK1\text{-}6MS2L} \div E^{\Delta CT\ TPI1/ACT1/ENO2}$
   b) $E^{\Delta CT\ PGK1} \div E^{\Delta CT\ TPI1/ACT1/ENO2}$

4) Calculation of relative enrichment of *PGK1-6MS2L* as compared to *PGK1*
$R_{PGK1\text{-}6MS2L\ (TPI1/ACT1/ENO2)} \div R_{PGK1\ (TPI1/ACT1/ENO2)} = x$
$R_{PGK1\ (TPI1/ACT1/ENO2)} \div R_{PGK1\ (TPI1/ACT1/ENO2)} = 1$

5) Calculation of average (corresponding to *TPI1*, *ACT1* and *ENO2*) relative enrichment of *PGK1-6MS2L*

Reverse transcription PCR (RT-PCR)

DNase treatment and cDNA synthesis

Input and Flow Through RNA samples. DNase treatment of total RNA was carried out as described above for qRT-PCR experiment and reverse-transcribed into cDNA using reagents from Fermentas (Thermo Scientific). All the incubation steps during DNase treatment and cDNA synthesis were performed in a PCR cycler with heated lid. 15 µl reaction containing 1 µg DNase treated RNA, 50 pmol Oligo(dT) Primer, 50 pmol Random Hexamer Primer and 1 µl 10 mM dNTPs was incubated for 5 minutes at 65°C in order to denature RNA secondary structures and rapidly cooled down on ice. 4 µl of 5x RT Buffer and 0.5 µl (20 U) of RiboLock RNase inhibitor were added to the reaction. Subsequently, 12 µl of the reaction was transfered into a new tube and 0.6 µl (120 U) RevertAid Premium Reverse Transcriptase was added. Reverse transcriptase was not added to the remaining 7.5 µl of reaction in order to have a control for genomic DNA contamination. cDNA syntheses was performed for 40 minutes at 55 °C followed by heat inactivation of the enzyme for 5 minutes at 85 °C.

Bead-captured RNA samples. DNase treatment and cDNA synthesis were performed using the same reagents and incubation conditions as described for I and FT samples. 2 µl bead-captured RNA was treated with 0.2 U of DNase in a total volume of 10 µl. 5 µl of the DNase-treated RNA was used for cDNA synthesis and 5 µl for a control reaction without reverse transcriptase.

Protein analysis

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

Sample preparation. 5x Laemmli Sample Buffer (Laemmli 1970) was added to lysate samples taken from I, FT and pellet material to obtain a 1x solution and proteins were denatured by heating for 10 min at 70°C. Vacuum dried RNase eluate containing the mRNP proteins was dissolved in 10 µl Solution A and heated for 5 min at 70°C, after which 10 µl Solution B was added and heated for an additional 10 min at 70°C. After removing the RNase eluate form the IgG-coupled beads, the beads were resuspended in 40 µl Solution A and B mix (1:1, v/v) and

heated for 10 min at 70°C. The beads were captured on a magnet and the protein-containing supernatant was transferred into a new 1.5 ml tube. Beads were resuspended in an additional 40 µl Solution A and B mix, heated for 2 min at 70°C and the supernatant was pooled with the previous supernatant to obtain a 4x diluted sample as compared to the RNase eluate. Prior to loading on SDS-PAGE, the samples were spun down at 16 000 x *g* for 2 min at RT.

Protein gel electrophoresis. SDS-PAGE was performed as described in (Sambrook and Russell 2001). Standard gels were run using Mini-PROTEAN Tetra Cell electrophoresis system (BioRad) at 100 V for 20 min, followed by 150 V until the dye front had reached the lower edge of the gel. NuPAGE Novex 4-12% Bis-Tris gels (Invitrogen™ Life Technologies) were run according to manufacturer's manual.

Western blot

Protein transfer onto membrane. Towbin Transfer Buffer without methanol was used for electrophoretic protein transfer onto PVDF membrane. Semi-dry transfer was carried using Trans-Blot SD (Bio-Rad) semi-dry transfer device at 12 V (1 gel) or 18 V (2 gels) for 35 min. If efficient transfer of large proteins (>100 kDa) was desired, transfer was performed in Mini Trans-Blot (Bio-Rad) tank transfer system for 1 h at 350 mA. Two types of PVDF membranes were used depending on the nature of the signal to be detected. Proteins were transferred onto Hybond-P (Amersham GE Healthcare Life Sciences) for chemiluminescent signal detection and onto Immobilon-FL (EMD Millipore) for fluorescent signal detection following the manufacturer's guidelines for membrane handling.

Solution A
0.5 M Tris-HCl (pH 8.0)
5% SDS (w/v)
Stored at RT

Solution B
75% glycerol (v/v)
124.5 mM DTT
0.05% bromphenol blue (w/v)
Stored at -20°C

Laemmli Sample Buffer (1x)
60 mM Tris-HCl (pH 6.8)
2% SDS (w/v)
10% glycerol (v/v)
5% β-mercaptoethanol, 710 mM
0.01% bromphenol blue
Stored at -20°C

Towbin Transfer Buffer
25 mM Tris
192 mM glycine

Protein detection. Membrane blocking and antibody incubation was performed using SNAP i.d. Protein Detection System (EMD Millipore) according to the manufacturer's manual. Membrane was blocked with phosphate-buffered saline (PBS) solution containing 0.2% (w/v) non-fat dry milk and 0.1% (v/v) Tween-20. Routinely, membrane was incubated with antibody solution for 20 min. After the final washing step in SNAP i.d., the membrane was removed from the blot holder and was washed with gentle rocking for an additional 10 min in PBS-0.1% (v/v) Tween-

20. Horseradish peroxidise enzymatic activity was detected with ECL Western Blotting Substrate (Pierce Thermo Scientific) and chemiluminescent image was captured with LAS-3000 or MultiImage II (Alpha Innotech) imaging system and quantified using FluorChem FC2 image analysis software (Alpha Innotech). Fluorescent signal was detected with Odyssey Infrared imaging system (LI-COR) and quantified using Image Studio (Li-COR).

Protein visualization by staining

In order to visualize mRNA co-purifying proteins, an RNase eluate volume corresponding to ~200 mg initial grindate used for mRNP affinity purification was separated on 10% SDS-PAGE and the proteins were stained using SilverQuest Silver Staining Kit (Invitrogen™ Life Technologies) according to the Basic Staining Protocol. For protein identification by mass spectrometry, an RNase eluate volume corresponding to ~1 g of initial grindate was separated on NuPAGE Novex 4-12% Bis-Tris Gel (Invitrogen™ Life Technologies) and stained using Colloidal Blue Staining Kit (Invitrogen™ Life Technologies).

Mass spectrometry-based quantitative proteomics

MS analysis of MS2L-tagged mRNA co-purifying proteins was conducted at Proteome Center Tübingen (http://www.pct.uni-tuebingen.de/index.php?id=2) by Dr. Mirita Frantz. Briefly, SILAC-labelled RNase eluate was separated by 1D SDS-PAGE and the gel was cut into several slices, which were subjected to in-gel enzymatic digestion of proteins with LysC endoproteinase. Liquid chromatography (LC) coupled to electrospray and tandem MS (MS/MS) analysis of peptide mixture was performed on Easy-nLC (Proxeon Biosystems) nanoscale chromatography system coupled to Linear Trap Quadrupole (LTQ)-Orbitrap XL mass spectrometer (Thermo Scientific). The raw MS spectra containing peptide mass and intensity information were processed and prepared for database search using MaxQuant software (Cox and Mann 2008). MS/MS spectra were searched using the Mascot search engine against a yeast database containing common contaminants and a reversed version of all sequences.

AIMS

In order to elucidate the mechanisms of mRNA regulation it is important to know which proteins interact with the mRNA during its lifetime. Methodological advances in high-throughput methods such as quantitative mass spectrometry have allowed to compile a comprehensive list of mammalian and yeast RNA-binding proteins (Baltz et al. 2012; Castello et al. 2012; S. F. Mitchell et al. 2013). However, the analysis of mRNA-bound proteome of specific cellular mRNPs has been hampered due to the lack of a suitable method that would combine fast and easy affinity purification of specific mRNPs with efficient identification of the captured proteins. Therefore, the aims of the current study were: (1) to develop an mRNP affinity purification method that can be used to capture *in vivo*-assembled mRNPs form *S. cerevisiae*; and (2) to test the applicability of the method for mRNA-bound proteome analysis using SILAC-based quantitative proteomics for the identification of mRNP proteins. Our goal was to study mRNP composition under normal yeast growth conditions with glucose as the carbon and energy source. We wished to get an idea of the full spectrum of mRNA-protein and protein-protein interactions mRNPs are involved in during their lifetimes. Therefore, the mRNP affinity purification method was optimized to prevent mRNA degradation and to preserve ribosome-association with the mRNA. In order to test whether the established mRNP affinity purification method is suitable for the analysis of mRNA-bound proteome, proteins co-purifying with the two abundant cellular mRNAs, *PGK1* and *ENO2*, were identified. Another goal of the study was to determine which proteins have the potential to interact with the RNA-tag that was chromosomally integrated after the translation termination codon to "mark" endogenous *PGK1* and *ENO2* mRNAs for affinity purification. Therefore, the proteins co-purifying with a plasmid-encoded mRNA-like transcript containing the RNA tag sequence were analysed.

RESULTS

Single-step mRNP affinity purification strategy

We isolate endogenously assembled mRNA-protein complexes from *Saccharomyces cerevisiae* via the mRNA component of the mRNP. In order to capture the mRNA of interest the mRNA is tagged before the 3' UTR with binding sites for bacteriophage MS2 coat protein (MS2CP), which is a 421 nt long sequence containing 6 stably folding stem-loop structures (MS2L) (Haim et al. 2007). mRNPs assembled on tagged messages are captured from cell lysate by using the wt version of MS2CP (Jou et al. 1972) fused with 4 IgG-binding Z domains of *Staphylococcus aureus* protein A (MS2CP-PrAx2) (B. Nilsson et al. 1987).

Conventional yeast cell lysis methods, such as glass bead milling and French press, result in rapid RNA degradation upon cell wall disruption (López de Heredia and Jansen 2004). Our strategy for preserving mRNP integrity includes cryolysis and fast mRNP capture by magnetic separation (Fig. 5). Previously, a similar strategy was used to isolate complexes containing protein A-tagged RNA binding proteins (Oeffinger et al. 2007). We adopted this method for mRNA-based mRNP affinity purification by replacing protein elution under denaturing conditions with selective RNA-associated protein release via ribonuclease (RNase) treatment. Finally, proteins co-isolating with MS2L-tagged mRNAs are identified by mass spectrometry.

Optimization of mRNP affinity purification

mRNA integrity during mRNP affinity purification

For efficient mRNP capture only minor degradation of MS2L-taggd mRNA during mRNP affinity purification is tolerable. Therefore, we first wanted to know if RNase inhibitors are needed to keep RNA intact while performing the experiment. We tested the effect of 3 different RNase inhibitors on mRNA integrity: (1) *E. coli* tRNA (500 μg/ml) as a competitor substrate for RNases; (2) heparin (500 μg/ml) as a non-specific RNase inhibitor; and (3) recombinant RNasin (50 U/ml) as a protein that inactivates RNases via non-covalent binding. In the negative control

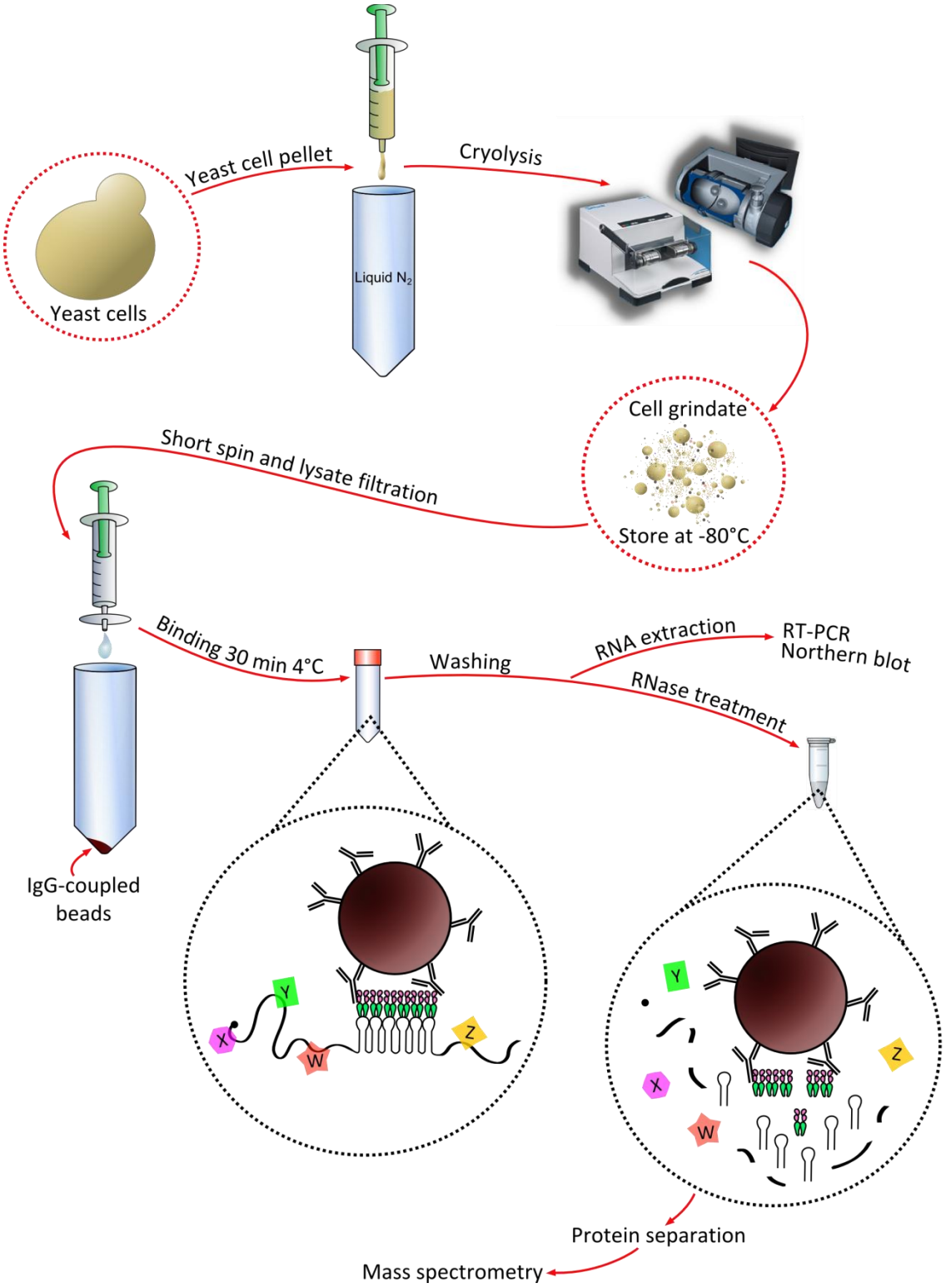


Figure 5. **Experimental workflow of mRNP affinity purification.** After harvesting, *S. cerevisiae* cells are frozen in liquid nitrogen and disrupted under cryo conditions using Retch mixer mill MM400. Cell grindates can be stored at -80°C until proceeding with mRNP isolation. Upon grindate resuspension in buffer, cell debris is spun down and the lysate is filtered. IgG-coupled superparamagnetic particles (Dynabeads) are incubated with the lysate for 30 min at 4°C, then captured by magnetic force, washed and used either for bead-captured RNA or protein extraction. RNA is isolated by Proteinase K digestion followed by PCI extraction and proteins are eluted by RNase A/T1 treatment. Isolated RNA and proteins are analysed.

RNase inhibitors were not added to the lysate. As a positive control for non-degraded RNA total RNA was extracted directly from yeast cell grindate by phenol-chlorophorm-isoamyl alcohol (PCI) extraction. The experiment was performed similarly to mRNP affinity purification except that the IgG-coupled beads were not added to the lysate. Samples for total RNA extraction were taken at different steps of the protocol, i.e. from crude lysate, from clarified lysate and from clarified lysate incubated at 4°C for 30 min and for 1 h.

We examined the stability of plasmid encoded *ASH1* (Fig. 6A) expressed under *GAL1* promoter control and genomically encoded *PGK1* (Fig. 6B). *ASH1* was chosen as a representative of an unstable transcript. The mRNA exhibits a half-life of around 3 min when expressed from the wild-type allele introduced at *ASH1* locus by gene replacement (Zheng et al. 2008). In contrast, *PGK1* is a very stable yeast transcript with a half-life of more than 60 min (Grigull et al. 2004; Y. Wang et al. 2002).

RNA samples were analysed by northern blot. Smear underneath the signal corresponding to the full-length transcript indicates mRNA degradation. *ASH1* degradation was observed already in the clarified lysate for samples not containing any RNase inhibitors (Fig. 6A, lane 7) or only tRNA (Fig. 6A, lane 8). Furthermore, rRNA was not entirely intact in these samples as shown by smearing of rRNA signal on methylene blue stained membrane. After 30 min of incubation, the full-length transcript seemed to be entirely degraded in the absence of RNase inhibitors (Fig. 6A, lane 11) and after 1 h also in the presence of tRNA (Fig. 6A, lane 16). Additionally, extensive rRNA degradation was observed for these samples. Remarkably, tRNA in combination with heparin or heparin and RNasin prevented *ASH1* degradation even after 1 h of lysate incubation (Fig. 6A, lane 17 and 18). Sharp 25S and 18S rRNA bands on methylene blue stained membrane also indicated intact rRNA.

Surprisingly, endogenous *PGK1* transcript seemed to be relatively stable throughout the whole experiment even in the absence of RNase inhibitors (Fig. 6B, lanes 2, 6, 10, 14) as no prominent smearing was observed. The lower signal intensity of the full-length mRNA in the clarified lysate that was incubated for 30 min in the absence of RNase inhibitors (Fig. 6B, lane 10) could be explained by less total RNA loaded as the signals for 25S and 18S rRNAs were also slightly weaker on methylene blue stained membrane.

We concluded that RNase inhibitors are crucial to preserve mRNA integrity if mRNP purification of overexpressed mRNAs with a short half-life, like *ASH1*, is performed. In this case a combination of tRNA and heparin should be used as tRNA alone did not allow efficient mRNA protection. Addition of RNasin is not essential; in the presence of tRNA and heparin RNasin did not seem to provide any additional mRNA stabilizing effect. However, in case of the stable endogenous *PGK1* transcript RNase inhibitors are not needed. Astonishingly, *PGK1* did

not show obvious degradation in any of the samples even after 1 h of lysate incubation (Fig. 6B lanes 14-17).

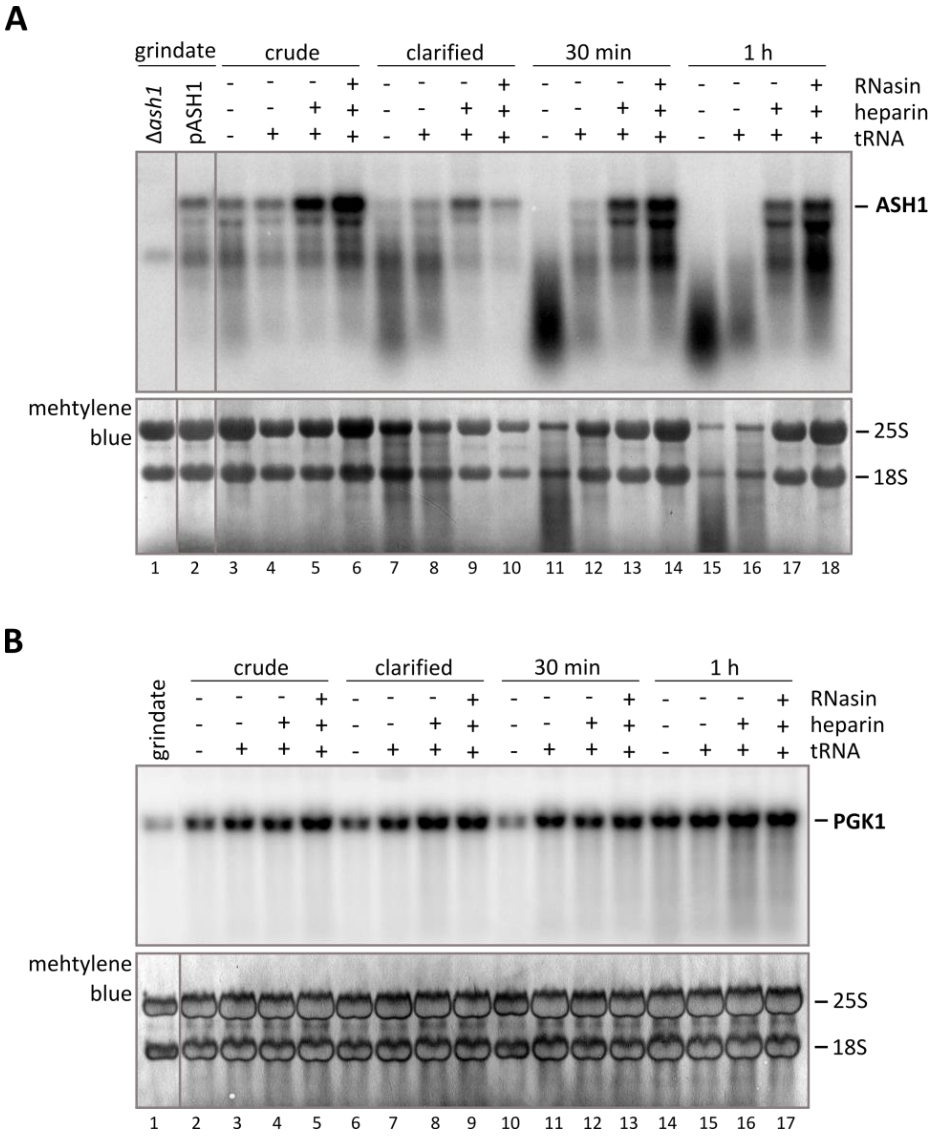


Figure 6. RNase inhibitors are needed to prevent degradation of overexpressed *ASH1* but not endogenously expressed *PGK1* during mRNP affinity purification. The effect of yeast tRNA (500 µg/ml), heparin (500 µg/ml) and recombinant RNasin ribonuclease inhibitor (50 U/ml) on mRNA stability was tested in two experiments imitating mRNP affinity purification. Yeast cell lysate was handled similarly to mRNP purification except that IgG-coupled beads were not added. Aliquots for total RNA extraction by PCI were taken after: (1) grindate resuspension in mRNP-isolation buffer (crude); (2) removal of cell debris by centrifugation at 4000 x g (clarified); (3) incubation of clarified lysate at 4°C for 30 min; (4) and for 1 h. RNA extracted by PCI directly from grindates was used as a control for intact RNA (grindate). 5 µg of total RNA was separated on 1.2% agarose-formaldehyde gels and transferred onto positively charged nylon membranes. After methylene blue staining, the membranes were hybridized with radiolabelled DNA probes against *ASH1* (A) or *PGK1* (B). The hybridization signal corresponding to the full-length transcripts and the position of 25S and 18S rRNAs on methylene blue stained membranes are indicated. (**A**) *ASH1* was expressed for 1 h from centromeric plasmid (RJP 1433) in response to 4% galactose in logarithmically growing Δ*ash1* strain (RJY 135) before cell harvesting. RNA extracted from strain RJY 135 not containing *ASH1*-encoding plasmid shows a non-specific crosshybridization band with *ASH1* probe (lane 1, Δ*ash1*). RNA extracted from grindate after 1h of galactose induction of *ASH1* (pASH1) (**B**) Δ*ash1* strain (RJY 135) expressing *PGK1* from the genomic locus.

TAP-She2p as a tool for mRNP affinity purification optimization

Starting point of mRNP affinity purification optimization – high non-specific mRNA and protein binding to IgG-coupled beads

Specificity is an important aspect of any affinity purification method. In case of mRNP pull-down the result should essentially be free of non-specific mRNAs to avoid contamination by RNA-binding proteins not belonging to the mRNP of interest. She2 is a yeast RNA-binding protein (Böhl et al. 2000) that targets several transcripts to the distal tip of the daughter cell (Long et al. 1997; Shepard et al. 2003). mRNA localization is achieved by She2 directly binding to the mRNA and linking it via the adaptor protein, She3, to the myosin Myo4 motor. Myo4 then delivers the cargo along actin cables to the bud tip (Müller et al. 2009; Müller et al. 2011). Öffinger et al. showed that affinity purification of PrA-tagged She2 resulted in co-purification of a very distinct set of proteins – She3, Myo4, Myo2 and Act1 (Oeffinger et al. 2007) – making She2 a perfect bite for accessing the specificity of an affinity tag-based RNA-protein complex purification method. Moreover, She2 affinity purification would also allow to characterize the method's specificity at the RNA level as more than 30 transcripts are known to specifically co-isolate with She2 (Shepard et al. 2003; Oeffinger et al. 2007).

In order to optimize our mRNP affinity purification protocol we first determined the level of non-specific adhesion of cellular mRNAs and proteins to the IgG-coupled beads. As an equivalent to She2-PrA used by Öffinger et al. (Oeffinger et al. 2007) we used TAP-She2. TAP is a double epitope tag (Rigaut et al. 1999) that, in our case, consists of an N-terminal PrA tag followed by the tobacco etch virus (TEV) protease cleavage site and a calmodulin binding peptide. TAP-She2 purification was performed under low stringency conditions (here and hereafter low stringency conditions refer to the use of 110 mM KAc as the only salt in RNP Buffer) in the presence of *E. coli* tRNA, heparin and RNasin. In a parallel control purification a lysate containing the untagged wt version of She2 was used. After washing, half of the beads were used to isolate bead-captured RNA by proteinase K digestion and PCI extraction, while the other half was used to elute proteins under denaturing conditions.

Western blot comparison of TAP-She2 signal in input and flow through samples using anti-She2 antibody showed that about 50% of TAP-She2 was isolated from the lysate after incubation with IgG-coupled beads (Fig. 7A, upper panel, compare lanes 2 and 4). TAP-She2 capture from lysate was confirmed by the analysis of the eluate sample with peroxidase anti-peroxidase soluble complex (PAP) antibody (Fig. 6A lower panel, lane 6). In the untagged strain She2 signal intensity in samples taken before and after lysate incubation with IgG-coupled beads remained constant (Fig. 7A, upper panel, compare lanes 1 and 3). This finding argues that She2 is

not non-specifically captured by the beads. Unfortunately, the eluate sample of mock purification could not be used to analyse the possible non-specific binding of She2 to IgG-coupled beads because the about 25 kDa rabbit IgG light chain present in the eluate cross-reacts with the primary and/or secondary antibody used for She2 detection, thereby resulting in a signal in 30-25 kDa region and masking the possible non-specifically bound She2 signal (Fig. 7A, upper panel, compare lanes 5 and 6).

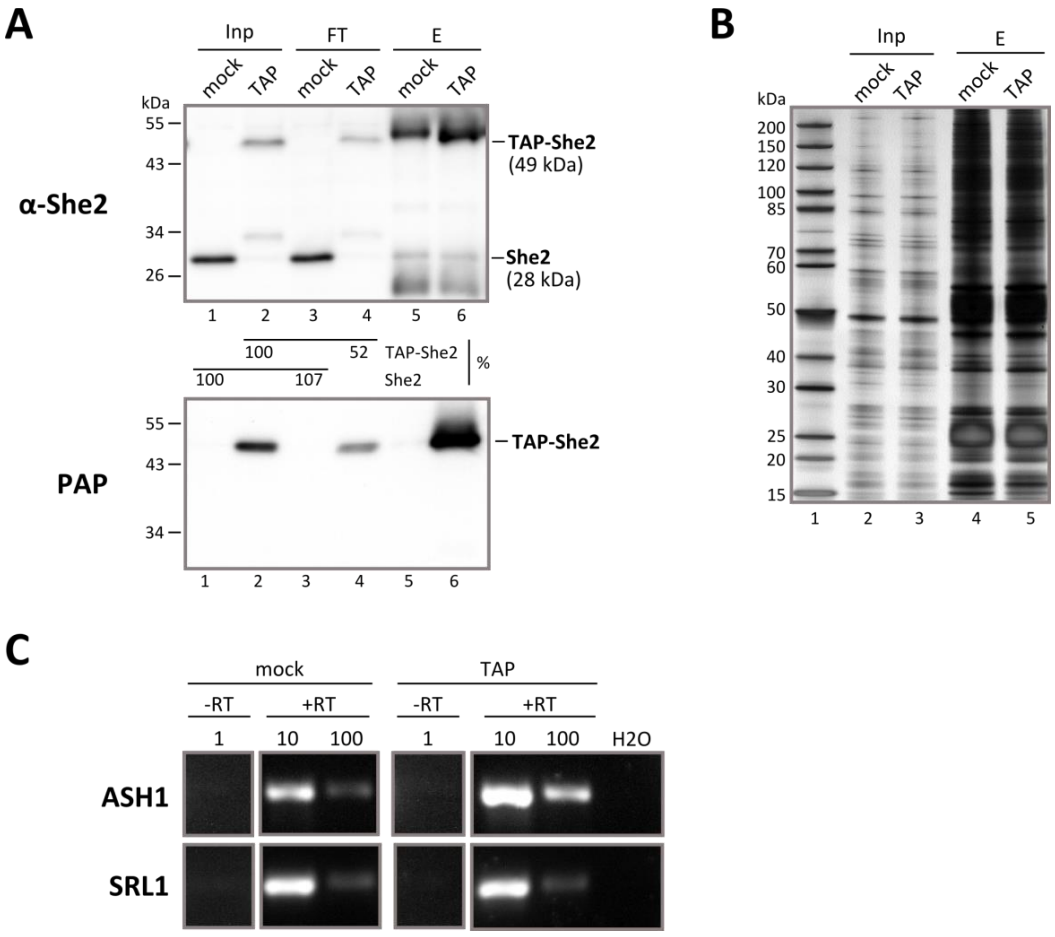


Figure 7. High non-specific RNA and protein binding to IgG-coupled beads as the starting point for mRNP affinity purification protocol optimization. Grindates of strains RJY 359 and RJY 3166 expressing the wt (mock) or TAP-tagged version of She2 (TAP), respectively, were subjected to TAP-She2 affinity purification using IgG-coupled superparamagnetic beads. mRNP capture and washing was performed in the presence of 110 mM KAc. (**A**) **Upper panel**: She2 and TAP-She2 immunodetection with α-She2 antibody 1C3-11. Quantification of western blot signal is indicated below the lanes. On lanes 2 and 4 a signal at about 34 kDa is visible. This signal may correspond to TAP-She2 proteolytic degradation product that has lost the protein A part of the tag because the signal is not detectable on the lower panel. **Lower panel**: TAP-She2 immunodetection with peroxidase anti peroxidase (PAP) soluble complex. Inp – input, lysate after removal of cell debris; FT – flow through, immunodepleted lysate; E – eluate, proteins eluted under denaturing conditions from IgG-coupled beads. Proteins were separated on 12% SDS-PAGE. (**B**) Silver staining of Inp and E samples separated on a 4-12% NuPAGE Novex BisTris gel. (**C**) RT-PCR analysis of RNA isolated from IgG-coupled beads by Proteinase K digestion and PCI extraction. TAP-She2 co-isolating mRNAs *ASH1* and *SRL1* were amplified from serially diluted cDNA. -RT, control for genomic DNA contamination (reverse transcriptase omitted); +RT, cDNA; H_2O, negative control lacking cDNA template.

The comparison of She2 signal in input and flow through samples suggested that She2 is not non-specifically captured by IgG-coupled beads (Fig. 7A, upper panel, compare lanes 1 and 3). However, silver staining of the affinity eluates revealed an identical protein pattern for both the TAP-She2 and the untagged strain (Fig. 7B, compare lanes 4 and 5), indicating high non-specific protein binding to the beads. Furthermore, RT-PCR uncovered that She2 target mRNAs *ASH1* and *SRL1* (Shepard et al. 2003; Oeffinger et al. 2007) were present at similar levels in bead-captured RNA samples of both the TAP-She2 and the untagged control strain (Fig. 7C). This was surprising as western blot analysis had suggested no non-specific She2 binding to the beads. The RT-PCR result could be explained by high levels of non-specific mRNA attachment to the beads, possibly via non-specifically bound polyribosomes. In this case the contribution of the low levels of specifically captured *ASH1* and *SRL1* to the signal intensity of semi-quantitative RT-PCR could be masked by high levels of non-specifically captured *ASH1* and *SRL1*.

Small changes can make a big difference – revised IgG coupling to Dynabeads

We assumed that the high non-specific mRNA binding to our IgG-coupled beads might arise from a small modification of the coupling protocol published by Öffinger et al. (Oeffinger et al. 2007). Namely, after coupling beads are washed extensively to remove non-covalently bound IgG. One of the steps includes washing 5 times for 5 min with PBS. During this step we added insulin (0.05% w/v) and *E. coli* tRNA (200 μg/ml) to the washing buffer to block the sites on the beads that have the potential to non-specifically interact with proteins and RNA. Analogous blocking agents are regularly used to block sepharose beads, which are incubated with low-immunogenic proteins such as BSA (C. Gilbert and Svejstrup 2012) or with tRNA (Slobodin and Gerst 2010) prior to using in immunoprecipitation experiments.

We prepared a new batch of IgG-coupled beads completely following the protocol by Öffinger et al. (Oeffinger et al. 2007). Neither insulin nor *E. coli* tRNA were used as blocking agents. In addition, a new magnetic separation rack for 15 ml tubes was used. This enabled to reduce the bead capture time after washing from about 2 min to 20 sec. Fast removal of two washing solutions, one containing 100 mM glycine and the other 100 mM triethylamine, is important according to Öffinger et al. (Oeffinger et al. 2007).

Using the new batch of IgG-coupled beads, TAP-She2 affinity purification was repeated under low stringency conditions. In parallel, we further wanted to test if the addition of tRNA, heparin and RNasin during mRNP capture was necessary for preserving mRNA integrity as had been observed earlier for overexpressed *ASH1* (Fig. 6A). Therefore, TAP-She2 affinity

purifications were performed either in the presence or in the absence of the above mentioned RNase inhibitors.

RT-PCR analysis of bead-captured RNA samples (Fig. 8A) indicated that *ASH1* was enriched to a comparable extent both in the presence and in the absence of tRNA, heparin and RNasin. The same was observed also for *SRL1*. In order to study the level of non-specific mRNA binding, PCR was performed with primers specific for *PGK1* and *SOD1*. These two mRNAs should not specifically co-isolate with TAP-She2. *PGK1* signal could be detected for undiluted cDNA sample if TAP-She2 purification had been performed in the absence of tRNA, heparin and RNasin. In contrast, *SOD1* signal was detected for undiluted and for 10-fold diluted cDNA if tRNA, heparin and RNasin had been present. Importantly, the signal of She2-target mRNAs was stronger than the signal of control mRNAs in all the tested cDNA dilution, indicating that non-specific RNA binding to the beads had not been as extensive as in the previous experiment (compare Fig. 7C and 8A). Therefore, IgG coupling to the beads was hereafter carried out using the new magnetic separation rack and excluding the blocking agents insulin and *E. coli* tRNA.

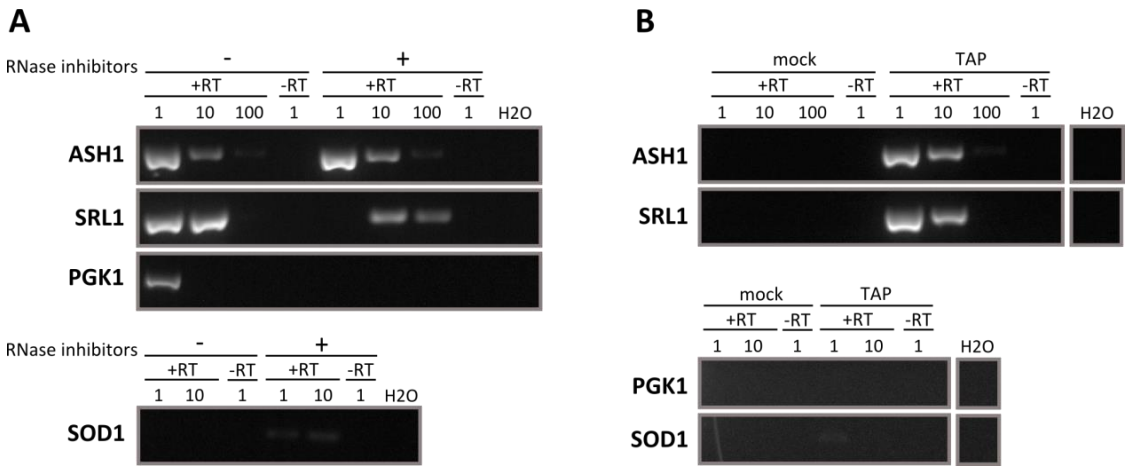


Figure 8. Optimization of mRNP affinity purification protocol to reduce non-specific RNA binding to IgG-coupled beads. (**A**) TAP-She2 co-isolating mRNAs are enriched to a similar extent after TAP-She2 affinity purification both in the absence (-) and presence (+) of RNase inhibitors. Using IgG-coupled beads, two parallel purifications from lysate containing TAP-She2 (strain RJY 3166) were carried out either in the absence of RNase inhibitors or in the presence of *E. coli* tRNA (500 µg/ml), heparin (500 µg/ml) and recombinant RNasin ribonuclease inhibitor (20 U/ml). mRNP capture and washing was performed in the presence of 110 mM KAc. Bead-captured RNA was isolated by Proteinase K digestion and PCI extraction and reverse transcribed. The levels of TAP-She2p target mRNAs *ASH1* and *SRL1* as well as negative control mRNAs *PGK1* and *SOD1* were determined by PCR from serially diluted cDNA. +RT, cDNA; -RT, control for genomic DNA contamination (reverse transcriptase omitted); H_2O, negative control lacking cDNA template. (**B**) TAP-She2 purification in the presence of 150 mM NaCl results in reduced non-specific RNA binding to IgG-coupled beads. Lysates containing untagged wt She2 (strain RJY 359, mock) or TAP-She2 (RJY 3166, TAP) were used for TAP-She2 affinity purification with IgG-coupled beads. mRNP capture and washing was performed in the presence of 150 mM NaCl and 110 mM KAc. Bead-captured RNA was isolated and subjected to RT-PCR as described in (A).

It is likely that TAP-She2-bound mRNAs are protected from RNases as *ASH1* and *SRL1* enrichment by TAP-She2 resulted in similar efficiencies independent of the presence of RNase inhibitors. Addition of tRNA, heparin and RNasin did not also seem to influence the extent of non-specific RNA capture as *PGK1* could be detected in the absence and *SOD1* in the presence of RNase inhibitors. Therefore, in the subsequent experiments tRNA, heparin and RNasin were omitted from the mRNP affinity purification.

Increased buffer stringency reduces non-specific RNA binding to IgG-coupled beads to minimum

In order to further reduce the non-specific RNA binding by IgG-coupled beads, we increased the stringency of mRNP capture conditions and performed the next TAP-She2 affinity purification in the presence of 150 mM NaCl (RNP Buffer 150, see Methods for buffer composition). Mock purification of wt untagged She2 served as the negative control.

Addition of 150 mM NaCl to TAP-She2 capture and washing steps resulted in a dramatic increase in affinity purification specificity (Fig. 8B). *ASH1* and *SRL1* could only be detected in bead-captured RNA samples of the TAP-She2 strain. Control mRNA *PGK1* could neither be detected in the eluate of the untagged nor of the TAP-She2 strain. A similar result was obtained also for *SOD1* except that a very weak signal was detected for the undiluted cDNA sample of the TAP-She2 strain.

PGK1 mRNA isolation via MS2L::MS2CP-PrA::IgG interaction

Having optimized mRNP affinity purification conditions, we wanted to test if MS2L-tagged *PGK1* could be specifically captured using our method. Lysates containing MS2CP-PrAx2 and either the wt untagged *PGK1* or the 6MS2L-tagged *PGK1* were subjected to mRNP affinity purification. Bead-captured RNA analysis by RT-PCR showed that *PGK1* was enriched only if the mRNA contained the MS2L tag (Fig. 9A, upper panel, lanes 4-6). No *PGK1* PCR product could be detected for mock purification of untagged *PGK1* (Fig. 9A, upper panel, lanes 1-2). In order to further analyse the levels of non-specific mRNA capture, RT-PCR was performed with primers for *SOD1* and *ADH1*. These mRNAs were chosen as controls because their transcript copy number is in the same range compared to *PGK1*, which on average has 177 copies per cell grown on YEPD (Miura et al. 2008). *ADH1* and *SOD1* have 306 and 93 copies per cell, respectively. PCR products could be detected for neither of the control mRNAs even after 30

PCR cycles (Fig. 9A lower panel, lanes 1-4), thus confirming low non-specific mRNA capture by IgG-coupled beads using the optimized mRNP affinity purification protocol (see Methods, "mRNP affinity purification protocol").

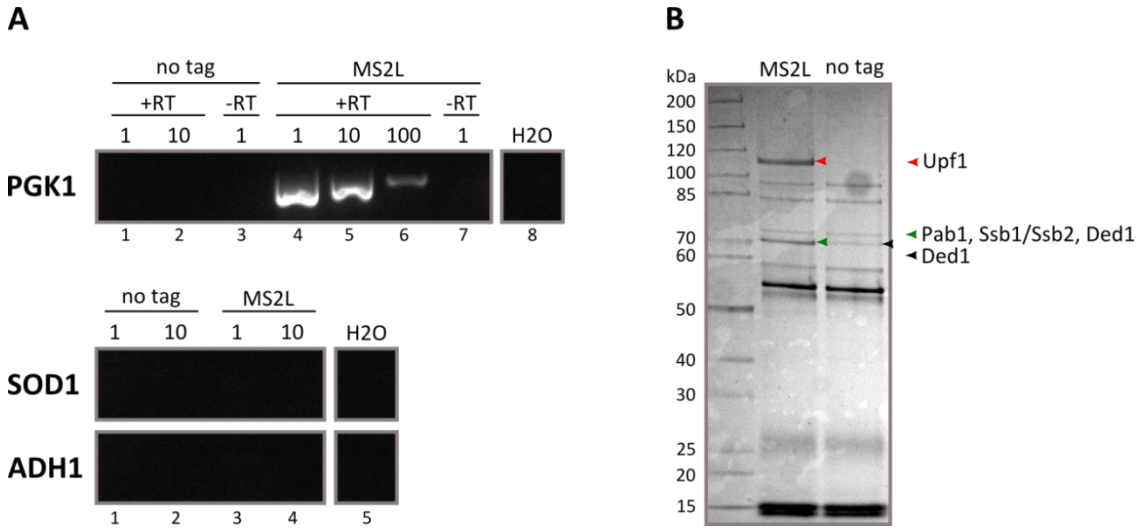


Figure 9. *PGK1-6MS2L* affinity purification – analysis of co-purifying RNAs and proteins. (**A**) *PGK1-6MS2L* is specifically enriched after mRNP affinity purification. Lysates containing MS2CP-PrAx2 and *PGK1-6MS2L* (MS2L, strain RJY 3683) or, as a control, MS2CP-PrAx2 and the untagged wt *PGK1* (no tag, strain RJY 3682) were subjected to mRNP affinity purification using IgG-coupled beads. Bead-captured RNA isolated by Proteinase K digestion and PCI extraction was used in RT-PCR analysis. *PGK1* (upper panel) and negative control mRNAs *SOD1* and *ADH1* (lower panel) were amplified from serially diluted cDNA. -RT, control for genomic DNA contamination (reverse transcriptase omitted); +RT, cDNA; H_2O, negative control lacking cDNA template. (**B**) mRNP protein elution via RNase treatment enables detection of specifically enriched proteins. Lysate containing *PGK1-6MS2L* and MS2CP-PrAx2 (strain RJY 3682) was subjected to mRNP affinity purification (MS2L). As a negative control, lysate expressing untagged wt version of *PGK1* and MS2CP-PrAx2 (strain RJY 3683) was used (no tag). RNA-associated proteins were eluted using RNase A/T1, eluates were resolved on 4-12% NuPAGE Novex BisTris gel and stained with Colloidal Blue. Two bands were specifically enriched for *PGK1-6MS2L* affinity purification. These bands, as well as the corresponding parts of the negative control lane, were analysed by mass spectrometry and the identified proteins are indicated by red (Upf1-), green (Pab1, Ssb1/Ssb2, Ded1) and black (Ded1) arrowheads.

RNase treatment of affinity-captured mRNPs enables identification of *PGK1-6MS2L* co-isolating proteins

After optimization of mRNP capture conditions our mRNP affinity purification method fulfilled two requirements for a reliable mRNP affinity purification method – specific enrichment of MS2L-tagged message and negligible background binding of non-specific mRNAs (Fig. 9A). Next, we wanted to test if *PGK1-6MS2L* purification would result in co-isolation of specifically enriched proteins. In order to release only RNA-associated proteins, we decided to use RNase A/T1 treatment for protein elution (Michlewski and Cáceres 2010).

PGK1-6MS2L affinity purification resulted in the detection of two specific bands compared to the negative control, the untagged wt *PGK1* (Fig. 9B). These bands were analysed by mass spectrometry and altogether four proteins were identified: Upf1, Pab1, Ssb1/Ssb2 and Ded1. The analysis of the corresponding parts of the negative control lane identified Ded1. Interestingly, all of the four proteins have a role in mRNA life cycle. Furthermore, three of them (Upf1, Pab1, Ded1) can directly bind RNA (Chakrabarti et al. 2011; Iost, Dreyfus, and Linder 1999; A. B. Sachs, Davis, and Kornberg 1987). This result implies that intact endogenous mRNPs can be isolated via MS2L-tagged mRNA.

Formaldehyde crosslinking and cycloheximide treatment as means of mRNP composition stabilization

Silver stained eluates from several *PGK1-6MS2L* affinity purification experiments revealed a similar protein pattern showing two specifically enriched bands in the upper molecular weight region and faint bands in the lower molecular weight region (Fig. 10A, lanes 1-2 and data not shown). Due to a weak signal it was not possible to spot clear differences in the protein patter between the MS2L-tagged *PGK1* and the untagged control below the 60 kDa marker band. Consequently, we addressed the question whether stabilization of the mRNP composition would allow us to detect more specifically enriched proteins in *PGK1-6MS2L* affinity purification. In addition, we wanted to confirm Upf1 co-isolation with *PGK1-6MS2L* by western blot analysis. For that, thee copies of c-myc epitope tag were inserted at the C-terminus of Upf1.

First, we tried out cross-linking with 0.05% formaldehyde (v/v) as described by (Slobodin and Gerst 2010) to stabilize mRNA-protein and protein-protein interactions (Fig. 10A). This resulted in the detection of more bands in the lower molecular weight region when compared to the untreated control. However, no additional specifically enriched bands for *PGK1-6MS2L* purification were observed after cross-linking. Therefore, it was decided not to use formaldehyde cross-linking in the subsequent experiments.

Western blot analysis clearly demonstrated that Upf1-3myc specifically co-purified with MS2L-tagged *PGK1* but not with the untagged control (Fig. 10C). Interestingly, a fraction of Upf1-3myc remained on the beads even after RNase treatment (Fig. 10C, lanes 4 and 8). This could be caused by incomplete RNA degradation during RNase treatment or, alternatively, by non-specific attachment of the released protein to IgG-coupled beads. The signal intensity of Upf1-3myc was comparable between the input and immunedepleted samples (Fig. 10B, uppermost panel, compare lanes 3-4 and 7-8). This is not surprising as probably only a minor

fraction of total cellular Upf1-3myc is bound to *PGK1-6MS2L*. The analysis of MS2CP-PrAx2 capture efficiency indicated that roughly 50% of the protein present in the lysate was bound to IgG-coupled beads during the 30 min of incubation time (Fig. 10B, middle panel). MS2CP-PrAx2 capture efficiency of around 50% was routinely observed if 15 mg of IgG-coupled beads per 1 g cell grindate was used (data not shown).

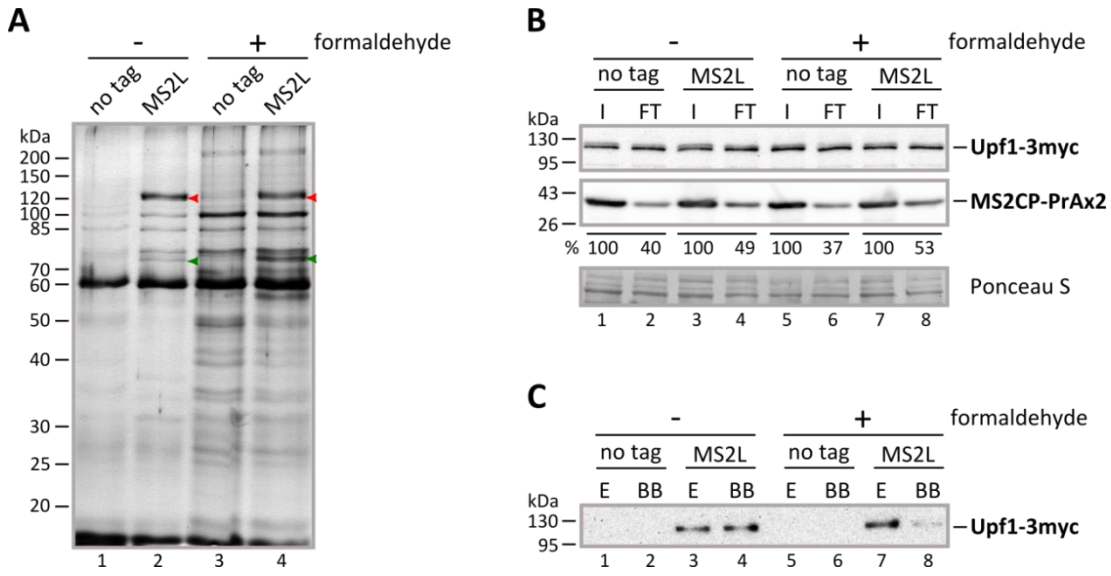


Figure 10. **Formaldehyde crosslinking does not result in the detection of additional specifically enriched proteins.** Lysates containing MS2CP-PrAx2 together with untagged wt *PGK1* (strain RJY 3740, no tag) or 6MS2L-tagged *PGK1* (strain RJY 3739, MS2L) were used for mRNP affinity purification. (**A**) Comparison of RNase eluate patterns of untreated and 0.05% formaldehyde cross-linked cells. During harvesting cells were cross-linked with 0.05% formaldehyde (v/v) for 10 min (+) or this step was omitted (-). After mRNP affinity purification RNA-associated proteins were released by RNase A/T1 treatment. Eluate volume corresponding to 185 mg initial grindate used for the experiment was loaded on each lane, separated on 10% SDS-PAGE and silver stained. Red arrowheads indicate the bands previously identified as Upf1, and green arrowheads as Pab1, Ssb1/Ssb2, Ded1. (**B**) Western blot analysis of Upf1-3myc levels with anti-myc antibody 9E10 (upper panel) and MS2CP-PrAx2 with PAP (lower panel). Quantification of MS2CP-PrAx2 signal is indicated below the lanes (100 = control band). I – input, lysate after removal of cell debris; FT – flow through, immunodepleted lysate. 5 µl of sample was loaded on each lane and separated on 10% gel. Equal loading was verified by Ponceau S staining of the membrane. (**C**) Western blot analysis of Upf1-3myc co-isolation with *PGK1-6MS2L* using anti-myc antibody 9E10. E – eluate, RNA-associated proteins eluted by RNase A/T1 treatment; BB – boiled beads, in order to remove proteins bound to beads after RNase treatment, beads were boiled in SDS-sample buffer.

Next, we used the protein synthesis inhibitor cycloheximide (CHX) to stabilize mRNPs that are associated with or part of translating ribosomes. CHX blocks the translocation step of elongation (Tatyana V. Pestova and Hellen 2003; Obrig et al. 1971; Schneider-Poetsch et al. 2010) and thereby stalls ribosomes on the transcript (Fig. 11). We reasoned that performing cell harvesting and mRNP capture in the presence of CHX (0.1 mg/ml) should result in more ribosomes co-isolating with *PGK1-6MS2L*. Most yeast ribosomal proteins have a size of 10-30 kDa (Michel, Traut, and Lee 1983). Upon CHX treatment it was therefore expected to find more proteins in that molecular weight range for *PGK1-6MS2L* affinity purification. In addition, if

translation is stalled during the pioneer round, CHX treatment can also prevent ribosomes from removing other mRNA-binding proteins from the transcript, possibly leading to the detection of additional specifically enriched bands (Dostie and Dreyfuss 2002; Lejeune et al. 2002; Sato and Maquat 2009).

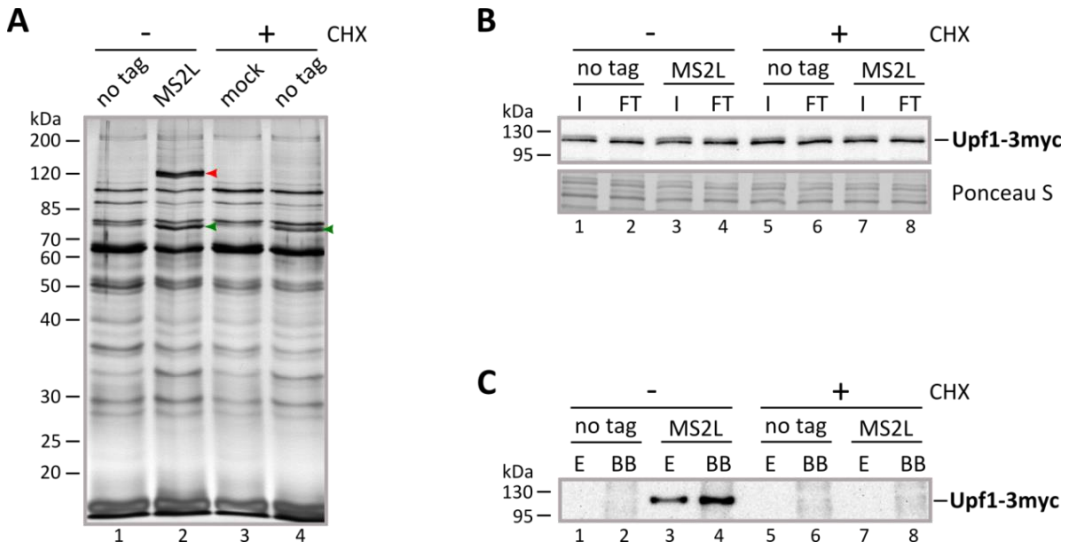


Figure 11. **Cycloheximide (CHX) treatment does not result in the detection of additional specifically enriched proteins.** Lysates containing MS2CP-PrAx2 together with untagged wt *PGK1* (strain RJY 3740, no tag) or 6MS2L-tagged *PGK1* (strain RJY 3739, MS2L) were used for mRNP affinity purification. (**A**) Comparison of RNase eluate patterns of untreated and 0.1 mg/ml CHX treated cells. In case of CHX treatment (+) cell harvesting and mRNP purification was carried out in the presence of 0.1 mg/ml CHX. For control cells CHX was omitted (-). Eluate volume corresponding to 360 mg initial grindate used for the experiment was loaded on each lane, separated on 10% SDS-PAGE and silver stained. Red arrowhead indicates the band previously identified as Upf1, and green arrowheads as Pab1, Ssb1/Ssb2, Ded1. (**B**) Western blot analysis of Upf1-3myc levels with α-myc antibody 9E10. I – input, lysate after removal of cell debris; FT – flow through, immunodepleted lysate. 5 µl of sample was loaded on each lane and separated on 10% gel. Equal loading was verified by Ponceau S staining of the membrane. (**C**) Western blot analysis of Upf1-3myc co-isolation with *PGK1-6MS2L* using α-myc antibody 9E10. E – eluate, RNA-associated proteins eluted by RNase A/T1 treatment; BB – boiled beads, in order to remove proteins still bound to beads after RNase treatment, beads were boiled in SDS-sample buffer.

Surprisingly, in this experiment we could detect distinct protein bands in the lower molecular weight region also for the control lysates, which were not treated with CHX (Fig. 11A, lanes 1-2). However, the protein pattern below 60 kDa did not significantly differ between the untagged control strain and *PGK1-6MS2L* strain. In fact, the pattern in that region was very similar among all four samples. The only clearly detectable difference between CHX-treated and -untreated lysates was that the band corresponding to Upf1 had disappeared upon CHX treatment (Fig. 11A, compare lanes 2 and 4). This could be confirmed also by western blot analysis (Fig. 11C, compare lanes 3-4 and 7-8). The total Upf1 level in the lysates remained constant upon CHX treatment (Fig. 11B). Altogether, CHX treatment did not lead to improved results and was therefore not used in the subsequent experiments.

mRNP affinity purification recapitulated

Before applying our affinity purification method to analyse the protein composition of *PGK1-6MS2L* containing mRNPs we further wanted to characterize the performance of the method. The individual aspects of the method's performance not yet studied included: (1) integrity of bead-captured *PGK1-6MS2L* mRNA; (2) precise enrichment levels of *PGK1-6MS2L* compared to control purification; (3) efficiency of *PGK1-6MS2L* affinity purification; (4) the levels of non-specific ribosome binding to IgG-coupled beads compared to specific ribosome enrichment via co-isolation with *PGK1-6MS2L*; and (5) the influence of cell harvesting method on *PGK1-6MS2L* affinity purification.

We addressed all these questions in one experiment. Starting from question no. 5, we prepared grindates for mRNP affinity purification following two different cell harvesting protocols. Previously we had been performing harvesting according to the protocol by Öffinger et al. (Oeffinger et al. 2007), where cells are collected and washed at 4°C in the absence on amino acids and glucose. Due to many washing and centrifugations steps it takes about 1.5 h before yeast cells are frozen in liquid N_2. This lengthy procedure is likely to alter the translational profile of the cells. Ashe et al. have shown that in yeast after 2.5 min of glucose deprivation actively translating polyribosomes almost entirely redistributed into 80S monosomes (Ashe, De Long, and Sachs 2000). However, polyribosomes could be preserved if harvesting was carried out in the presence of glucose and amino acids (Inada et al. 2002; Ashe, De Long, and Sachs 2000). Our goal was to study mRNP composition under physiological conditions. Therefore, we tested a new protocol that allowed the completion of the whole harvesting process in only 20 min. Cells were collected by vacuum filtration and washed once. This washing step was performed in the presence of glucose and amino acids at room temperature as described by Inada et al. (Inada et al. 2002). Grindates prepared from cells harvested according to the above-mentioned protocols we subjected to 4 parallel mRNP affinity purifications. In order to answer the questions about *PGK1-6MS2L* integrity, enrichment and capture-efficiency, as well as ribosomal RNA levels, lysate samples for total RNA extraction before and after mRNP isolation were collected and bead-captured RNA was extracted.

Affinity purified *PGK1-6MS2L* integrity and enrichment level

Northern blot analysis using a probe complementary to a 154 nt long sequence in the second half of *PGK1* ORF (Fig. 12A) indicated specific capture of the ~2 kb long *PGK1-6MS2L* full-length mRNA (Fig. 12B, uppermost panel). Mock purifications with lysates containing untagged wt *PGK1* mRNA (~1.6 kb) did not result in any detectable hybridization signal (Fig. 12B, uppermost

panel, lanes 9-11 and 15-17). In contrast, *PGK1-6MS2L* could be detected in up to 100-fold diluted bead-captured RNA samples (Fig. 12B, upper panel, lane 14 and 20). No extensive smear beneath the signal corresponding to the full-length transcript was observed suggesting affinity purification of mostly intact *PGK1-6MS2L*. However, hybridization with a probe complementary to 120 nt in the MS2 stem-loop region (Fig. 12A) revealed the presence of a large fraction of 5'→3' shortened *PGK1-6MS2L* RNA species (Fig. 12B, lower panel). Interestingly, the intensity of the smear appeared higher if cells had been harvested according to Öffinger et al. (Oeffinger et al. 2007) (Fig 12B, lower panel, compare lanes 13 and 19). Full-length *PGK1-6MS2L* quantification indicated that about 1.3-times more of the tagged transcript was captured if cells had been harvested according to Inada et al. (Inada et al. 2002).

Interestingly, the level of total *PGK1-6MS2L* was considerably lower than that of *PGK1* (Fig. 12B, upper panel, compare lanes 1 and 3, 5 and 7). This does not seem to influence cell fitness, as the tagged and untagged strains have similar growth rates (data not shown). Minor mRNA degradation was observed for both *PGK1* and *PGK1-6MS2L* in the input samples. The degradation levels did not seem to increase during the 30 min of mRNP capture as the intensity of the smear underneath the full-length transcript remained about equal between input and flow through samples. This observation is in good agreement with the previous result showing that endogenous *PGK1* is stable even in the absence of RNase inhibitors (Fig. 12B, lower panel).

By the time of performing the described experiment quantitative real-time reverse transcription PCR (qRT-PCR) analysis had become available in our laboratory. This enabled us to determine the precise *PGK1-6MS2L* level after affinity purification (Fig. 13A). Relative enrichment analysis (see Methods, "Relative quantification of qRT-PCR results") indicated more than 1000-fold enrichment of *PGK1-6MS2L* compared to *PGK1*. Slightly more *PGK1-6MS2L* was affinity-captured if cells had been harvested according to Inada et al. (Inada et al. 2002). The 1.52-fold difference between the two harvesting methods correlates well with northern blot quantification results (Fig. 12B, compare lanes 13 and 19).

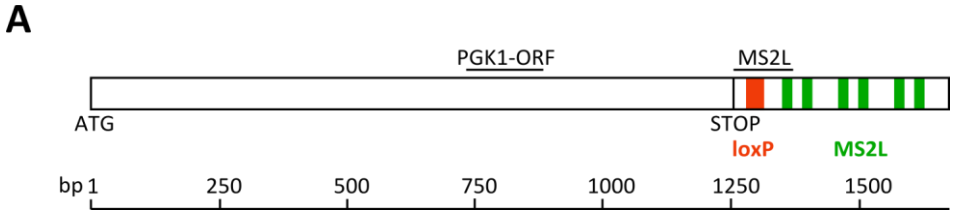
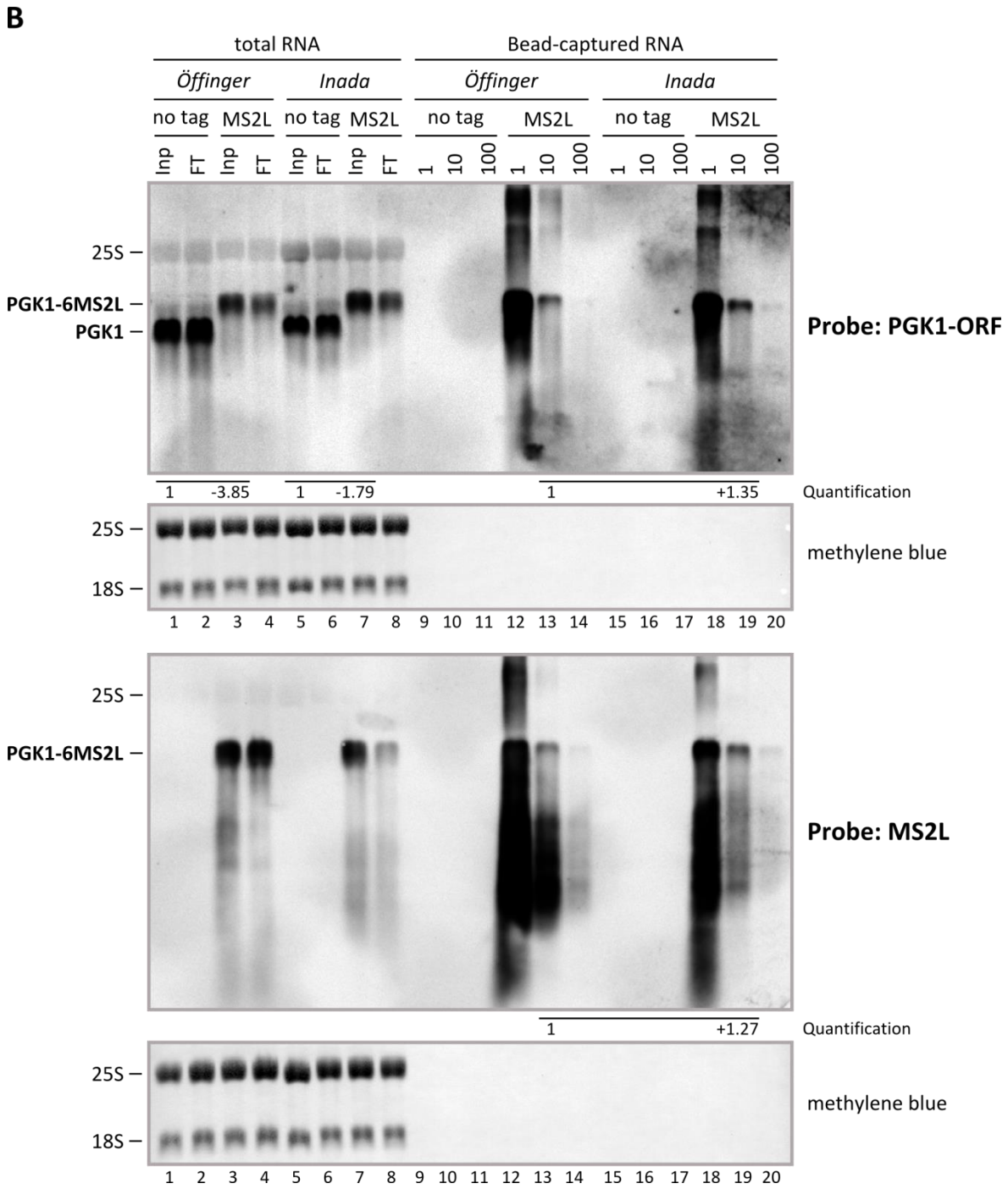


Figure 12. (A) In scale diagram of *PGK1* ORF and 6MS2L region. Annealing sites for hybridization probes (PGK1-ORF and MS2L) are indicated as well as start codon (ATG), stop codon (STOP), loxP site (orange bar) and six MS2-loops (green bars). Figure 12B, see next page.

Figure 12. (B) Northern blot analysis of affinity captured *PGK1-6MS2L* integrity (see next page). Yeast cells containing MS2CP-PrAx2 together with *PGK1-6MS2L* (MS2L, strain RJY 3827) or *PGK1* (no tag, strain RJY 3828) were harvested following two different protocols. The first protocol (*Öffinger*) involves harvesting at 4°C in the absence of glucose and amino acids, whereas in the second protocol (*Inada*) cells are collected at growth temperature in the presence of the above mentioned nutrients. Samples for total RNA analysis were taken before (I – input) and after (FT – flow through) *PGK1* mRNP isolation. Bead-captured RNA was isolated form IgG-coupled beads by Proteinase K and PCI treatment. 1.5 µg total RNA as well as 1/6th (1), 1/60th (10) and 1/600th (100) of bead-capture RNA were separated on 1.3% agarose-formaldehyde gels and blotted onto positively charged nylon membranes. After methylene blue staining, the membranes were hybridized with DIG-UTP-labelled antisense RNA probes complementary to *PGK1* ORF (upper panel) or beginning of 6MS2L-tag (MS2L, lower panel). The hybridization signal corresponding to the full-length transcript, as well as non-specific cross-reaction with 25S rRNA, is indicated. On a methylene blue stained membrane, 25S and 18S rRNAs are marked. Quantification of the selected hybridization signals is indicated below the lanes (1 = control band).



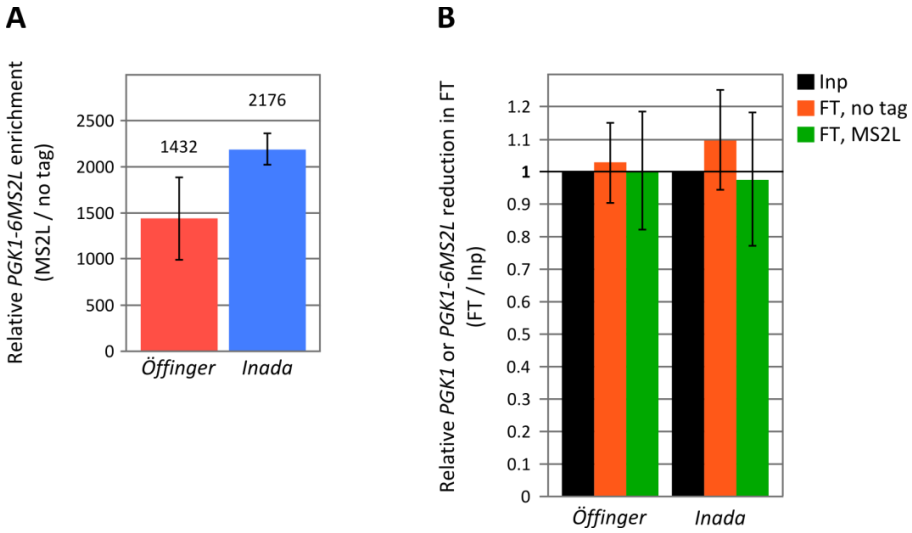


Figure 13. Relative enrichment and capture efficiency of *PGK1-6MS2L* after *PGK1*-mRNP affinity purification. Yeast cells expressing MS2CP-PrAx2 together with wt untagged *PGK1* (no tag, strain RJY 3828) or 6MS2L-tagged *PGK1* (MS2L, strain RJY 3827) were collected for mRNP affinity purification following 2 different harvesting protocols (*Öffinger*, *Inada*). (**A**) mRNP affinity purification results in more than 1000-fold enrichment of *PGK1-6MS2L* compared to *PGK1*. Affinity-purified RNA was isolated form IgG-coupled beads by Proteinase K digestion and PCI extraction and subjected to qRT-PCR analysis. The level of affinity-captured *PGK1-6MS2L* or *PGK1* was normalized to the level of the corresponding transcript in input RNA sample. The same normalization was applied to control mRNAs *TPI1*, *ACT1* and *ENO2*. Data is presented as the arithmetic mean (indicated above the bars) of three relative enrichment values (corresponding to 3 control mRNAs) ± standard deviation, n=1. (**B**) Only negligible amount of total *PGK1-6MS2L* is affinity captured. qRT-PCR analysis of *PGK1* and *PGK1-6MS2L* levels before (Inp – input, lysate after removal of cell debris) and after (FT – flow through, immunodepleted lysate) mRNP isolation. *PGK1* and *PGK1-6MS2L* levels in Inp and FT were normalized to *TPI1*, *ACT1* and *ENO2*. The normalized *PGK1* and *PGK1-6MS2L* levels in Inp (value 1) were compared to the levels in FT. Data is presented as the arithmetic mean of three relative *PGK1* or *PGK1-6MS2L* levels in FT (corresponding to 3 control mRNAs) ± standard deviation, n=1.

In order to get an idea about the capture-efficiency of the tagged *PGK1*, its level in the lysate before and after incubation with IgG-coupled beads was determined by qRT-PCR (Fig. 13B). The same analysis was performed for untagged *PGK1* where a reduction in the level of *PGK1* after mRNP capture step should reflect the fraction of non-specifically bound and/or degraded *PGK1*. Compared to the input, the untagged *PGK1* level did not significantly change after mRNP capture. Unexpectedly, the same was observed also for *PGK1-6MS2L*. Northern blot analysis had suggested that a considerable fraction of cellular *PGK1-6MS2L* had been captured on IgG-coupled beads as a reduction in the hybridization signal intensity could be observed by visual comparison of input and flow through samples (Fig. 12B, upper panel, compare lanes 3 and 4, 7 and 8; lower panel, compare lanes 7 and 8). However, after repeating the northern blot several times a similar signal reduction in flow through sample was observed only for some of the experiments, whereas in other experiments the signal intensity of *PGK1-6MS2L* appeared equal for input and flow through samples (Fig. 12B, lower panel, lanes 3-4 and data not shown). The inconsistencies in northern blot results, as well as the large variability of qRT-PCR data, make it impossible to precisely assess the mRNP capture efficiency. However,

these data suggest that only a minor fraction of total cellular *PGK1-6MS2L* is captured onto IgG-coupled beads.

The level of total cellular *PGK1* or *PGK1-MS2L* mRNA and the corresponding protein

Northern blot quantification indicated a 3.85- and 1.79-fold reduction in total *PGK1-6MS2L* level compared to *PGK1* for cells harvested according to Öffinger et al. (Oeffinger et al. 2007) and Inada et al. (Inada et al. 2002), respectively (Fig. 12B, upper panel). qRT-PCR confirmed reduced total *PGK1-6MS2L* levels. However, compared to *PGK1*, the reduction was about 2-fold for both of the cell harvesting protocols (Fig. 14A). Interestingly, the analysis of Pgk1 protein level showed no difference between the untagged control and *PGK1-6MS2L* strain (Fig. 14B). Given that *PGK1* is an essential gene, this finding can explain the similar growth rates of *PGK1* and *PGK1-6MS2L* strains (data not shown). As expected, the harvesting method did not have any significant effect neither on the total level of *PGK1* nor *PGK1-6MS2L* (Fig. 14C).

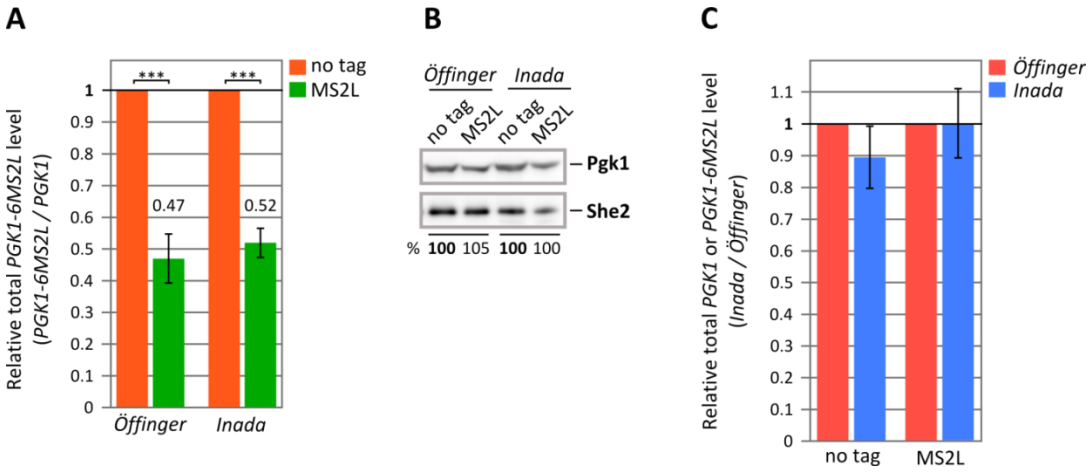


Figure 14. **Steady-state level of *PGK1* or *PGK1-6MS2L* mRNA and the corresponding protein.** Yeast cells were collected for mRNP affinity purification following 2 different harvesting protocols (*Öffinger*, *Inada*). Total RNA or protein was extracted from cell lysate of strain RJY 3828, containing untagged *PGK1* (no tag), or RJY 3827 containing *PGK1-6MS2L* (MS2L). (**A**) Total *PGK1-6MS2L* level is significantly reduced compared to *PGK1*. qRT-PCR analysis of relative steady-state *PGK1-6MS2L* level compared to *PGK1*. Untagged or tagged *PGK1* level in bead-captured RNA sample was normalized to input level of these transcripts. The same normalization was applied to control mRNAs *TPI1*, *ACT1*, and *ENO2*. Data are presented as the arithmetic mean (indicated above the bars) of three relative enrichment values (corresponding to 3 control mRNAs) ± standard deviation, n=1. ***, $P < 0.001$. (**B**) Pgk1 protein level is not reduced in strain expressing 6MS2L-tagged *PGK1* compared to untagged *PGK1*. Western blot analysis of total cell lysates with anti-Pgk1 and anti-She2 antibodies. Pgk1 signal was normalized to She2. Quantification of normalized signal is indicated below the lanes (control band in bold). (**C**) *PGK1* or *PGK1-6MS2L* level is not significantly influenced by the cell harvesting method. qRT-PCR analysis of relative *PGK1* expression level in cells harvested according to Öffinger et al. compared to Inada et al. Untagged or 6MS2L-tagged *PGK1* level in bead-captured RNA sample was normalized to input level of these transcripts. The same normalization was applied to control mRNAs *TPI1*, *ACT1*, and *ENO2*. Data are presented as the arithmetic mean of three relative enrichment values (corresponding to 3 control mRNAs) ± standard deviation, n=1.

Ribosomal RNA detection in bead-captured RNA samples

Since mRNA translation is part of the mRNA life cycle, mRNA-based mRNP affinity purification should result in co-isolation of ribosomal RNA and proteins. However, ribosomal proteins also belong to common contaminants of affinity purifications (Trinkle-Mulcahy et al. 2008), suggesting non-specific ribosome binding to various types of affinity matrices. Therefore, we were interested to compare the level of ribosomes specifically co-isolating with *PGK1-6MS2L* to the level of ribosomes captured due to non-specific attachment to IgG-coupled beads. As polyribosomes can easily be lost while cells are collected (Ashe, De Long, and Sachs 2000) we also wanted to compare harvesting methods from Öffinger et al. (Oeffinger et al. 2007) and Inada et al. (Inada et al. 2002) for the levels of rRNA co-isolation with *PGK1-6MS2L*.

Assuming that a higher enrichment level of rRNA after *PGK1-6MS2L* affinity purification compared to mock purification of *PGK1* is an indicative of active translation, we first performed northern blot analysis with hybridization probes complementary to 25S and 18S rRNA (Fig. 15A). rRNA could easily be detected in bead-captured RNA samples both for *PGK1-6MS2L* affinity purification and for mock purification of untagged *PGK1*. However, independent of the used harvesting protocol, hybridization signal quantification indicated that rRNA levels were lower for mock purification (Fig 15A, compare lanes 10 and 13, 16 and 19), suggesting specific ribosome co-isolation with *PGK1-6MS2L*. Comparison of the cell harvesting protocols by Öffinger et al. (Oeffinger et al. 2007) and Inada et al. (Inada et al. 2002) showed that the enrichment level of both 25S and 18S rRNA was higher after *PGK1-6MS2L* affinity purification if the latter harvesting protocol was used (Fig. 15A, compare lanes 13 and 19). Remarkably, in the mock purification the level of captured rRNA was comparable between the two cell harvesting methods (Fig. 15A, compare lanes 10 and 16). This result indicate that the non-specific binding of ribosomes to the beads is, in contrast to specific co-isolation with *PGK1-6MS2L*, not influenced by the cell harvesting method. Not surprisingly, a higher level of ribosome co-isolation with *PGK1-6MS2L* was observed for cells harvested in the presence of glucose and amino acids.

Next, in order to more precisely determine the levels of rRNA, we performed qRT-PCR on the same bead-captured RNA samples as used for northern blot analysis (Fig. 15B). Even though the absolute enrichment values determined by northern blot quantification and qRT-PCR analysis differed, the same trend was observed for both methods. Compared to mock purification, rRNA was significantly enriched for *PGK1-6MS2L* affinity purification for cells harvested according to Inada et al. (Inada et al. 2002). A 2.36- and 3.73-fold increase for 25S and 18S rRNA, respectively, was determined. Surprisingly, the same comparison for cells harvested according to Öffinger et al. (Oeffinger et al. 2007) showed almost identical 25S rRNA levels for *PGK1-6MS2L* and mock purification and a nonsignificant increase of 1.79-fold for 18S rRNA.

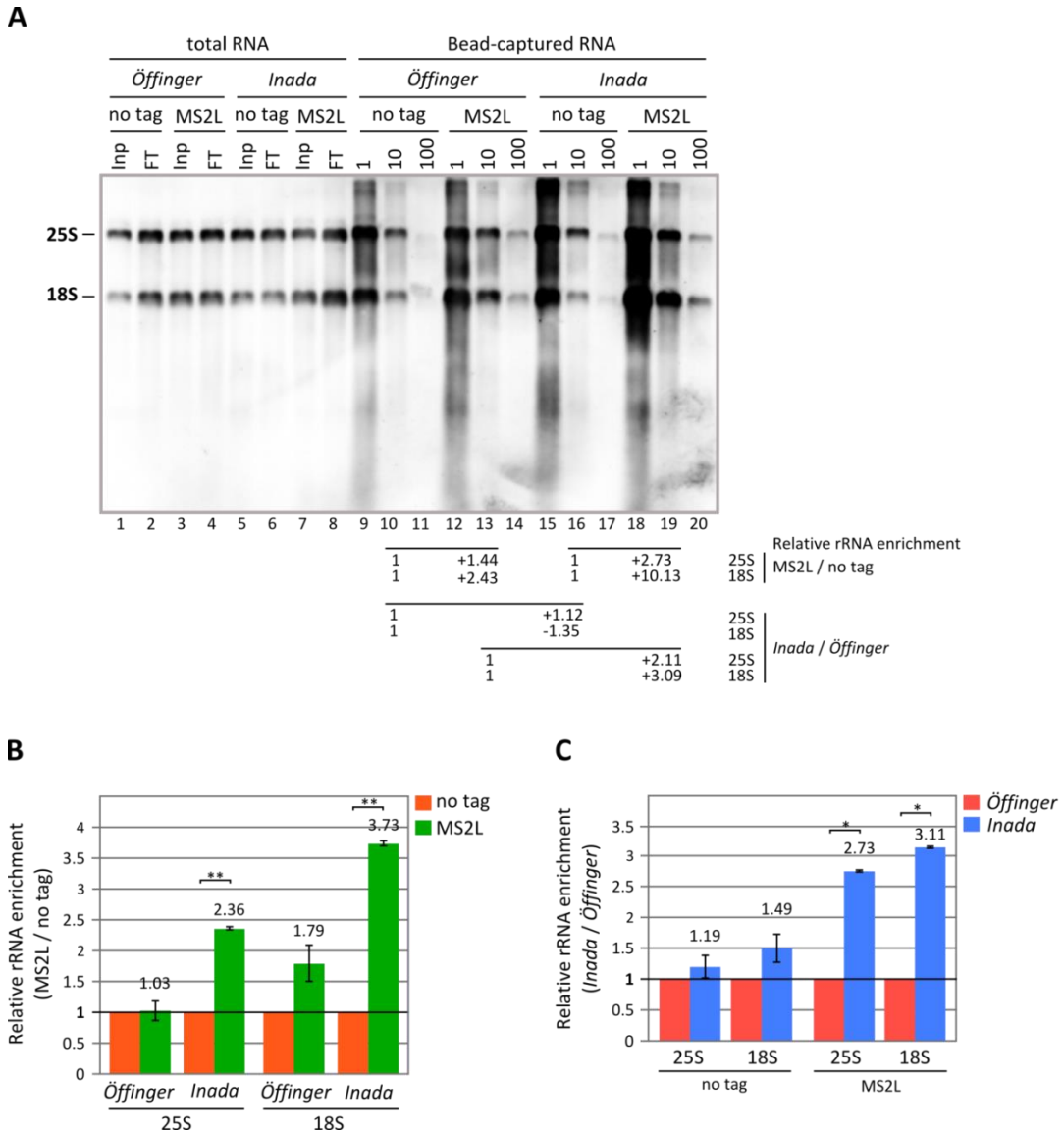


Figure 15. The level of ribosome co-isolation with *PGK1* and *PGK1-6MS2L*. Yeast strain expressing MS2CP-PrAx2 and wt untagged *PGK1* (no tag, strain RJY 3828) or 6MS2L-tagged *PGK1* (MS2L, strain RJY 3827) were collected for mRNP affinity purification following 2 different harvesting protocols (*Öffinger*, *Inada*). Total RNA from lysate samples taken before (Inp – input, lysate after removal of cell debris) and after (FT – flow through, immunodepleted lysate) mRNP affinity purification was extracted by PCI. Affinity-purified RNA was isolated from IgG-coupled beads by Proteinase K digestion and PCI extraction. (**A**) *PGK1-6MS2L* affinity purification as well as mock purification of *PGK1* results in ribosome capture. Northern blot analysis of 25S and 18S rRNA in total RNA and bead-captured RNA samples. 1 ng total RNA as well as 1/6th (1), 1/60th (10) and 1/600th (100) of bead-capture RNA was separated on 1.3% agarose-formaldehyde gel and blotted onto positively charged nylon membrane. 25S and 18S rRNA was detected using DIG-UTP-labelled antisense RNA probes. Hybridization signal quantification of 10x diluted samples is shown below the lanes (1 = control band). (**B**) Cell harvesting according to Inada et al. results in significant rRNA enrichment upon *PGK1-6MS2L* affinity purification. qRT-PCR comparison of strains containing *PGK1-6MS2L* or *PGK1* for rRNA enrichment in bead-captured RNA samples. rRNA levels in bead-captured RNA samples were normalized to input levels of rRNA. The same normalization was applied to control mRNAs *TPI1*, *ACT1*, and *ENO2*. Data is presented as the arithmetic mean (indicated above the bars) of three relative enrichment values (corresponding to 3 control mRNAs) ± standard deviation, n=1. **, P < 0.01. (**C**) Cell harvesting method does not influence non-specific ribosome attachment to IgG-coupled beads. However, it significantly influences the level of ribosome co-isolation with *PGK1-6MS2L*. qRT-PCR comparison of two cell harvesting methods for rRNA levels in bead-captured RNA samples. Data are presented as the arithmetic mean (indicated above the bars) of three relative enrichment values (corresponding to control mRNAs *TPI1*, *ACT1*, and *ENO2*) ± standard deviation, n=1. *, P < 0.05.

Similarly to northern blot analysis, qRT-PCR results suggest specific ribosome co-isolation with *PGK1-6MS2L* for cells harvested according to Inada et al. (Inada et al. 2002). However, in contrast to northern blot, qRT-PCR indicated no significant rRNA enrichment for cells harvested according to Öffinger et al. (Oeffinger et al. 2007). It should be noted that the northern blot signal of 25S and 18S rRNA could not be normalized against a reference gene, whereas qRT-PCR results were normalized against three reference genes and should therefore represent a more reliable quantification.

The same qRT-PCR dataset was used for a second comparison to determine if the cell harvesting method influenced bead-captured rRNA levels (Fig. 15C). As already observed by northern blot analysis (Fig. 15A, compare lanes 9-11 and 15-17), the cell harvesting method did not significantly influence the background binding of rRNA to IgG-coupled beads. Specifically, *PGK1* mock purification from the cells harvested according to Inada et al. (Inada et al. 2002) resulted in a non-significant increase of 1.19- and 1.49-fold for 25S and 18S rRNA, respectively, as compared to cells harvested according to Öffinger et al. (Oeffinger et al. 2007). In contrast, when MS2L-tagged *PGK1* was affinity purified, the harvesting protocol by Inada et al. (Inada et al. 2002) enabled to capture 2.73- and 3.11-times more 25S and 18S rRNA, respectively, as compared to the harvesting protocol by Öffinger et al. (Oeffinger et al. 2007). qRT-PCR thus clearly demonstrated that ribosomes co-isolation with *PGK1-6MS2L* was more efficient if cells were harvested in the presence of nutrients. Consequently, in the following experiments the harvesting method by Inada et al. (Inada et al. 2002) was used.

TEV protease cleavage as a possible alternative to RNase treatment for mRNP protein release

Protein elution via RNase treatment does not only release mRNA-associated proteins, but also ribosomal and ribosome-associated proteins via the disintegration of rRNA. A high level of non-specifically bound ribosomes can therefore pose a problem for mRNP protein composition analysis by mass spectrometry because it would reduce the signal to noise ratio, i.e. low abundance proteins specifically co-isolating with *PGK1-6MS2L* could be detected as false-negative due to the signal overlap with background proteins. We reasoned that proteolytic cleavage could confer specific mRNP protein release yet prevent the elution of proteins associated with non-specifically attached ribosomes because the rRNA would remain intact during proteolytic cleavage. Therefore, a tobacco etch virus (TEV) protease cleavage site was

inserted between MS2CP and PrAx2. Upon treatment with TEV protease MS2CP should be released from affinity matrix together with the bound mRNP (Fig. 16).

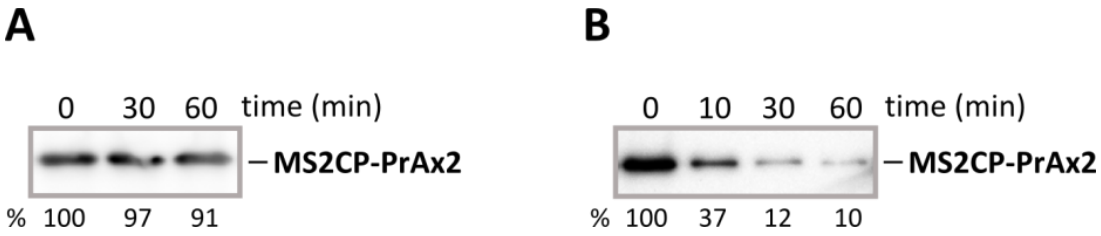


Figure 16. TEV protease cleavage as an alternative to RNase treatment for releasing affinity purified mRNA-associated proteins. Yeast lysates containing MS2CP-PrAx2 and an MS2L-tagged transcript were subjected to mRNP affinity purification using IgG-coupled magnetic beads. (**A**) mRNP release from IgG-coupled beads by TEV cleavage is inefficient in the presence of bead-captured RNA. After mRNP capture IgG-coupled beads were incubated in the presence of 0.2 U/µl TEV protease (Roboklon). An aliquot of beads was taken 0, 30 and 60 min after incubation. Bead-bound proteins were released by boiling in SDS-sample buffer and separated on 12% SDS-PAGE. MS2CP-PrAx2 was immunodetected with PAP. Signal quantification is indicated below the lanes (100 = control band). (**B**) MS2CP-PrAx2 cleavage by TEV protease is efficient after RNase treatment. Following mRNP capture IgG-coupled beads were incubated in the presence of RNase for 30 min and then TEV protease was added to a final concentration of 0.2 U/µl. Bead aliquots were taken after 0, 10, 30 and 60 min of TEV protease cleavage. Samples were used for MS2CP-PrAx2 immunodetection as described under (A).

In the first TEV protease cleavage time course experiment the protease was added to the IgG-coupled beads after mRNP capture to a final concentration of 0.2 U/µl. Western blot analysis of cleavage efficiency indicated that at least 90% of bead-bound MS2CP-PrAx2 remained uncleaved even after 60 min of incubation (Fig. 16A). Repeating the experiment using a higher final TEV protease concentration (0.5 U/µl) did not result in more efficient cleavage (data not shown). We assumed that the low proteolytic cleavage efficiency could be possibly due to the masking the TEV protease cleavage site. To further investigate this possibility, the time course experiment was repeated by first degrading bead-captured RNA by RNase treatment followed by TEV protease cleavage (0.2 U/µl, Fig. 16B). In contrast to the previous experiments, a clear time-dependent reduction in bead-captured MS2CP-PrAx2 signal intensity was observed. Western blot quantification indicated that already after 10 min 63% of MS2CP-PrAx2 had been cleaved. After 1 h, the cleavage efficiency had risen to 90%. This result clearly demonstrates that in our experimental setup the prerequisite for efficient proteolytic cleavage is the removal of intact mRNPs by RNase treatment. Consequently, TEV protease cleavage cannot be used as an alternative to RNase treatment for mRNP protein release.

Quantitative proteomic analysis of *in vivo*-assembled mRNA-protein complexes

In order to obtain quantitative information about mRNP protein composition we employed stable isotope labelling by amino acids in cell culture (SILAC) (Ong et al. 2002). SILAC enables the labelling of newly synthesized proteins through normal metabolic process with either the natural "light" isotope-containing (i.e. $_{12}C$, $_{14}N$, H) amino acids or with "heavy" SILAC amino acids containing stable isotopes (i.e. $_{13}C$, $_{15}N$, $_{2}H$). Compared to peptides generated from proteins containing light amino acids, incorporation of heavy amino acids into proteins results in a mass shift of the corresponding peptides. This mass shift can be detected by mass spectrometry and upon mixing heavy and light samples in 1:1 ratio, used for the detection of differences in the relative protein abundance in these samples. The general workflow of SILAC-based relative quantification of mRNP proteins is depicted on figure 17. In the "forward" experiment the proteome of the MS2L-tagged yeast strain is labelled with heavy lysine (Lys8) and of the untagged control strain with light non-labelled lysine (Lys0, see Methods, "Metabolic labelling by SILAC for mass spectrometry-based quantitative proteomics"). Consequently, proteins specifically co-purifying with MS2L-tagged mRNAs are enriched from the heavy lysate resulting in a heavy (H) to light (L) SILAC ratio >1, whereas the non-specific background binders display a H/L ratio around 1. In order to be able to detect dynamic protein-protein interactions, mRNP affinity purification is performed separately from the two SILAC states (see Methods, "mRNP affinity purification protocol"). Mixing heavy and light lysates prior to affinity purification has been shown to result in the exchange of dynamically interacting proteins between the heavy and light labelled protein complexes, leading to the erroneous identification of dynamically interacting proteins as background binders (X. Wang and Huang 2008). To avoid this, two parallel purifications are carried out and IgG-coupled beads are combined immediately prior to RNase elution. In order to increase the specificity of relative quantification, each SILAC mRNP affinity purification is also carried out in "reverse", i.e. the amino acid labelling conditions are switched so that the proteome of the untagged control strain is labelled with Lys8 and of the MS2L-tagged strain with Lys0. The H/L ratios of the two biological replicate experiments determined for each identified protein are manually analysed to single out proteins specifically co-isolating with the tagged mRNAs. Specific binders are characterized by a high H/L ratio (H/L ratio >1) in the forward experiment, whereas in the reverse experiment the H/L ratio should have a reciprocal value to the forward experiment H/L ratio.

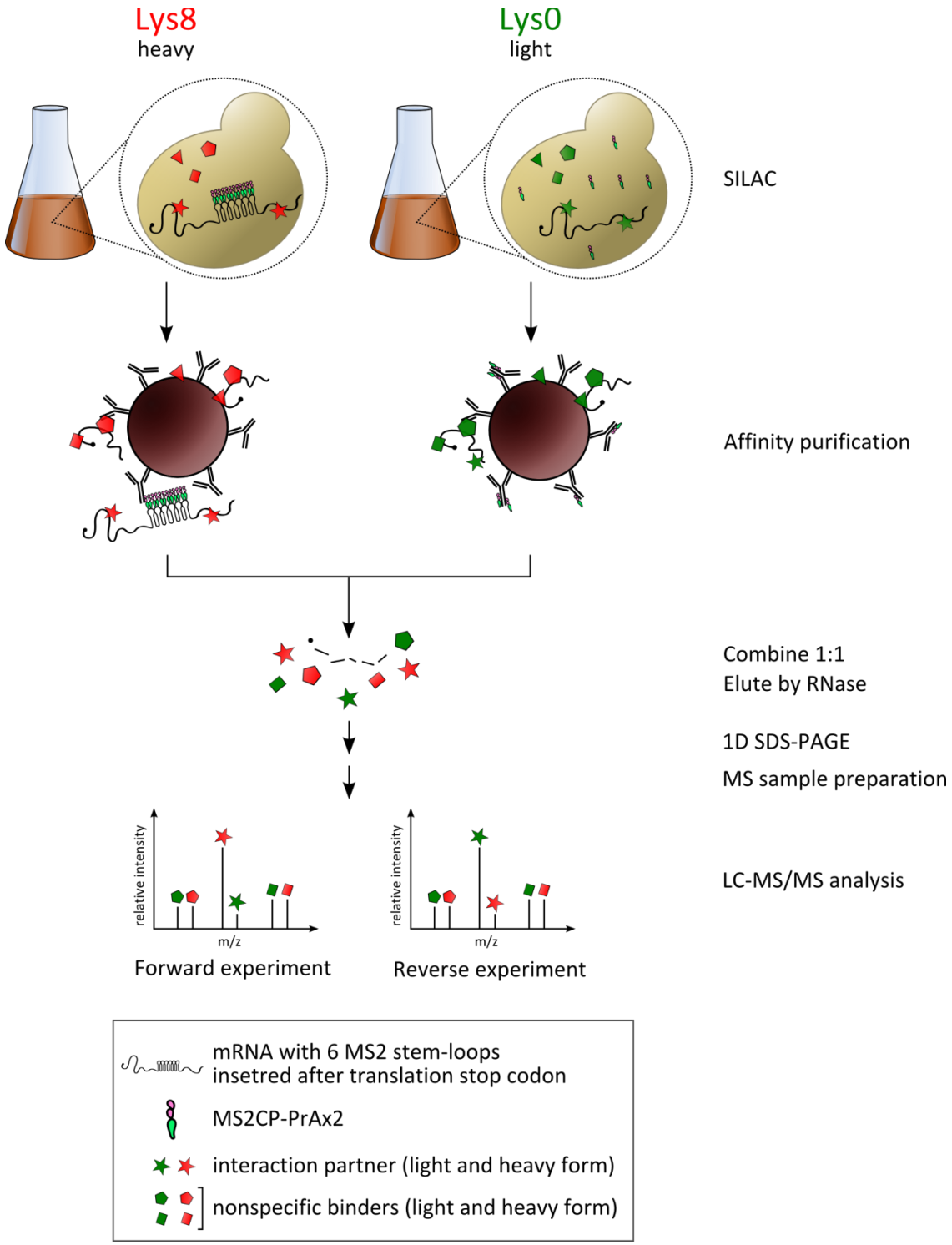


Figure 17. Experimental workflow of SILAC-based quantitative proteomic analysis of *in vivo*-assembled mRNA-protein complexes. In the forward experiment the proteome of *S. cerevisiae* strain expressing MS2L-tagged mRNA is metabolically labelled with "heavy" isotope-containing lysine (Lys8), whereas the untagged control strain is labelled with "light" isotope-containing lysine (Lys0). mRNP affinity purification is carried out in parallel from Lys8- and Lys0-labelled cells. IgG-coupled beads with the captured complexes are combined immediately before RNase digestion. The protein composition of RNase eluate is analysed by LC-MS/MS. In the reverse experiment the amino acid labelling condition are swapped. Proteins specifically co-purifying with MS2L-tagged mRNA are characterized by high heavy-to-light ratio in the forward experiment and a low heavy-to-light ratio in the reverse experiment. Non-specific background binders show 1:1 heavy-to-light ratio under both labelling conditions. m/z – mass-to-charge ratio.

Using our mRNP affinity purification strategy combined with SILAC-based quantitative proteomics, we have analysed the protein composition of mRNPs containing endogenously expressed MS2L-tagged *PGK1* and *ENO2*. Besides *PGK1*, *ENO2* was chosen to test the applicability of the established mRNP affinity purification method for quantitative analysis of mRNP protein composition because, similarly to *PGK1*, it is a stable and abundant yeast transcript (Y. Wang et al. 2002; Grigull et al. 2004; Miura et al. 2008). Affinity purification of *ENO2-6MS2L*-containing mRNPs could therefore be carried out without further optimization of the mRNP affinity purification protocol. In addition, we reasoned that *PGK1*- and *ENO2*-containing mRNPs would share similarities in their mRNP protein composition because both mRNAs encode enzymes that participate in the same metabolic pathways, glycolysis and glyconeogenesis (Lam and Marmur 1977; McAlister and Holland 1982; Hitzeman, Clarke, and Carbon 1980). By studying the composition of *PGK1*- and *ENO2*-containing mRNPs we wished to determine the general mRNP composition of glycolytic enzymes and thereby shed light on the proteome of mRNPs translated on cytosolic ribosomes under normal yeast growth conditions (Reid and Nicchitta 2012).

In order to study what proteins might directly bind to the MS2L tag, we have also analysed what proteins co-purify with an exogenously expressed RNA containing the 6 MS2 stem-loops (6MS2L-RNA). The *in silico* predicted structural and experimentally determined functional properties of 6MS2L-RNA will be discussed in the following chapter.

Control RNA to determine the effect of MS2L tag on mRNP protein composition

In order to identify the proteins co-purify with the MS2L tag, the 421 bp long loxP-6MS2L sequence identical to the MS2L tag present at the genomic loci of *PGK1*- and *ENO2-6MS2L* genes (Haim et al. 2007) was cloned into a yeast centromeric plasmid under the control of *PGK1* promoter and iso-1-cytochrome c (*CYC1*) transcriptional terminator (see Materials, Plasmids, RJP 1783). The *PGK1* promoter was chosen to control 6MS2L-RNA expression in order to ensure comparable transcriptional gene expression regulation to *PGK1* mRNA. Important aspects of 6MS2L-RNA expression that we reasoned would be similar to *PGK1* due to *PGK1*-promoter controlled transcription were 6MS2L-RNA expression level and transcriptional start site (TSS) selection. The cloned *PGK1* promoter fragment extended from position –947 to position –1 upstream of *PGK1* initiator AUG (here and hereafter A is assigned as +1) and contained all identified transcription factor binding sites (Chambers et al. 1989; Packham,

Graham, and Chambers 1996). The well characterized *CYC1* transcriptional terminator sequence was used to ensure efficient termination of 6MS2L-RNA transcript (Osborne and Guarente 1989; Zaret and Sherman 1982; Russo et al. 1993; Guo and Sherman 1996).

As both the promoter and transcriptional terminator of 6MS2L-RNA are derived from genes transcribed by RNA PolII, the transcript is predicted to carry a 5' cap and 3' poly(A) tail (reviewed in Houseley and Tollervey 2009). The predicted size of 6MS2L-RNA is around 680 nt (Fig. 18A). *PGK1* transcriptional start sites in *S. cerevisiae* have been mapped to a region spanning positions –48 to –27 (van den Heuvel et al. 1989; Hitzeman et al. 1982; Mellor et al. 1985; Z. Zhang and Dietrich 2005) with the major TSS mapped to position –40 (Z. Zhang and Dietrich 2005). We assumed that 6MS2L-RNA transcription is initiated from the same promoter region as for *PGK1* mRNA, likely at position –40. Several polyadenylation sites in *CYC1* gene have been mapped to a 58 bp region downstream of position +468 (Russo et al. 1993). Assuming that polyadenylation of 6MS2L-RNA would take place at the major poly(A) site spanning positions +503 to +505 (Russo et al. 1993; Russo and Sherman 1989), the length of *CYC1* 3' UTR present in 6MS2L-RNA would be around 150 nt. The average poly(A) tail length of *CYC1* mRNA is 60 nt (Christine E. Brown and Sachs 1998; Dheur et al. 2005), which we reasoned would be similar for 6MS2L-RNA.

In order to determine if 6MS2L-RNA carries coding potential, the predicted transcript sequence was analysed for the presence of open reading frames with ORF Finder (Sequence Manipulation Suit, Version 2). A thorough analysis of 6MS2L-RNA's coding potential was necessary in order to be able to better interpret the SILAC-based quantitative proteomics results. The 6MS2L-RNA contains 3 full-length ORFs (containing a start and a stop codon) of at least 30 codons and 2 full-length ORFs of at least 10 codons, all of which are located in the MS2L-tag region. The preferred nucleotide sequence around the initiation codon in *S. cerevisiae* is reported to be AAAAAAAAA<u>AUG</u>UC (<u>AUG</u> represents the translation initiation codon) (Cavener and Ray 1991). Nucleotide bias around the initiation codon in yeast is especially strong at positions –3, +4 and +5 with the most frequent appearance of A, U and C, respectively (Nakagawa et al. 2008). *PGK1* with the sequence UAUAAAACA<u>AUG</u>UC matches the preferred initiation codon context at 11 positions out of 14, including positions –3, +4 and +5. The first AUG triplet of the predicted 6MS2L-RNA transcript is located within the loxP site. Importantly, the sequence xxxxxxAxA<u>AUG</u>Ux (x denotes a mismatch compared to the preferred yeast initiation codon context) contains the preferred nucleotides A and U at positions –3 and +4, respectively. The nucleotides at positions –3 and +4 are known to have a strong effect on translation initiation efficiency (Kozak 1986a) and therefore the presence of the preferred nucleotides at these positions is likely to promote translation initiation from the first AUG triplet in 6MS2L-RNA.

The predicted ORF is 8 codons long. There are two additional AUG triplets 5' to the 6 MS2 stem-loops, which, if used as translation initiation sites would encode for 7 and 8 aa long peptides. However, as the surrounding sequence of these AUG triplets does not match the preferred translation initiation site context in any of the 3 important positions, these AUG triplets are not likely to be used as translation initiation codons.

Each 19 nt long MS2 stem-loop contains 2 AUG triplets. The analysis of the surrounding nucleotides revealed several matches with the preferred *S. cerevisiae* initiation codon context. Importantly, in the sequence xxAxAAAxAUGxx an A is present at position –3 and in the sequence xxAxxAxxxAUGUC a U and a C are present at positions +4 and +5, respectively. It should be noted that in the RNA genome of bacteriophage MS2 the initiation codon of the replicase gene is located in the MS2 stem-loop and corresponds to the AUG triplet in the sequence xxAxxAxxxAUGUC (Borisova et al. 1979).

Considering the context of the two AUG triplets present in MS2L sequence, it seems possible that both of them could serve as translation initiation codons. However, this would mean that the 43S pre-initiation complex would have to scan past at least 3 upstream AUG codons. Furthermore, the interaction between MS2 stem-loop and MS2 coat protein is likely too strong to be dissociated by the scanning 43S PIC. The physiological role of MS2L-MS2CP interaction in the bacteriophage MS2 RNA genome is to repress translation of the viral replicase gene (Fouts, True, and Celander 1997; Bernardi and Spahr 1972). To our knowledge, the effect of a 5' UTR-located MS2 stem-loop on the translational efficiency of yeast genes in the presence of MS2CP has not been studied. However, in *E. coli* the expression of MS2CP resulted in a 30-50-fold repression of protein synthesis from MS2 replicase-β-galactosidase reporter gene (Peabody 1990). In our experimental setup 6MS2L-RNA is expressed in the presence of MS2CP-PrAx2. Therefore, the AUG triplets present in MS2L sequence are not likely accessible for translation initiation.

Besides the translation initiation codon context, RNA secondary structures upstream of the translation initiation site can have a dramatic effect on translation initiation efficiency (Kozak 1986b; Babendure et al. 2006). Translation efficiency can be reduced to minimum by a stem-loop with thermal stability above –35 kcal/mol, whereas a stem-loop with thermal stability up to –25 kcal/mol, if not placed directly downstream of the 5' cap, does not significantly influence translation efficiency (Babendure et al. 2006). The predicted 6MS2L-RNA transcript was analysed for RNA folding with RNAfold from ViennaRNA Web Service (Gruber et al. 2008). As could be expected, the first 40 nt of 6MS2L-RNA that are transcribed from the *PGK1* promoter did not contain any stable RNA stem-loops. The following loxP sequence, where the first AUG triplet of 6MS2L-RNA is located, forms a stem-loop with minimum free energy of –13.7 kcal/mol and

should therefore be efficiently removed by 43S PIC-associated RNA helicase activity (Tatyana V Pestova and Kolupaeva 2002; S. F. Mitchell et al. 2010; Rogers et al. 2001; Marintchev et al. 2009). The MS2 stem-loop has a minimum free energy of –5.3 kcal/mol, which the 43S PIC is likely able to unwind only in the absence of MS2CP.

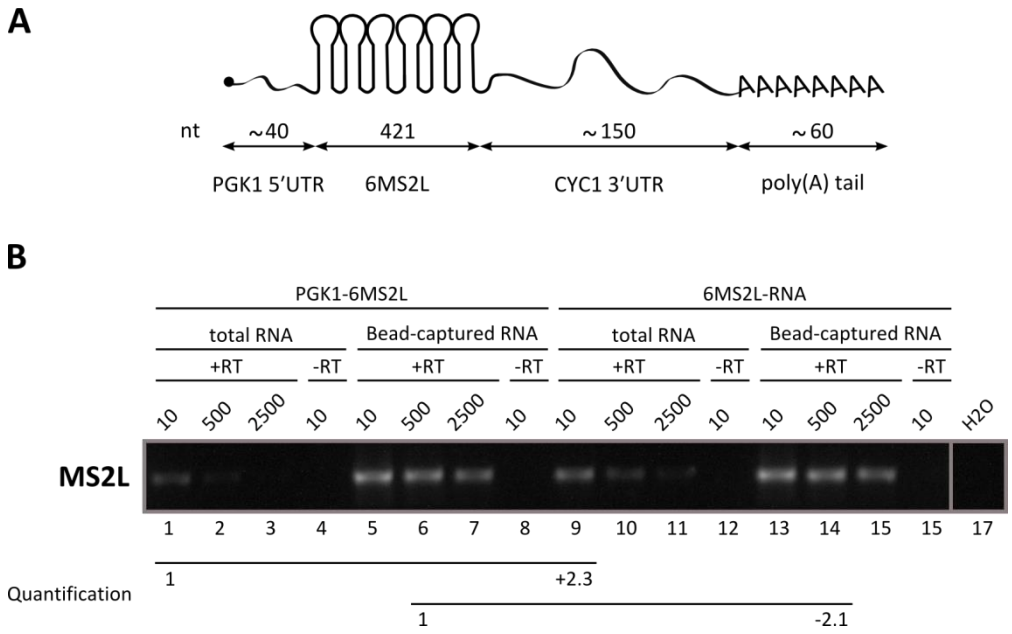


Figure 18. 6MS2L-RNA is expressed and enriched on IgG-coupled beads after mRNP affinity purification. (**A**) Schematic representation of predicted 6MS2L-RNA transcript. (**B**) A yeast strain containing plasmids encoding for 6MS2L-RNA and MS2CP-PrAx2 (RJY 3989) was used for mRNP affinity purification. Strain RJY 3827, which contains plasmid encoded MS2CP-PrAx2 and genomically encoded *PGK1-6MS2L*, served as positive control. RNA extracted from input material (total RNA) and from IgG-coupled beads (bead-captured RNA) was used in RT-PCR analysis. 6MS2L tag was amplified from serially diluted cDNA. Quantification of RT-PCR signal is indicated below the lanes. To determine the total level of 6MS2L-RNA as compared to *PGK1-6MS2L* (1 = control band), the signals corresponding to total RNA (10x cDNA dilution) were directly compared. To determine the level of beads-captured 6MS2L-RNA as compared to *PGK1-6MS2L*, the signal corresponding to beads-captured RNA (500x cDNA dilution) was first normalized to signal corresponding to total RNA (10x cDNA dilution). -RT, control for genomic DNA contamination (reverse transcriptase omitted); +RT, cDNA; H_2O, negative control lacking cDNA template.

Experimental characterization of 6MS2L-RNA involved testing if the predicted transcript would be expressed and if it could be captured from yeast cell lysate via MS2L::MS2CP-PrA::IgG interaction. For this, a yeast strain transformed with plasmids encoding for 6MS2L-RNA and MS2CP-PrAx2 was subjected to mRNP affinity purification. As a positive control, a parallel *PGK1-6MS2L* affinity purification was performed. Indeed, RT-PCR analysis of total RNA extracted from input material showed that 6MS2L-RNA is expressed (Fig. 18B, lanes 9-11). Quantification of RT-PCR signal indicated that 6MS2L-RNA level in total RNA is 2.3-fold higher than that of *PGK1-6MS2L* mRNA. Analysis of bead-captured RNA demonstrated that 6MS2L-RNA can also be captured from yeast cell lysate by IgG-coupled beads (Fig. 18B, lanes 13-15), albeit 2.1-times less efficiently than *PGK1-6MS2L*.

The analysis of quantitative MS data: enrichment criteria and distribution of H/L ratios

Using our mRNP affinity purification strategy combined with SILAC-based quantitative proteomics, the proteome of mRNPs containing endogenously expressed 6MS2L-tagged *PGK1* or *ENO2* were analysed. In addition, proteins with the potential to co-purify with the 6MS2L-tag were identified by affinity capture of plasmid-encoded 6MS2L-RNA. Our first quantitative proteomic analysis was performed on *PGK1-6MS2L*-containing mRNPs. Besides identifying proteins that co-purify with *PGK1-6MS2L* mRNA, we analysed the efficiency of mRNP protein release from IgG-coupled beads during RNase treatment. mRNP proteins were first eluted by RNase treatment; the remaining proteins were subsequently release by heating the IgG-coupled beads in SDS sample buffer at 70°C and both protein samples were analysed by LC-MS/MS. In case of *ENO2-6MS2L* and 6MS2L-RNA affinity purification, only the RNase eluate was analysed by LC-MS/MS. The following chapter gives an overview about MS data analysis to identify the enriched proteins among MaxQuant-quantified proteins as well as discusses the quality of the data. The next chapter will focus on the comparison of proteins enriched in *PGK1-6MS2L* RNase eluate or BB sample to analyse the efficiency of RNase elution.

The number of proteins identified at a false discovery rate (FDR) of 1% (Käll et al. 2008) in the 8 analysed samples is listed in table 8. The enriched proteins were identified by integrating the MS data from the forward and reverse labelling experiments. In order to be classified as an enriched interaction partner the quantified proteins, i.e. proteins with MaxQuant-assigned H/L ratios, had to meet two criteria: (1) proteins identified by a single peptide were considered enriched only if in one of the biological replicate experiments the protein was identified by more than 1 peptide; (2) the H/L ratio had to meet the set threshold criteria in both forward and reverse labelling experiment. In order to facilitate the comparison of H/L ratios of the two biological replicate experiments, all normalized H/L ratios were first converted into $\log_2$ space. We applied two arbitrarily defined thresholds to classify the quantified proteins as enriched: (1) a less stringent threshold of $\log_2$ (H/L) >0.5 or <–0.5; and (2) a more stringent threshold of $\log_2$ (H/L) >1 or <–1. The thresholds $\log_2$ (H/L) >0.5 and >1, which correspond to H/L ratio of 1.41 and 2, respectively, were applied to identify the enriched proteins in forward labelling experiment. The thresholds $\log_2$ <–0.5 and <–1, which correspond to H/L ratio of 0.71 and 0.5, respectively, were applied to identify the enriched proteins in reverse labelling experiment.

Using two thresholds of different stringencies enabled us to define two sets of enriched proteins: firstly, a set containing specific interaction partners with likely few if any contaminating proteins; and, secondly, a set containing also low abundance and/or low affinity interaction

partners including possible contaminating proteins. Depending on the MS data set, the low abundance proteins comprised 20-40% of all enriched proteins. Figure 19 shows the distribution of quantified proteins in each LC-MS/MS run as well as the two subsets of enriched proteins identified by comparing $\log_2$ (H/L) ratios determined for the forward and reverse experiment. Importantly, ~70-90% of quantified proteins in each MS data set did not classify as enriched, emphasising the importance of robust quantitative analysis based on SILAC metabolic labelling to distinguish between specific interactors and contaminating proteins. The number of proteins >1.41-fold enriched (threshold $\log_2$ (H/L) >0.5 or <–0.5) in *PGK1-6MS2L* RNase eluate and BB sample, respectively, was 78 and 64. By applying the more stringent threshold of $\log_2$ (H/L) >1 or <–1, which corresponds to >2-fold enrichment, the number of enriched proteins in both data sets was reduced roughly by 40% to 45 in RNase eluate and to 39 in BB sample. The number of proteins >1.41-fold enriched after *ENO2-6MS2L* and 6MS2L-RNA affinity purifications was 103 and 90, respectively. The number of proteins >2-fold enriched was reduced by ~20% to 83 and to 71 for *ENO2-6MS2L* and 6MS2L-RNA, respectively.

Table 8. Number of identified proteins in each LC-MS/MS run. The data were processed with a setting of 1% for the FDR, i.e. with an estimation that 1% of all identifications are false-positive.

PGK1 – Boiled Beads		PGK1 – RNase eluate		ENO2		6MS2L	
for	rev	for	rev	for	rev	for	rev
614	384	688	380	404	312	454	363

Table 9. Number of significantly enriched (significance B <0.01) proteins in each LC-MS/MS run.

PGK1 – Boiled Beads		PGK1 – RNase eluate		ENO2		6MS2L	
for	rev	for	rev	for	rev	for	rev
28	19	26	18	1	7	14	13

Table 10. SILAC mixing error – median of unnormalized heavy-to-light ratios of each LC-MS/MS run.

PGK1 – Boiled Beads		PGK1 – RNase eluate		ENO2		6MS2L	
for	rev	for	rev	for	rev	for	rev
0.99	0.67	1.39	0.64	5.21	0.53	0.74	0.92

The largest number of proteins was classified as enriched for *ENO2-6MS2L* affinity purification. However, the number of statistically significant H/L ratio changes, as expressed through a quantity termed significance B (Cox and Mann 2008), was the lowest in the corresponding forward and reverse experiment MS data sets (Table 9). Notably, in the two MS data sets the distribution of $\log_2$ (H/L) ratios of proteins that did not classify as enriched appears more scattered as compared to other MS data sets (Figure 19). Indeed, box plot statistics

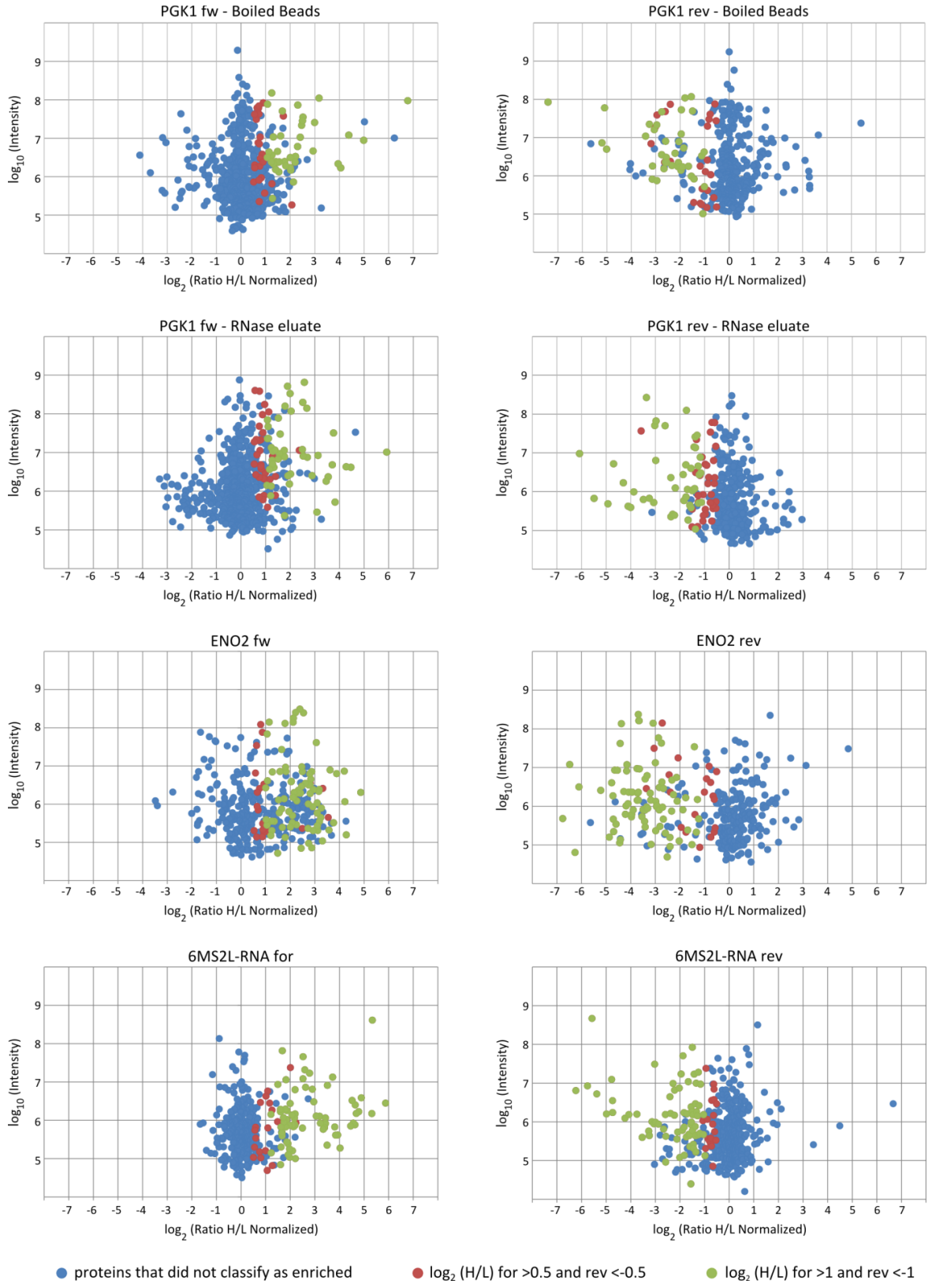


Figure 19. SILAC-based quantitative mass spectrometry of mRNP proteome. Plots display $\log_2$ values of normalized heavy-to-light ratios (H/L, x axis) and $\log_{10}$ values of summed peptide intensities (y axis) for all identified proteins (FDR 1%). Blue dots represent proteins that did not classify as enriched by comparing the H/L ratios determined in two biological replicate experiments. Red dots represent proteins that, at least in one of the biological replicate experiments, were >1.41-fold but <2-fold enriched. Green dots represent protein that were >2-fold enriched in both biological replicate experiments. for – forward labelling, rev – reverse labelling.

confirms that the $\log_2$ (H/L) values are more dispersed in *ENO2-6MS2L* forward and reverse MS data sets as compared to other MS data sets (Figure 20). The reason for higher variability in the spread of H/L ratios in the above mentioned data sets could lie in unequal mixing of heavy and light protein populations. Namely, from the 8 analysed samples the largest SILAC mixing errors, 5.21 and 0.53, were determined for *ENO2-6MS2L* forward and reverse experiment, respectively. Table 10 shows the values of SILAC mixing errors, i.e. the median of all unnormalized H/L ratios determined for each LC-MS/MS run. Assuming that heavy and light protein populations are mixed in 1:1 ratio and that most of the proteins captured during affinity purification are non-specific background binders, the sample's peptide median H/L ratio should be around 1. Inequality in the heavy and light protein populations introduced by unequal protein mixing or other imperfections during mRNP affinity purification can result in a median H/L ratio that considerably deviates from 1. In order to remove mixing errors, the H/L ratios determined in each LC-MS/MS run are normalized so that the mean of all $\log_2$-transformed H/L ratios is zero (Cox and Mann 2008). Despite the possibility to correct for mixing errors of total protein amounts by normalization, inaccurate mixing of heavy and light protein populations may affect the dynamic range over which accurate peptide masses can be determined and thus reduce the overall accuracy of the LC-MS/MS analysis (reviewed in Bantscheff et al. 2012; Bantscheff et al. 2007).

A step in our mRNP affinity purification protocol that could possibly introduce inequality between the pools of heavy and light proteins is lysate preparation. The preparation of lysates of the same protein concentrations relies on weighing in the same amount of yeast cell grindate per each analysed strain (see Materials and Methods, "mRNP affinity purification protocol"). The grindate has to be filled into tubes cooled down in LN_2 to avoid grindate thawing. Determining the exact weight of the grindate filled into a cooled tube might be imprecise because of the water vapour condensing on the walls of the cooled tube. Even though the tube is cooled several times during weighing in the grindate to keep a constant temperature, it is possible that this step in the mRNP affinity purification protocol can lead to differences in the amount of grindate used for lysate preparation between the MS2L-tagged strain and the wt control strain. In order to test if the protein concentration of heavy and light lysates had been the same in the experiment with the largest SILAC mixing error, the same volume of heavy and light lysate from *ENO2-6MS2L* forward experiment was separated on SDS-PAGE and stained with Coomassie dye (Ulrike Thieβ, unpublished data). Densitometric analysis of the stained gel confirmed that the two lysates in this case had had exactly the same protein concentrations. Therefore, in *ENO2-6MS2L* forward experiment the imbalance between the heavy and light protein populations must have been introduced at some other step than weighing in the grindate.

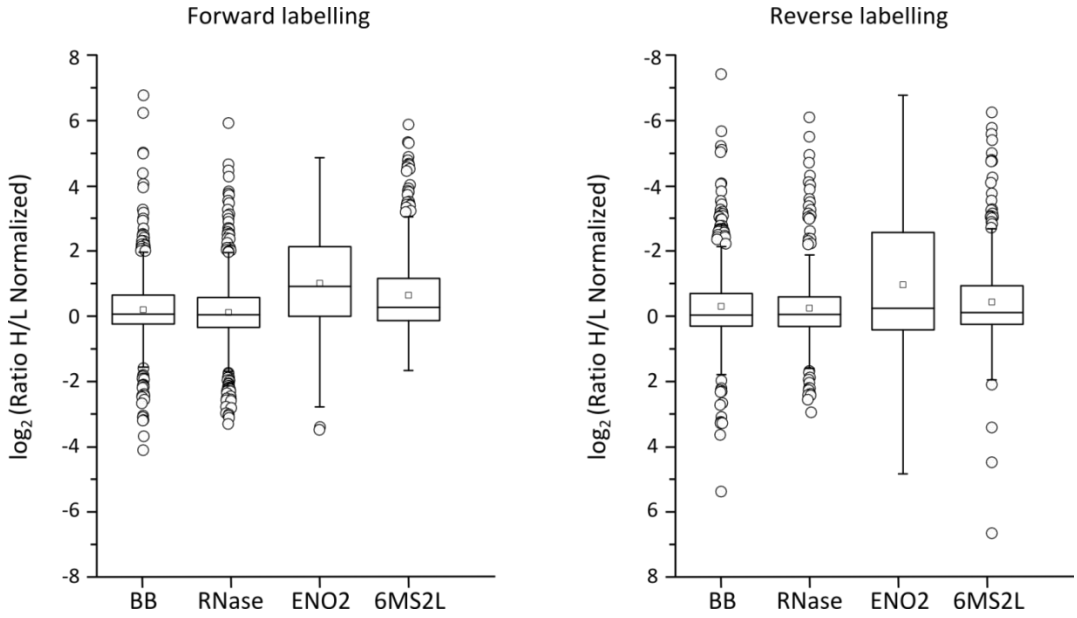


Figure 20. Box plots showing the distribution of SILAC ratios determined for mRNP affinity purification experiments shown in figure 19. Box plots demarcate the median (stripe), the mean (open rectangle), the 25th to 75th percentile (interquartile range, box), 1.5× the interquartile range (whiskers) and outliers (open circles).

Two of the three sets of mRNP affinity purification experiments were performed without technical problems as indicated by the spread of the determined H/L ratios (Fig. 19 and 20) as well as by SILAC mixing errors (Table 10), which were within an acceptable error range for *PGK1-6MS2L* and 6MS2L-RNA affinity purifications. However, the high SILAC mixing errors determined for *ENO2-6MS2L* forward and reverse experiment suggest that during the course of the experiment an imbalance in the heavy and light protein pools can be introduced, underlying the importance of careful and precise performance of the experiment.

RNase elution efficiency

Western blot analysis had shown that Upf1, which specifically co-purified with *PGK1-6MS2L* mRNA (Fig. 9B), was present both in the RNase eluate and in the BB sample (Fig. 10C). This result hinted at the possibility that the 30 min of RNase treatment might not be sufficient to release all mRNP proteins because of insufficient RNA degradation. However, northern blot analysis of *PGK1-6MS2L* mRNA integrity after RNase treatment suggested the opposite – efficient degradation of bead-captured *PGK1-6MS2L* (Fig. 21B and data not shown). In this experiment, the sample was split after *PGK1-6MS2L* affinity purification. Half of the IgG-coupled beads were treated with proteinase K followed by PCI RNA extraction to isolate the bead-captured RNA (Fig. 21A). The other half of the beads was first treated with RNase and the

RNase eluate, as well as the RNase-treated IgG-coupled beads, was subjected to RNA isolation as described above. Northern blot hybridization with *PGK1*-ORF (Fig. 21B) and MS2L (data not shown) antisense RNA probes resulted in a detectable hybridization signal only if RNase treatment had been omitted (Fig. 21B, compare lanes 7-9, 12-14 and 17-19 and data not shown). The 30 min of RNase treatment seems not only sufficient to degrade bead-captured *PGK1-6MS2L* but also rRNA (Fig 21B, methylene blue staining and data not shown). *PGK1-6MS2L* but not untagged *PGK1* affinity purification resulted in the detection of two bands on methylene blue stained membrane that migrated at the same height as 25S and 18S rRNA. However, these bands could only be detected if bead-captured *PGK1-6MS2L* had not been treated with RNase (Fig. 21B, methylene blue staining, compare lanes 7, 12 and 17).

Unable to detect any *PGK1-6MS2L* degradation products after RNase treatment, we reasoned that Upf1 is efficiently released from RNA during RNase treatment but non-specifically reattaches to the IgG-coupled beads. During RNase treatment other proteins might behave similarly to Upf1 and therefore in our first quantitative proteomic analysis we wished to identify the proteins that were enriched both in the RNase eluate and in the BB sample. In addition, we were interested to determine if some proteins were exclusively enriched in BB sample. Therefore, the proteins >1.41-fold enriched in *PGK1-6MS2L* RNase eluate and BB sample were grouped into three categories: (1) "Unique; (2) "Possible common; and (3) "Common". Categories "Unique" and "Common" contain proteins that were unambiguously identified as enriched only in one or in both of the data sets, respectively. Proteins that were classified as enriched in one of the data sets but failed partly to fulfil the set threshold criteria to be classified as enriched in the other data set, for example due to a missing H/L ratio in one of the biological replicate experiments, were classified as "Possible common" (Appendix, Table 2). From the 78 proteins enriched in RNase eluate, 27 (35%) classified as "Unique", 21 (26%) as "Possible common" and 30 (39%) as "Common". 18 (28%) of the 64 enriched proteins in BB sample classified as "Unique" and 16 (25%) as "Possible common". The 30 proteins classified as "Common" comprised 47% of the enriched proteins in BB sample.

The comparison of the enriched proteins in RNase eluate and BB sample revealed that besides Upf1, 29 proteins were enriched in both data sets. Assuming that *PGK1-6MS2L* mRNA was efficiently degraded as suggested by northern blot analysis (Fig. 21B), this finding demonstrates that many eluted proteins could only be partly removed from the IgG-coupled beads. 60% of the proteins classified as "Common" were present among the top 50% of enriched proteins in both RNase eluate and BB sample if the data was sorted according to $\log_2$ (H/L) values determined for forward experiment (if the data was sorted according to $\log_2$ (H/L) values determined for reverse experiment 70% and 63% of "Common" proteins mapped to top 50% of

enriched proteins in RNase eluate and in BB sample, respectively). The distribution of the proteins enriched both in RNase eluate and BB sample thus revealed that the more highly enriched proteins are more likely to be only partly removed from the IgG-coupled beads.

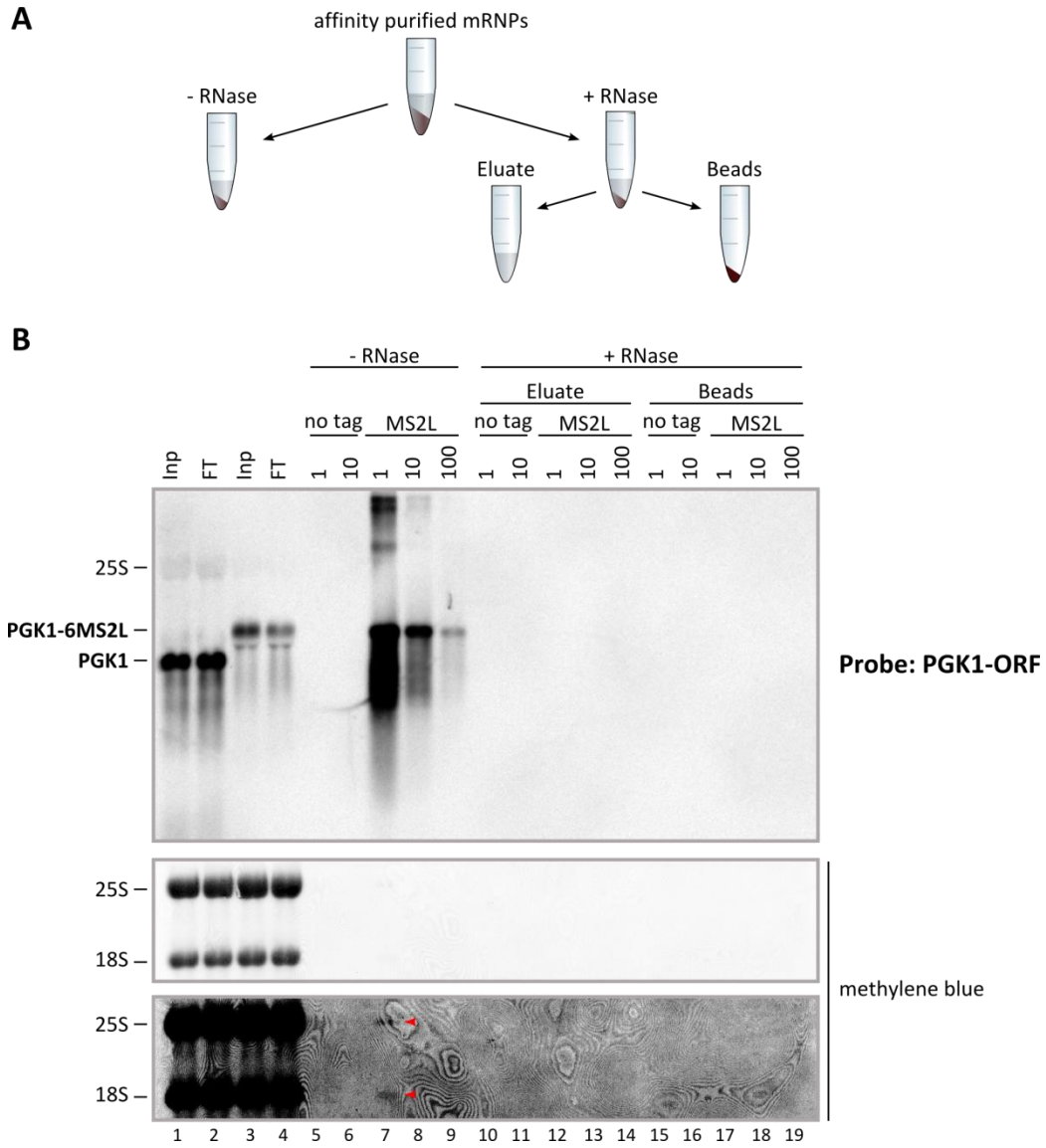


Figure 21. Northern blot analysis of *PGK1-6MS2L* mRNA integrity after RNase digestion. (**A**) Schematic representation of sample preparation for northern blot analysis. After mRNP affinity purification the sample was divided into two. One part of the sample was subjected to RNA extraction, whereas the other part was first treated with RNase. After RNase treatment RNA was extracted from RNase eluate and RNase-treated IgG-coupled beads. + RNase – RNase digestion; prot K – proteinase K treatment; PCI – phenol-chlorophorm-isoamyl alcohol RNA extraction. (**B**) Northern blot hybridization with DIG-UTP-labelled antisense RNA probes complementary to *PGK1* ORF. mRNP affinity purification was performed from yeast cells containing MS2CP-PrAx2 together with *PGK1-6MS2L* (MS2L, strain RJY 3827) or *PGK1* (no tag, strain RJY 3828). Samples for total RNA analysis were taken before (I – input) and after (FT – flow through) *PGK1* mRNP isolation. 1.5 µg total RNA and 10-fold serial dilutions of RNA extracted after mRNP affinity purification (1/6th to 1/600th of total sample) were separated on 1.3% agarose-formaldehyde gels and blotted onto positively charged nylon membranes. The hybridization signal corresponding to the full-length *PGK1* and *PGK1-6MS2L*, as well as non-specific cross-reaction with 25S rRNA, is indicated. On methylene blue stained membrane 25S and 18S rRNAs are marked. Red arrowheads indicate two bands corresponding in size to 25S and 18S rRNA that became visible after adjusting image contrast.

Slightly more than a quarter of the enriched proteins in BB sample classified as "Unique", suggesting that not all *PGK1-6MS2L* co-purifying proteins could be efficiently eluted from IgG-coupled beads by RNase treatment. Remarkably, 7 of the 18 proteins in this category are involved in rRNA processing. According to Saccharomyces Genome Database (Cherry et al. 2012) proteins encoded by *RRP7, UTP22, KRI1, KRR1, NOP4, RPP1* and *NUG1* are all predominantly nucleolar proteins participating in various aspects of ribosome biogenesis. Besides the 7 aforementioned rRNA processing factors unique for BB sample, the two data sets include only 3 additional proteins with the function in rRNA processing – the gene products of *CBF5* and *DIM1* in BB sample and of *NOP58* in RNase eluate – all classified as "Possible common".

The second largest group of functionally related proteins among the proteins exclusively enriched in BB sample is comprised of 4 large ribosomal subunit proteins. Further analysis of the distribution of 60S ribosomal subunit proteins in the two data sets of enriched proteins raised the possibility that 60S rRNA might not have been completely degraded during the 30 min of RNase treatment. Specifically, 11 60S ribosomal proteins were enriched in BB sample, whereas only 5 were enriched in RNase eluate. In contrast, the small ribosomal subunit proteins were relatively more abundant among the enriched proteins in RNase eluate. RNase eluate contained 19 and BB sample 13 enriched 40S ribosomal proteins. Notably, in addition to the 19 40S ribosomal subunit proteins detected as enriched after RNase treatment, only 6 additional 40S ribosomal subunit proteins were detected as enriched after heating the RNase-treated IgG-coupled beads in SDS sample buffer. This is in contrast to the results obtained for 60S ribosomal proteins – only 5 60S proteins were detected as enriched after RNase treatment, whereas heating the RNase-treated beads resulted in the detection of 9 additional enriched 60S ribosomal proteins. These results suggest that the less complex 40S rRNA might be more efficiently degraded during RNase treatment than the more complex 60S rRNA, thereby leading to the detection of relatively more 40S ribosomal subunit proteins in RNase eluate. 60S ribosomal subunit proteins, on the other hand, seem to have more efficiently dissociated from rRNA during protein denaturation by heating in SDS sample buffer.

Due to financial limitations we did not wish in the subsequent SILAC mRNP affinity purification experiments to determine the composition of BB sample. Due to time limitations it was unfortunately also impossible to further optimize RNase treatment conditions to ensure full rRNA degradation. Therefore, it can be expected that not all *ENO2-6MS2L* and 6MS2L-RNA co-purifying proteins have been detected. The MS2L-tagged mRNA co-purifying proteins that might not be efficiently released by RNase treatment from IgG-coupled beads under current experimental conditions include nucleolar rRNA processing factors and large ribosomal subunit

proteins; the latter escaping detection possibly due to incomplete rRNA degradation during RNase treatment.

The proteome of *in vivo*-assembled mRNPs is enriched for proteins involved in mRNA biology

In order to facilitate the comparison of proteins enriched after *PGK1-6MS2L*, *ENO2-6MS2L* and 6MS2L-RNA affinity purification, *PGK1-6MS2L* co-purifying proteins identified in RNase eluate or BB sample were integrated into one data set. The classification of the enriched proteins according to Protein Class using PANTHER classification system (P. D. Thomas et al. 2003; Mi, Muruganujan, and Thomas 2012) revealed a close functional similarity among proteins specifically co-purifying with *PGK1-6MS2L*, *ENO2-6MS2L* and 6MS2L-RNA (Fig. 22). Within each of the three quantitative MS data sets PANTHER classification results were very similar between proteins >1.4-fold and >2-fold enriched (Table 11). Independent of the set threshold, more than 50% of the enriched proteins in the three data sets classified as nucleic acid binding proteins (Fig. 22A). Within this category 76-83% of proteins were classified as RNA binding proteins (Fig. 22B). The most prominent class of proteins among RBPs were ribosomal proteins with 42-49% of RBPs classifying under this category in the three MS data sets (Fig. 22C). The enrichment of ribosomal proteins in all three MS data sets indicates that not only *PGK1-6MS2L* and *ENO2-6MS2L* but also 6MS2L-RNA can be engaged in translation. In addition to translation, the three MS2L-tagged RNAs seem to undergo largely the same processes. With only one exception – a single protein classified as DNA-directed RNA polymerase in *ENO2-6MS2L* data set – the enriched RBPs in the three data sets classified to the same 8 subcategories (Fig. 22C). Furthermore, the fraction of enriched proteins mapped to a specific RBP subcategory was very similar for *PGK1-6MS2L*, *ENO2-6MS2L* and 6MS2L-RNA.

Table 11. PANTHER Protein Class ontology classification (version 8.1) of enriched proteins. Table depicts percent of gene hit against total number of Protein Class hits.

MS2L-tagged RNA	$\log_2$[a]	Nucleic acid binding	RNA binding protein	Ribosomal protein
PGK1	0.5	54	80	46
	1	51	76	42
ENO2	0.5	58	79	48
	1	55	77	44
6MS2L	0.5	60	79	48
	1	69	83	49

[a] $\log_2$ 0.5 corresponds to threshold $\log_2$ (H/L) >0.5 or <-0.5; $\log_2$ 1 corresponds to threshold $\log_2$ (H/L) >1 or <-1.

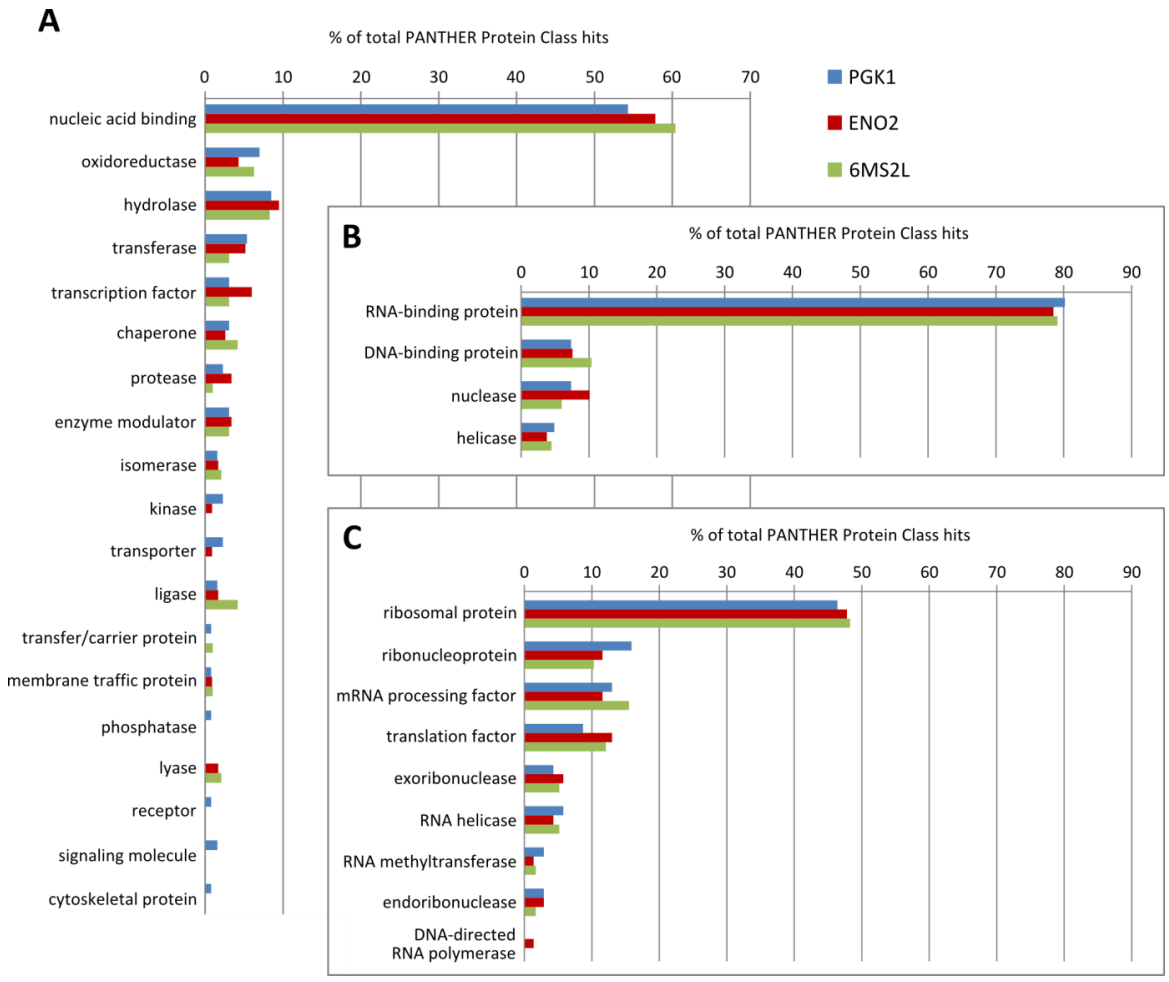


Figure 22. PANTHER Protein Class ontology classification of proteins that classified as enriched by applying the threshold $\log_2$ (H/L) >0.5 or <-0.5. Figures depict percent of gene hit against total number of Protein Class hits. (**A**) Initial classification of all enriched proteins in the three MS data sets: *PGK1-6MS2L* (proteins enriched in RNase eluate and BB sample were combined into one data set), *ENO2-6MS2L* and 6MS2L-RNA co-purifying proteins (**B**) Further classification of proteins in the category "nucleic acid binding". (**C**) Further classification of proteins in the category "RNA-binding protein".

PANTHER Protein Class category "RNA-binding protein" contained not only known mRNA-binding proteins but also proteins interacting with other cellular RNAs such as rRNA and tRNA. Consequently, several proteins classified as RBPs have previously not been implicated in mRNA biology. In order to estimate how many of the enriched proteins are annotated mRNA-binding proteins or proteins known to play a role in mRNA biology, a manual literature-based analysis of the three MS data sets was performed (Appendix, Table 3). Depending on the data set, 60-67% of the >1.41-fold enriched proteins belonged to one of the following categories: mRNA nuclear maturation, export, localization, decay and translation. The latter category contained translation initiation factors, ribosomal proteins and proteins involved in mRNA translational control or co-translational nascent peptide maturation. Among the proteins >2-fold enriched, 67-72% of the proteins could be classified under one of the above-mentioned categories. The prevalence of proteins with a role in mRNA biology among the MS2L-tagged

RNA co-purifying proteins clearly demonstrates that 6MS2L::MS2CP-PrAx2::IgG interaction can be employed to capture *in vivo*-assembled mRNPs for quantitative MS analysis. The identification of proteins involved in various stages of mRNA life cycle both in the nucleus and in the cytoplasm indicates that a variety of mRNPs from diverse cellular compartments could be affinity purified (see table 12 for examples). However, the repertoire of the MS2L-tagged RNA co-purifying proteins also suggests that the efficiency of detecting certain mRNP proteins is influenced by mRNP abundance in different cellular compartements (see Discussion, Part 2 "The analysis of mRNA-bound proteome is likely influenced by mRNP abundance in different cellular compartments").

In order to better visualize how proteins that specifically co-purified with an MS2L-tagged RNA are related to each other, the lists of enriched proteins were analysed using STRING database, which integrates the information about known and predicted physical and functional protein-protein interactions to create a protein interaction network (L. J. Jensen et al. 2009). Figures 23-25 depict the STRING networks obtained for *PGK1-6MS2L*, *ENO2-6MS2L* and 6MS2L-RNA. Proteins >1.41-fold enriched are shown.

A common feature of the three STRING networks is the high degree of connectivity between the proteins in each network even when using the highest confidence score (0.900) for generating the networks. In each data set only 10-14% of all proteins >1.41-fold enriched are not linked to any other protein in the core network (Fig. 23-25). Among the proteins >2-fold enriched the fraction of such unconnected proteins is remarkably similar – 9-12% (data not shown). This result suggests that the threshold $\log_2$ (H/L) >0.5 or <–0.5 can efficiently filter out non-specific background binding proteins. Non-specific background binders are expected to be less enriched compared to proteins specifically interacting with MS2L-tagged RNAs. In addition, they would be more likely to carry out functions unrelated to mRNA metabolism and therefore would be less likely linked to proteins forming the core of the STRING network. The finding that for each MS2L-tagged RNA the number of unconnected proteins in the STRING network is very similar among proteins >1.41-fold and >2-fold enriched thus suggests that the proteins that do not classify as enriched if the more stringent threshold is applied are mostly specific interaction partners of MS2L-tagged RNAs.

An overall functional similarity among proteins specifically co-purifying with *PGK1-6MS2L*, *ENO2-6MS2L* and 6MS2L-RNA suggested by PANTHER classification (Fig. 22) becomes evident through the STRING interaction networks. Distinct clusters within the networks common to all three MS2L-tagged RNAs are formed by ribosomal proteins and by proteins involved in cytoplasmic mRNA decay. Translation and mRNA turnover thus appear to be the two central processes in the life cycles of the studied MS2L-tagged RNAs. Each STRING

network, however, also shows unique features, revealing some interesting differences in the physiology of the studied RNAs. The following chapter will give an overview of the enriched proteins in the context of the determined protein interaction networks.

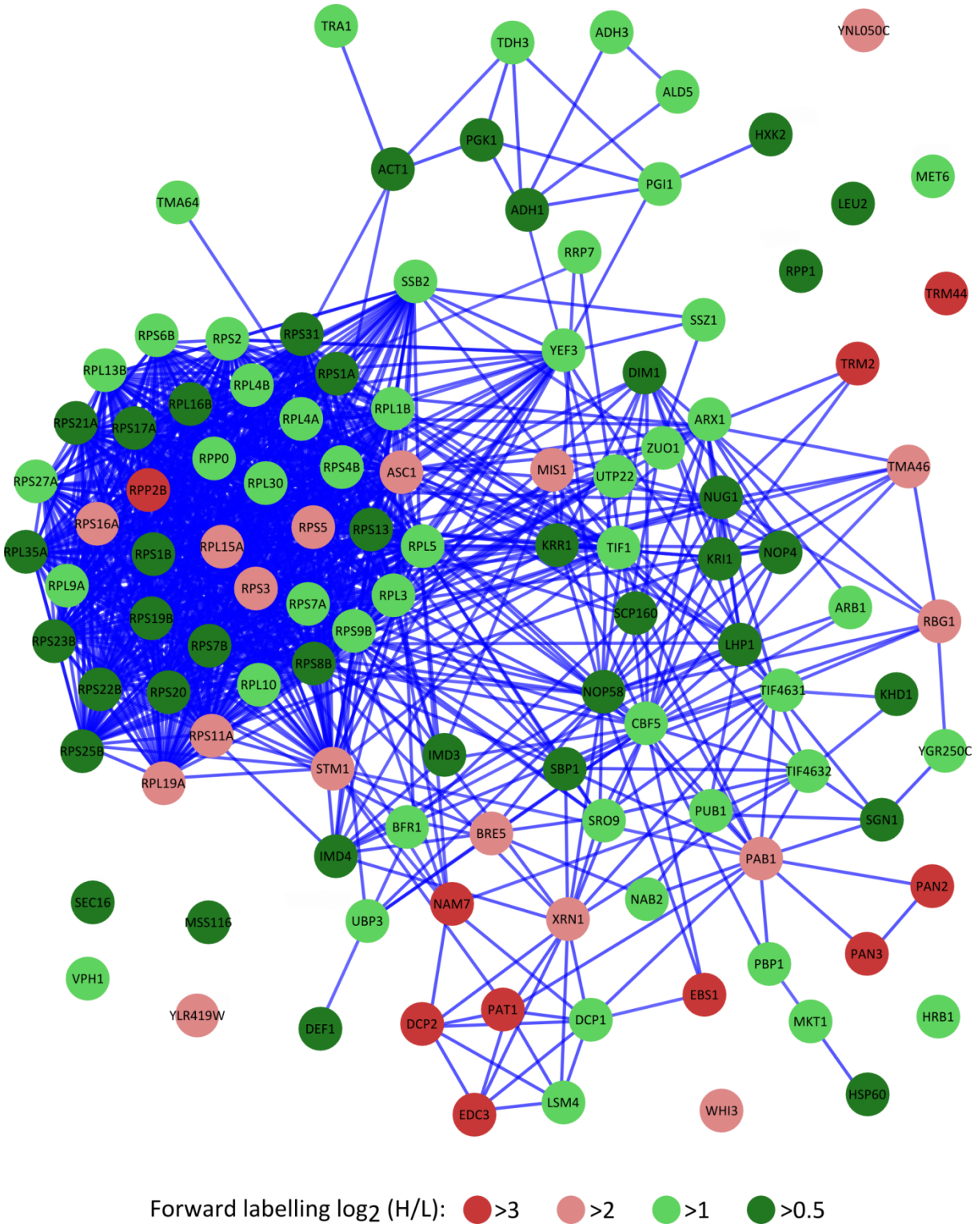


Figure 23. Protein-protein interaction network of proteins enriched after *PGK1-6MS2L* affinity purification. The enriched proteins in two MS data sets – RNase eluate and BB sample – are combined. Image modified from the image created by STRING database (v.9.1) using the highest confidence score (0.9). Each circle represents

an individual protein with the standard name depicted. The colour coding corresponds to the H/L ratios determined in the forward labelling experiment. If a protein was enriched both in RNase eluate and BB sample, the colour was chosen according to the H/L ratio determined for RNase eluate. Connecting lines represent association.

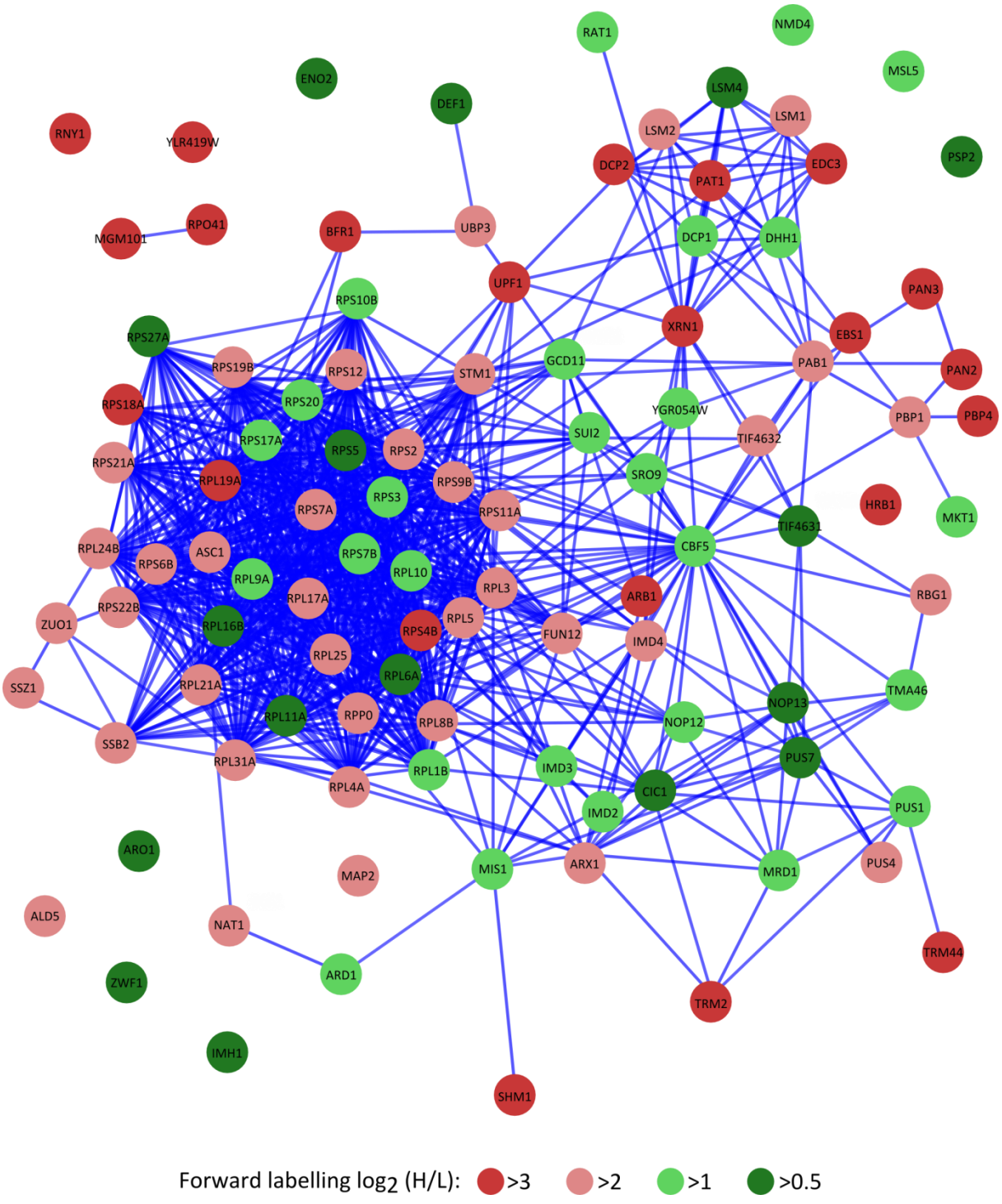


Figure 24. Protein-protein interaction network of proteins enriched after *ENO2-6MS2L* affinity purification. Image modified from the image created by STRING database (v.9.1) using the highest confidence score (0.9). Each circle represents an individual protein with the standard name depicted. The colour coding corresponds to the H/L ratios determined in the forward labelling experiment. Connecting lines represent association.

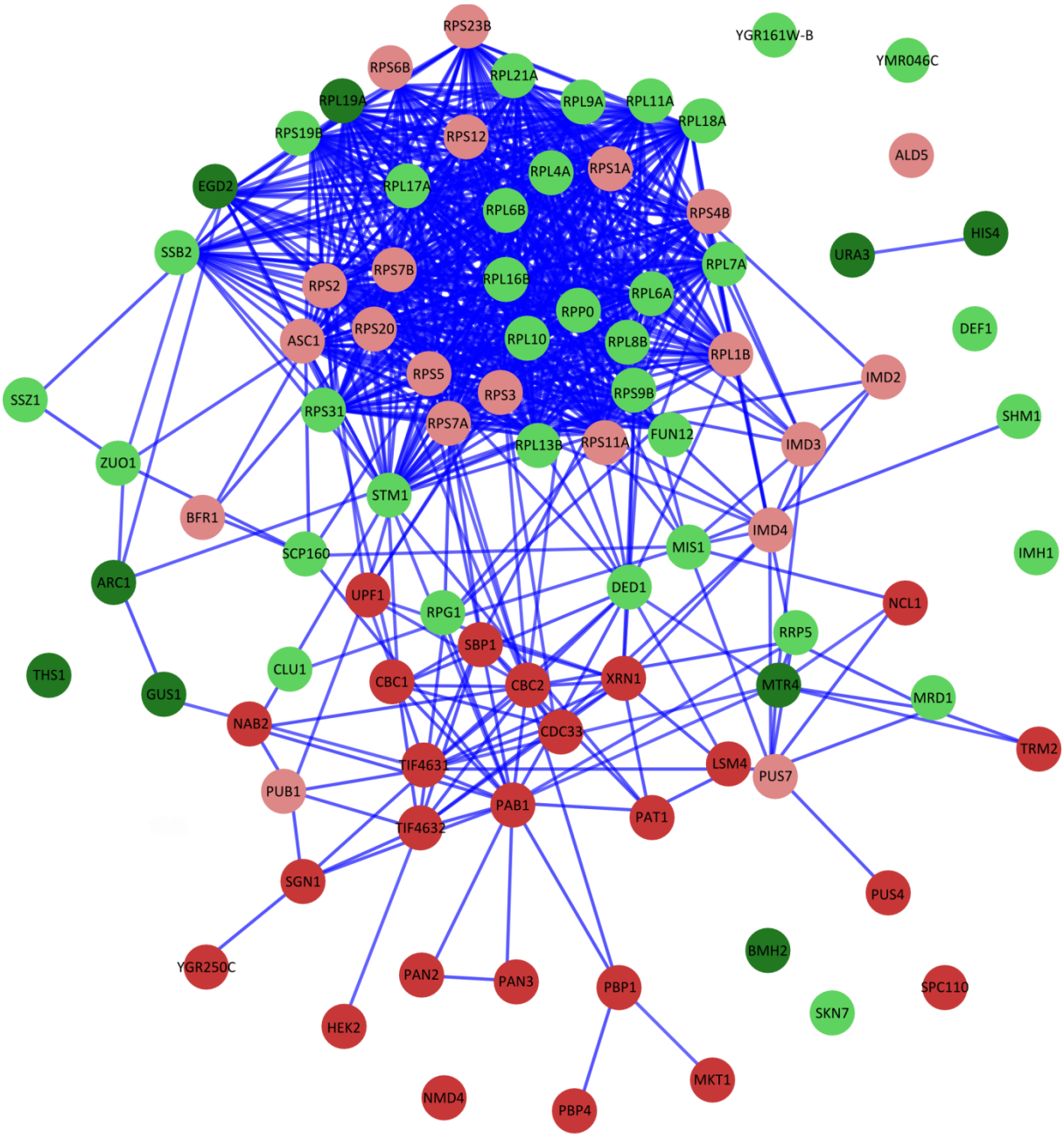


Figure 25. Protein-protein interaction network of proteins enriched after 6MS2L-RNA affinity purification. Image modified from the image created by STRING database (v.9.1) using the highest confidence score (0.9). Each circle represents an individual protein with the standard name depicted. The colour coding corresponds to the H/L ratios determined in the forward labelling experiment. Connecting lines represent association.

Overview of MS2L-tagged RNA co-purifying proteins

Literature-based analysis of the enriched proteins indicated that 26-35% of proteins in each MS data set (Appendix, Table 4) are mRNA-binding proteins or components of multiprotein

complexes with known mRNA-binding subunits (examples shown in Table 12). Most of the detected mRNA-binding or mRNA-associated proteins are part of cytoplasmic mRNPs engaged in translation or in mRNA decay. The enriched proteins involved in translation include translation initiation factors (Table 13), the major yeast poly(A) binding protein Pab1, components of polyribosome-associated mRNPs (Scp160, Bfr1) and proteins involved in translation repression (e.g. Sbp1, Dhh1, see also Table 15). Several enriched proteins of the latter class are functionally linked to mRNA degradation as decapping activators (e.g. Dhh1, Pat1, see also Table 16). Besides decapping activators, the enriched mRNA decay factors include Dcp1-Dcp2 decapping complex and 5'→3' exonuclease Xrn1. Remarkably, most mRNA decay factors were among the highly enriched proteins (>5-fold enrichment), suggesting that a large subpopulation of cellular MS2L-tagged RNA-containing mRNPs are involved in mRNA degradation. The co-purification of one protein – the nonsense-mediated mRNA decay factor Upf1 – with all three MS2L-tagged RNAs suggests that the studied mRNAs may be targeted by NMD and thus subjected to accelerated mRNA decay leading to high enrichment levels of mRNA decay factors (see Discussion, Part 1, "MS2L-tagged RNAs may be targeted by nonsense-mediated decay"). MS2L-tagged RNAs also co-purified with several proteins implicated in stress granule formation (e.g. Pbp1, Pub1, see also Table 19), hinting at the possibility that a subpopulation of mRNPs might be stalled in the process of translation initiation in these cytoplasmic mRNP granules. The nuclear stage of the mRNA life cycle is reflected by co-purification of MS2L-tagged RNAs with several RBPs know to be loaded on mRNPs in the nucleus (e.g. Nab2, Sro9, Cbc2-Cbc1, see also Table 20).

The largest group of MS2L-tagged RNA co-purifying proteins is comprised of rRNA-binding proteins; 38-43% of the enriched proteins in each MS data set classified under this category. The majority of the rRNA-binding proteins are ribosomal proteins of the 40S or 60S subunit. The enrichment of ribosomal proteins of both the small and large subunit strongly suggests that the MS2L-tagged RNAs have been captured while bound to 80S ribosomes and thus engaged in translation. The minor group among the enriched rRNA-binding proteins is composed of 17 ribosome biogenesis factors (e.g. Mrd1, Arx1, see also Table 21). Most of the ribosome biogenesis factors were enriched only in single MS data sets. Furthermore, the enrichment level of these proteins was mostly moderate (<3-fold enrichment), raising the question whether ribosome biogenesis factors might have co-purified with contaminating ribosomes. Even though we cannot rule out that the enriched ribosome biogenesis factors represent false-postitive interactors in our experiments, recent findings from other groups suggest that mRNA binding by ribosome biogenesis factors might be a common phenomenon both in yeast and in mammalian cells (S. F. Mitchell et al. 2013; Castello et al. 2012; Baltz et al.

2012) (see Discussion, Part 1, "Ribosome biogenesis factors co-purifying with MS2L-tagged RNAs").

Ribosome biogenesis factors were not the only group of proteins that are known to bind RNA but not known to associate with mRNA among the MS2L-tagged RNA co-purifying proteins. We also identified 3 tRNA methyltransferases (Trm44, Trm2, Ncl1) and 3 tRNA pseudouridine synthases (Pus1, Pus4, Pus7). Most of these proteins were highly enriched (>5-fold enrichment) and co-purified with at least two MS2L-tagged RNAs (Table 23). Remarkably, the human homologs of 4 enriched tRNA-modifying enzymes out of 5 conserved proteins (Pus4 is not conserved between yeast and human) have been found to co-purify with poly(A)$^+$ RNA from mammalian cells (Castello et al. 2012; Baltz et al. 2012), suggesting that Trm2, Ncl1, Pus1 and Pus7 play a jet undefined but conserved role in mRNA biology.

The studied MS2L-tagged RNAs co-purified with several proteins which have been implicated in RNA-binding but whose RNA-interaction partners remain unknown or whose function in general or in the context of mRNA biology is not well understood. Such proteins include, for instance, the putative RBP Ygr250c and the GTPase Rbg1 together with its interaction partner Tma46, all three proteins have a possible role in translation initiation, or the deubiquitinase Ubp3 and its positive regulator, a putative RBP Bre5, or the putative homolog of DEAH-box family of RNA-dependent ATPases Ylr419w. All the mentioned proteins and their human homologs have been identified in several studies to co-purify with poly(A)$^+$ RNA or with Pab1 (S. F. Mitchell et al. 2013; Castello et al. 2012; Baltz et al. 2012; Klass et al. 2013; R. Richardson et al. 2012). Hence, our results confirm previous observations and identify Ygr250c, Rbg1, Tma46, Ubp3, Bre5 and Ylr419w as mRNP proteins. Three proteins, the general vacuolar RNAse Rny1 involved in rRNA and tRNA decay, the mitochondrial RNA polymerase Rpo41 and the intronic branchpoint binding complex component Msl5 were highly enriched after *ENO2-6MS2L* affinity purification. Even though all the mentioned proteins are RBPs, their role in the context of *ENO2* mRNA, which is transcribed from an intronless gene and encodes a cytoplasmic protein, remains unknown.

14-22% of MS2L-tagged RNA co-purifying proteins have previously not been identified as RBPs. This group include proteins that have likely co-purified with mRNA in association with mRNA-bound ribosomes; however, in case of many of the enriched proteins with no known RNA-binding activity the mechanism underlying co-purification with MS2L-tagged RNAs remain elusive. Ribosome-association of proteins involved in nascent peptide maturation can easily explain why the MS2L-tagged RNAs have co-purified, for instance, with the components of the ribosome-associated chaperone triad (Leidig et al. 2013) or the NatA N-terminal acetyltransferase (Matthias Gautschi et al. 2003; Polevoda et al. 2008) (Table 14). Ribosome-association is also a

plausible reason for the co-purification of three proteins involved in tRNA aminoacylation (Arc1, Gus1, Ths1, Table 32) with 6MS2L-RNA. Interestingly, several aminoacyl-tRNA synthetases have been found to associate with ribosomes in organisms as diverse as humans and archaea (Kaminska et al. 2009; Raina et al. 2012; David et al. 2011; Godinic-Mikulcic et al. 2014). The enrichment of Pgk1 protein after *PGK1-6MS2L* affinity purification and enolase 2 after *ENO2-6MS2L* affinity purification but not *vice versa* suggests that the nascent peptide has co-purified with the mRNA it is encoded by as an mRNA-ribosome-nascent peptide complex (see Discussion, Part 1 "Ribosomal proteins, translation factors, nascent peptide modifying enzymes and proteins involved in translation regulation – mRNP proteome reveals translation and a network of translation-associated molecular events as part of MS2L-tagged RNA life cycle"). This chain of interactions might also contribute to the co-purification of several glycolytic enzymes with *PGK1-6MS2L* (Table 31). Namely, several lines of evidence indicate that glycolytic enzymes associate into multi-enzyme complexes presumably for optimal pathway activity (Campanella, Chu, and Low 2005; Puchulu-Campanella et al. 2013; Araiza-Olivera et al. 2013; Araiza-Olivera et al. 2010). The co-purification of *PGK1-6MS2L* with Pgk1 protein and additional glycolytic enzymes thus hints at the possibility of co-translational glycolytic enzyme complex formation (see Discussion, Part 1 "*PGK1-6MS2L* co-purifies with several glycolytic enzymes – co-translational formation of a supramolecular glycolytic enzyme complex?").

Besides the glycolytic enzymes that were enriched after *PGK1-6MS2L* affinity purification, 13 additional metabolic enzymes co-purified with the MS2L-tagged RNAs (Table 25). None of these proteins is known to have RNA-binding activity, however, the high enrichment level and co-purification with more than one of the tested RNAs suggests that the association of at least 6 of the identified metabolic enzymes with the MS2L-tagged RNAs is specific. Remarkably, 5 of these 6 proteins have previously been found to co-purify with poly(A)$^+$ RNA or Pab1, including the inosine monophosphate dehydrogenase isozymes Imd2, Imd3 and Imd4, the mitochondrial trifunctional C1-tetrahydrofolate synthase Mis1 and the mitochondrial serine hydroxymethyltransferase Shm1 (S. F. Mitchell et al. 2013; Castello et al. 2012; Klass et al. 2013; R. Richardson et al. 2012). Our results are thus in agreement with previous observations and suggest that the above mentioned proteins play a jet undefined role in mRNA biology (see Discussion, Part1, "Metabolic enzymes co-purifying with MS2L-tagged RNAs"). The sixth protein in this group, the mitochondrial aldehyde dehydrogenase Ald5, might specifically interact with the 6MS2L-tag. Even though the protein has not been found to co-purify with poly(A)$^+$ RNA (S. F. Mitchell et al. 2013; Castello et al. 2012; Baltz et al. 2012) or with Pab1 (Klass et al. 2013; R. Richardson et al. 2012), Ald5 was highly enriched after the affinity purification of all three MS2L-tagged RNAs. The remaining 7 metabolic enzymes co-purified only with single

MS2L-tagged RNAs and were mostly <2-fold enriched, suggesting that at least some of these enriched proteins might represent false-positive interactors. Additional moderately enriched proteins in single MS data sets included Vph1, a subunit of the vacuolar ATPase V0 domain; Sec16, COPII coat assembly protein; Tra1, a subunit of SAGA and NuA4 histone acetyltransferase complexes; Hsp60, a mitochondrial chaperonin; Bmh2, a 14-3-3 protein; Ymr046c, the Gag nucleocapsid protein of retrotransposon Ty1, etc. While non-specific interaction with MS2L-tagged RNA-containing mRNPs might contribute to the isolation of some of these proteins, other proteins such as Bmh2 and Ymr046c possibly represent *bona fide* interaction partners (see Discussion).

Table 12. Examples of MS2L-tagged RNA co-purifying proteins identified by quantitative MS. An MS2L-tagged RNA co-purifying protein was considered enriched if it fulfilled the threshold criteria $\log_2$ (H/L) > 0.5 or <0.5 (+).

Category	Gene	Protein ID	Function	BB[a]	RNase	ENO2	6MS2L
mRNA-binding protein	TIF4631	P39935	Translation initiation	+	+	+	+
	SCP160	P06105	Translation regulation	+	+		+
	DHH1	P39517	Translation repression, Decay			+	
	DCP2	P53550	Decay	+	+	+	
	XRN1	P22147	Decay	+	+	+	+
	NAB2	P32505	Export		+		+
	KHD1	P38199	Localization		+		+
	WHI3	P34761	Unknown		+		
rRNA-binding protein/ ribosome-associated protein	RPL4A	P10664	Ribosomal 60S subunit protein	+	+	+	+
	MRD1	Q06106	Ribosome biogenesis			+	+
	ARX1	Q03862	pre-60S subunit export	+	+	+	
	ZUO1	P32527	Ribosome-associated chaperone	+	+	+	+
	GUS1	P46655	Cytosolic glutamyl-tRNA synthetase				+
	PGK1	P00560	Glycolytic enzyme	+	+		
tRNA-binding protein	TRM2	P33753	tRNA methyltransferase		+	+	+
	PUS7	Q08647	tRNA, snRNA, rRNA pseudouridine synthase			+	+
Undefined RNA-binding protein	YGR250C	YGR250C	Putative RBP		+		+
	UBP3	Q01477	Ubiquitin-specific protease	+	+	+	
	RPO41	P13433	Mitochondrial RNA polymerase			+	
Not annotated as RBP	IMD4	P50094	Inosine monophosphate dehydrogenase		+	+	+
	ALD5	P40047	Mitochondrial aldehyde dehydrogenase		+	+	+
	VHP1	P32563	Subunit of vacuolar ATPase	+			

[a] Abbreviations here and hereafter: BB – Boiled Beads sample of *PGK1-6MS2L* affinity purification, RNase – RNase eluate of *PGK1-6MS2L* affinity purification.

DISCUSSION

mRNP affinity purification: our strategy and the obtained results at a glance

In order to analyse the proteome of *in vivo*-assembled mRNA-protein complexes in *S. cerevisiae*, we have established an affinity purification method that enables to capture specific mRNAs together with the mRNA-associated proteins. Our approach makes use of two naturally occurring high affinity interactions: (1) the interaction between the bacteriophage MS2 coat protein and its RNA binding site (Lago et al. 1998); and (2) the interaction between IgG and the IgG-binding Z domains of *Staphylococcus aureus* protein A (Cedergren et al. 1993). The mRNA of interest is genomically tagged with MS2 stem-loops (Haim et al. 2007) and co-expressed with MS2 coat protein fused to IgG-binding domains. mRNPs assembled on the tagged mRNAs are isolated using IgG-coupled beads (Oeffinger et al. 2007). Quantitative analysis of the mRNP proteome is achieved by using SILAC metabolic labelling technique in combination with sample analysis by liquid chromatography-tandem mass spectrometry. Using this system, we have analysed the proteins co-purifying with 6MS2L-tagged *PGK1* and *ENO2*. In addition, we have identified the proteins that associate with an mRNA-like transcript containing the 6MS2L tag.

Our approach should provide an overall picture of the various interactions an mRNP is involved in during its life cycle. In order to preserve the native structure of the mRNP, we used optimized yeast cell harvesting and lysis conditions. Harvesting was done in the presence of glucose and amino acids, which enabled to preserve ribosome binding to the tagged mRNA (Fig. 15A and 15B). mRNA degradation during cell lysis could be prevented by breaking the cells under cryo conditions (Fig. 6B). Removal of cell debris by a short centrifugation step followed by lysate filtration, the use of superparamagnetic IgG-coupled beads as the affinity matrix and a short capture time of the tagged mRNPs (30 min) enabled to finish the whole affinity purification procedure in about 70 min. The relatively fast completion of the affinity purification should further help to maintain the native structure of the mRNPs.

We did not include a cross-linking step to stabilize mRNP composition. On the one hand, excluding a cross-linking step helps to avoid possible changes in mRNP protein composition that might occur in response to cross-linking. The two commonly used cross-linking approaches to stabilize RNA-protein interactions – cross-linking by 254 nm UV light or by

formaldehyde – often involve removal of the cells from their natural growth environment (Slobodin and Gerst 2010; S. F. Mitchell et al. 2013). This likely alters the mRNP composition of mRNAs engaged in translation; removal of the carbon source has been shown to result in rapid inhibition of translation in *S. cerevisiae* which is paralleled by the loss of polyribosomes (Ashe, De Long, and Sachs 2000). Cross-linking may also induce a stress response and thereby influence the abundance of mRNPs in different subcellular compartments. For instance, exposure to UV light has been shown to increase the accumulation of P-body marker proteins in P-bodies (Teixeira et al. 2005), indicating an increase in the translationally repressed pool of mRNPs. On the other hand, omitting a cross-linking step may result in the loss of weak or transient interaction partners during mRNP affinity purification. Indeed, in several cases we observed that only certain subunits of well defined heteromeric protein complexes were enriched after MS2L-tagged RNA affinity purification. In a test experiment the treatment of yeast cells with formaldehyde (0.05% v/v) (Slobodin and Gerst 2010) did not enable us to detect additional specifically enriched proteins compared to the purification of untagged control mRNA. In this experiment the protein pattern of RNase eluates was compared on silver stained SDS-PAGE (Fig. 10A). We cannot rule out that by using a more sensitive method such as mass spectrometry to analyse the protein composition of the RNase eluate samples we would have been able to detect a beneficial effect of formaldehyde cross-linking on mRNP stability.

Despite not using cross-linking to stabilize mRNP composition, a relatively large number of proteins were enriched after MS2L-tagged RNA affinity purification. The number of proteins that were >2-fold enriched in both biological replicate experiments was 58, 83 and 71 for *PGK1-6MS2L* (combined proteins enriched in RNase eluate and boiled beads samples), *ENO2-6MS2L* and 6MS2L-RNA, respectively. PANTHER Protein Class analysis indicated that >50% of the enriched proteins in each MS data set were nucleic acid binding proteins (Table 11). A manual literature-based analysis identified 26-35% of the enriched proteins as previously known mRNA-binding proteins or as components of protein complexes containing annotated mRNA-binding subunits (Appendix, Table 3 and 4). Importantly, 60-72% of the MS2L-tagged RNA co-purifying proteins perform a function related to mRNA biology (Appendix, Table 3). The repertoire of the proteins co-purifying with the three MS2L-tagged RNAs reflects both nuclear and cytoplasmic steps of mRNP life cycle. Many of the enriched proteins in each MS data set have a role in mRNA translation or decay, suggesting that a large subpopulation of the MS2L-tagged RNAs is engaged in these two processes. The large number of enriched proteins involved in mRNA biology as revealed by PANTHER analysis and the literature-based analysis thus indicates that the established mRNP affinity purification method is successful at capturing *in vivo*-assembled mRNPs. This notion is also supported by STRING analysis, which demonstrated that the

proteins co-purifying with a specific MS2L-tagged RNA are largely physically or functionally linked to each other (Fig. 23-25).

In addition to the enriched proteins with a well defined role in mRNP life cycle, many of the MS2L-tagged RNA co-purifying proteins participate in cellular processes not connected to mRNA function. Interestingly, in several such cases we found that the protein has previously been implicated in association with mRNA or the major yeast poly(A) binding protein Pab1 (S. F. Mitchell et al. 2013; Castello et al. 2012; Baltz et al. 2012; Tsvetanova et al. 2010; Scherrer et al. 2010; Klass et al. 2013; R. Richardson et al. 2012). Collectively, our mRNA-associated proteome analysis suggests a role for several ribosome biogenesis factors, tRNA modifying enzymes and metabolic enzymes in mRNA biology. Our results also point to the possibility of co-translational glycolytic enzyme complex formation. However, each MS data set also contains a few proteins which possibly represent false-positive interactors. Non-specific association of cellular proteins with MS2L-tagged RNA-containing mRNPs likely takes place during the 30 min of mRNP capture when proteins released from different cellular compartments can form interactions that normally would not occur. Candidate false-positive interactors include some cytoplasmic metabolic enzymes and mitochondrial proteins that have previously not been found to associate with mRNA and also some RBPs that might non-specifically interact with the MS2L-tagged RNAs due to deregulated RNA-binding activity upon cell lysis. During mRNP capture the *bona fide* mRNP proteins, especially those with fast association and dissociation rates, might also rearrange between mRNPs assembled on different mRNAs (X. Wang and Huang 2008). However, these rearrangements should not lead to changes in protein SILAC ratios since mRNP affinity purification from heavy and light labelled lysate is carried out separately. The IgG-coupled beads with the captured mRNPs from two parallel purifications are only mixed prior to mRNP protein release by RNase treatment (Fig. 17), thus preventing the exchange of heavy and light labelled forms of dynamically interacting mRNP proteins between MS2L-tagged RNA-containing mRNPs and the untagged control RNA-containing mRNPs.

The focus of the first part of the discussion is on the analysis of the mRNA-bound proteome and the possible molecular mechanisms responsible for the co-purification of a certain set of proteins with each tested MS2L-tagged RNA. The second part of the discussion tries to find answers to the questions that stem from the results of mRNP proteome analysis. For instance, what features of mRNA-protein and protein-protein interactions determine their efficient capture using our mRNP affinity purification method? What are the limitations of the MS2L system for mRNP affinity purification and how these limitations could be overcome? In the final part of the discussion unanticipated findings regarding mRNP protein composition will be recapitulated.

Part 1

Ribosomal proteins, translation factors, nascent peptide modifying enzymes and proteins involved in translation regulation – mRNP proteome reveals translation and a network of translation-associated molecular events as part of MS2L-tagged RNA life cycle

The analysis of mRNP proteome of *PGK1-6MS2L*, *ENO2-6MS2L* and 6MS2L-RNA revealed that ribosomal proteins comprise the most abundant group of enriched proteins in the three MS data sets. Depending on the data set, 49-70% of the 33 *S. cerevisiae* small ribosomal subunit proteins and 30-37% of the 46 large ribosomal subunit proteins classified as enriched (reviewed in D. N. Wilson and Cate 2012). The relative abundance of enriched 40S ribosomal proteins compared to 60S ribosomal proteins is thus not specific for *PGK1-6MS2L* affinity purification (see Results, "RNase elution efficiency"), supporting the notion that 60S rRNA might have been less efficiently degraded than 40S rRNA during RNase treatment.

The presence of both 40S and 60S ribosomal proteins among the enriched proteins suggest that all three MS2L-tagged RNAs can be captured while bound to 80S ribosomes. This finding is in agreement with northern blot and qRT-PCR results showing that 18S and 25S rRNAs are enriched after *PGK1-6MS2L* affinity purification compared to the mock purification of untagged *PGK1* (Fig. 15). The enrichment of 80S ribosomal components after affinity purification of the three MS2L-tagge RNAs thus suggests that a subset of *PGK1-6MS2L*, *ENO2-6MS2L* and 6MS2L-RNA transcripts has been engaged in translation at the moment of flash freezing the yeast cells in LN_2. Enrichment of several canonical translation factors and ribosome-associated proteins involved in translational control or nascent peptide maturation further supports the notion that we have been able to capture mRNPs that in living cells were engaged in protein synthesis. In addition, the protein product of *PGK1-6MS2L* or *ENO2-6MS2L* is among the enriched proteins in the respective MS data set. The simplest explanation to this finding is that the nascent peptide has co-purified with the mRNA it is encoded by as a nascent peptide-ribosome-mRNA complex. Alternatively, Pgk1 and enolase 2 could directly bind to the mRNA the respective protein is encoded by. While there is no experimental evidence suggesting that enolase 2 would possess RNA-binding activity (Castello et al. 2012; Baltz et al. 2012; Tsvetanova et al. 2010; Scherrer et al. 2010), Pgk1 has been identified as a candidate RBP in mammalian cells by poly(A)$^+$ RNA affinity purification combined with co-purifying protein identification by label-free quantitative MS (Castello et al. 2012). However, in a similar study in mammalian cells the protein was not detected after poly(A)$^+$ RNA affinity purification (Baltz et al. 2012). Furthermore, Pgk1 also failed to be identified as an RBP in *S. cerevisiae* in two separate studies

where RBPs were identified by probing high-density protein microarrays with mRNA (Tsvetanova et al. 2010) or with different types of cellular RNA (Scherrer et al. 2010). These latter findings suggest that Pgk1 is not an RBP and thus the likely reason for Pgk1 protein co-purification with *PGK1-6MS2L* is the association between the Pgk1 nascent peptide, the ribosome and the mRNA. Identification of Pgk1 as a candidate RBP by Castello et al. could in fact also be due to the above-mentioned chain of interactions. In this study, many ribosomal proteins were identified as RBPs, suggesting poly(A)$^+$ RNA co-purification with translating ribosomes (Castello et al. 2012). Since UV crosslinking was used to covalently crosslink direct RNA-protein interactions (Castello et al. 2012), Pgk1 nascent peptide could have been crosslinked to ribosomal exit tunnel (Bhushan et al. 2010), ultimately leading to Pgk1 identification as a candidate RBP.

The only translation factors enriched in all three MS data sets are the two eIF4G isoforms in *S. cerevisiae*, eIF4G1/*TIF4631* and eIFG2/*TIF4632* (Goyer et al. 1993) (Table 13). During translation initiation eIF4G acts as a scaffold protein, which coordinates the interaction between the mRNA 5' cap and the 3' poly(A) tail, as well as promotes the recruitment of 43S PIC to the mRNA (reviewed in Hinnebusch 2011). Among its direct interaction partners are the cap-binding protein eIF4E (Goyer et al. 1989; J. D. Gross et al. 2003) and the RNA helicase eIF4A (Dominguez et al. 1999; Neff and Sachs 1999; P. Schütz et al. 2008). Both proteins were found among the enriched proteins: eIF4E/*CDC33* was enriched in 6MS2L-RNA affinity purification and eIF4A/*TIF1* was enriched in *PGK1-6MS2L* affinity purification.

eIF5B/*FUN12*, the ribosome-dependent GTPase that mediates ribosomal subunit joining, was found enriched in two MS data sets (*ENO2-6MS2L* and 6MS2L-RNA). Other identified translation initiation factors were enriched in single MS data sets. 6MS2L-RNA co-purified with eIF3a/*RPG1* (also known as *TIF32*), which is one of five eIF3 core subunits in *S. cerevisiae* (Phan et al. 1998). Besides eIF3a, a possible eIF3 subunit encoded by *CLU1/TIF31* (Vornlocher et al. 1999) was also found enriched. *ENO2-6MS2L* co-purified with eIF2 subunits eIF2α/*SUI2* and eIF2γ/*GCD11*. The third eIF2 subunit eIF3β/*SUI3* was enriched in the forward experiment but was not quantified in the reverse experiment, suggesting that the subunit might have dissociated from eIF2 heterotrimer during affinity purification. Besides eIF2, which delivers the initiator methionyl tRNA to the 40S ribosome during canonical translation initiation, *ENO2-6MS2L* co-purified with a second protein with the potential to direct the binding of Met-tRNA$_i^{Met}$ to small ribosomal subunit – eIF2A/*YGR054W* (Merrick and Anderson 1975). Importantly, the requirements of the two proteins for Met-tRNA$_i^{Met}$ binding to 40S ribosome are different – eIF2 requires GTP (Safer et al. 1975) whereas eIF2A the presence of an AUG codon (Merrick and Anderson 1975). Consistent with a role in translation initiation, eIF2A genetically

interacts with several yeast translation initiation factors (Wilmes et al. 2008; Komar et al. 2005) and binds to 40S ribosomal subunit and 80S ribosome (Komar et al. 2005); however, the exact molecular function of the protein remains elusive (Komar et al. 2005; Reineke et al. 2011). Among the proteins enriched in single MS data sets was also the only identified translation elongate factor – eEF3 (encoded by paralogous genes *YEF3* and *HEF3*), which was enriched after *PGK1-6MS2L* affinity purification.

Table 13. MS2L-tagged RNA co-purifying translation factors. Log_2 (H/L) ratios of enriched proteins are in bold.

Protein ID	Protein name	Gene	BB		RNase		ENO2		6MS2L	
			for	rev	for	rev	for	rev	for	rev
P39935	eIF4G1	TIF4631	**1.42**	**-2.08**	**1.23**	**-1.76**	**0.92**	**-2.39**	**4.51**	**-4.78**
P39936	eIF4G2	TIF4632	**1.78**	**-2.34**	**1.76**	**-2.37**	**2.04**	**-3.70**	**5.31**	**-5.76**
P07260	eIF4E	CDC33			-0.68	0.21	-1.73	1.37	**3.72**	**-3.03**
P38912	eIF4A	TIF1	1.29		**1.40**	**-0.54**	2.38		-0.32	-1.29
P39730	eIF5B	FUN12	0.46		0.36	0.16	**2.09**	**-3.41**	**1.60**	**-1.37**
P38249	eIF3a	RPG1	0.20	-0.01	1.26				**1.22**	**-0.99**
Q03690	eIF3 p135	CLU1	0.31	-0.04	0.12	0.08	-0.07	-2.08	**1.67**	**-1.20**
P20459	eIF2α	SUI2	-0.19	0.19	-0.18	0.29	**1.25**	**-1.25**	0.26	0.09
P32481	eIF2γ	GCD11	0.17		-0.07	0.15	**1.02**	**-1.34**	0.30	-0.05
P09064	eIF2β	SUI3	-0.64	0.73	-0.32	0.68	1.13			
P53235	eIF2A	YGR054W	0.27		0.10	0.06	**1.94**	**-2.82**		-0.72
P16521; P53978	eEF3	YEF3; HEF3	**0.73**	**-0.82**	**1.52**	**-1.38**	0.63	-0.21	0.10	-0.66

Co-translational nascent peptide maturation steps reflected in mRNP proteome

The nascent polypeptide emerging from the ribosomal exit tunnel undergoes several co-translational maturation steps, which may include N-terminal enzymatic processing, chaperone-assisted protein folding and targeting to endoplasmatic reticulum (ER). The ribosome plays an important role in the spatial and temporal coordination of these maturation steps by acting as a binding platform for the processing enzymes (reviewed in Kramer et al. 2009; Jha and Komar 2011). The proteome of the studied mRNPs contains several enzymes participating in co-translational maturation of the nascent peptide (Table 14). Among the proteins enriched in all three MS data sets are Ssb2, Ssz1 and Zuo1, which form a functional ribosome-associated chaperone triad (Matthias Gautschi et al. 2002; Leidig et al. 2013; Albanèse et al. 2006). Ssb2 and its functionally interchangeable isoform Ssb1 belong to the Hsp70 family of proteins (Boorstein, Ziegelhoffer, and Craig 1994) whose members participate in various protein folding processes in the cell (reviewed in Mayer and Bukau 2005). The chaperone function of Hsp70 proteins depends on ATP binding and hydrolysis. The J-domain co-chaperones promote stable Hsp70

interaction with the substrate by stimulating Hsp70 ATPase activity, whereas nucleotide exchange factors control the duration of Hsp70 interaction with the substrate by stimulating ADP-ATP exchange on Hsp70 (reviewed in Kampinga and Craig 2010). The co-chaperone function in the Ssb/Ssz1/Zuo1 ribosome-associated chaperone triad is performed by Ssz1 and Zuo1, which form the so-called ribosome-associated complex (RAC) (M Gautschi et al. 2001; P. Huang et al. 2005; Conz et al. 2007). RAC dynamically associates with the ribosome in the vicinity of the peptide tunnel exit through Zuo1-mediated interactions with ribosomal proteins and rRNA (Raue, Oellerer, and Rospert 2007; Peisker et al. 2008; Leidig et al. 2013). Ssb associates with the ribosome independently of RAC (Rakwalska and Rospert 2004); however, the direct binding site of Ssb remains unknown (reviewed in Peisker, Chiabudini, and Rospert 2010). Unlike RAC, the co-immunoprecipitation of nascent polypeptides with Ssb is not abolished by ribosome release, suggesting a stable interaction between Ssb and the newly synthesised proteins (Albanèse et al. 2006). Collectively, these findings indicate that Ssz1 and Zuo1 interaction with the MS2L-tagged RNAs is ribosome-mediated. Ssb2 interaction with the MS2L-tagged RNAs is also likely ribosome-mediated but interactions with the nascent peptide may contribute to Ssb2 co-purification with *PGK1-6MS2L* and *ENO2-6MS2L* mRNAs.

6MS2L-RNA co-purified with the α-subunit of the heterodimeric nascent polypeptide-associated complex (NAC). The prevalent cellular version of yeast NAC is composed of an α-subunit encoded by *EGD2* and a β-subunit encoded by *EGD1* (Reimann et al. 1999). The exact molecular function of NAC remains unknown; however, the complex interacts both with the nascent peptide and the ribosome (Reimann et al. 1999; Wiedmann et al. 1994; Beatrix, Sakai, and Wiedmann 2000; Wegrzyn et al. 2006) and has therefore been proposed to act as a molecular chaperone (reviewed in Kramer et al. 2009; Jha and Komar 2011). Ribosome-association of NAC is mediated by the β-subunit, which has been shown to bind to the large ribosomal subunit protein L25 located adjacent to peptide tunnel exit (Wegrzyn et al. 2006). The complex can be dissociated from ribosomes by high salt wash and purified as an intact heterodimer from both yeast and mammalian cells, thus suggesting a stable interaction between the α- and β-subunit (Wiedmann et al. 1994; Reimann et al. 1999). Considering the high salt concentration needed to release NAC from ribosomes (500 mM KAc) as well as the salt-resistance of the α- and β-subunit interaction, it is surprising that only one of the NAC subunits was enriched after 6MS2L-RNA affinity purification. Similarly, in *ENO2-6MS2L* MS data set the α- and β-subunit of NAC were enriched for only one of the biological replicate experiments. These results hint at the possibility that in our experimental setup the ribosome- and possibly also nascent peptide-mediated interaction between the MS2L-tagged RNA and the NAC complex might have been prone to dissociation.

Nascent polypeptide emergence from the peptide tunnel exit is for the majority of proteins accompanied by the removal of the N-terminal methionine (reviewed in Giglione, Boularot, and Meinnel 2004; Kramer et al. 2009; Jha and Komar 2011). The enrichment of methionine amonopeptidase-2/*MAP2* (MetAP-2) after *ENO2-6MS2L* affinity purification suggests that the enolase 2 protein is subjected to N-terminal methionine excision (NME). Whether a protein undergoes NME or not is determined by the second residue; an N-terminal methionine followed by a small and uncharged residue is removed (Boissel et al. 1985; Tsunasawa, Stewart, and Sherman 1985; Flinta et al. 1986; Ben-Bassat et al. 1987; S. Huang et al. 1987; Hirel et al. 1989; Moerschell et al. 1990). Importantly, the second residue in enolase 2, alanine, belongs to the group of amino acids known to elicit NME (Boissel et al. 1985; Tsunasawa, Stewart, and Sherman 1985; Flinta et al. 1986), thus suggesting that *ENO2-6MS2L* has been captured in complex with the nascent peptide undergoing NME. Pgk1 with serine as the second residue should also be subjected to NME; however, neither of the two N-terminal aminopeptidases expressed in yeast was enriched after *PGK1-6MS2L* affinity purification. Assuming that Pgk1 is an NME substrate, this result suggests that the interaction between Pgk1 nascent peptide and the N-terminal aminopeptidase might have been lost during mRNP affinity purification.

Co-purification of *ENO2-6MS2L* with the two subunits of the N-terminal acetyltransferase NatA reveals another nascent peptide enzymatic modification step. N-terminal acetylation is a very common protein modification in eukaryotic cells; more than 50% of yeast and 80% of mammalian proteins are N-terminally acetylated (Arnesen et al. 2009). Since in most of the yeast proteins the N-terminal methionine is removed, the majority of the proteins are acetylated by NatA – the N-terminal acetyltransferase family member whose activity depends on prior NME (Soppa 2010; Starheim, Gevaert, and Arnesen 2012; Kramer et al. 2009). The transfer of the acetyl moiety form acetyl coenzyme A to the N-terminal α-amino group is catalyzed by NatA subunit Ard1 (also known as Naa10); the auxiliary subunit Nat1 (also known as Naa15) mediates NatA ribosomal association (Matthias Gautschi et al. 2003; Polevoda et al. 2008) likely in the vicinity of the peptide tunnel exit (reviewed in Kramer et al. 2009). Surprisingly, enolase 2 has been shown not to be N-terminally acetylated (Polevoda and Sherman 2003; Arnesen et al. 2009), raising the question why an enzyme mediating N-terminal acetylation would co-purify with *ENO2-6MS2L*. The answer to this question may lie in the finding that Nat1 does not only cross-link to nascent peptides that are NatA substrates but also efficiently cross-links to nascent peptides that are not subjected to N-terminal acetylation (Matthias Gautschi et al. 2003). Cross-link formation between Nat1 and the nascent peptide is abolished after puromycin treatment, which leads to premature nascent chain release from the ribosome, indicating that Nat1

interaction with the nascent peptide can be ribosome-mediated (Matthias Gautschi et al. 2003). A likely explanation for Nat1 and Ard1 co-purification with *ENO2-6MS2L* would therefore be that *ENO2-6MS2L* mRNA is translated on ribosomes that are associated with NatA. Unlike enolase 2, Pgk1 is N-terminally acetylated (Polevoda and Sherman 2003; Arnesen et al. 2009). Even though NatA subunits were not classified as enriched after *PGK1-6MS2L* affinity purification, Nat1 with $\log_2$ (H/L) ratio 0.41 in the forward experiment and -0.6 in the reverse experiment almost fulfilled the set threshold criteria for enriched proteins. Therefore, it seems likely that at least the ribosome-binding subunit of NatA complex has co-purified also with *PGK1-6MS2L*.

Table 14. MS2L-tagged RNA co-purifying proteins involved in co-translational nascent peptide maturation. $\log_2$ (H/L) ratios of enriched proteins are in bold.

Protein ID	Gene	BB		RNase		ENO2		6MS2L	
		for	rev	for	rev	for	rev	for	rev
Ribosome-associated chaperone triad									
P40150	SSB2	**1.08**	**-1.55**	**1.89**	**-1.74**	**2.13**	**-3.70**	**1.68**	**-1.49**
P38788	SSZ1	**0.90**	**-0.89**	**1.08**	**-1.42**	**2.83**	**-3.75**	**1.08**	**-0.63**
P32527	ZUO1	**0.75**	**-1.45**	**1.10**	**-1.37**	**2.75**	**-3.65**	**1.01**	**-0.62**
Nascent polypeptide-associated complex									
P38879	EGD2			0.02	-0.28	0.39	-1.36	**0.79**	**-0.52**
N-terminal methionine excision									
P38174	MAP2			-0.31	0.54	**2.25**	**-2.99**		-0.48
N-terminal acetylation by NatA									
P07347	ARD1	-0.91				**1.67**	**-2.41**		-0.56
P12945	NAT1	0.49		0.41	-0.60	**2.08**	**-2.52**		

mRNP proteome reveals complex translation regulation of MS2L-tagged RNAs

Several proteins enriched in the three MS data sets are ribosome-associated proteins implicated in translation regulation (Table 15). The repertoire of these proteins suggests that the translation of the studied MS2L-tagge RNA is dynamically regulated to enable both active translation on polyribosomes as well as translational repression to promote mRNA decapping and decay.

A highly enriched protein (>5-fold enrichment) in all three MS data sets involved in both translation-promoting and -repressing molecular events is Asc1 (Ceci et al. 2003; Shor et al. 2003; Chantrel et al. 1998; Sezen, Seedorf, and Schiebel 2009; Rachfall et al. 2013). Asc1 is a core protein of the 40S ribosomal subunit believed to mediate signals from the cellular signalling pathways to the ribosome (reviewed in J. Nilsson et al. 2004). Consistent with a role in signal transduction, the loss of *ASC1* leads to defects in multiple cellular processes (Valerius et al. 2007; Rachfall et al. 2013; Zeller, Parnell, and Dohlman 2007; Melamed et al. 2010). This evolutionarily

highly conserved protein is located on the 40S subunit in close proximity to the mRNA exit site and is proposed to form a binding platform for the simultaneous recruitment of multiple factors (Sengupta et al. 2004; Taylor et al. 2009; Ullah et al. 2008). One such protein whose ribosome-association seems to be partly mediated by Asc1 is Scp160 (Baum et al. 2004) – a protein also enriched after *PGK1-6MS2L* and 6MS2L-RNA affinity purification. Scp160 is a KH domain RNA-binding protein (Weber et al. 1997) proposed to bind most if not all cellular mRNAs (Hogan et al. 2008a). Scp160 has been shown to associate with cytosolic as well as membrane-bound polyribosomes (Frey, Pool, and Seedorf 2001). The protein likely associates with polyribosomes as a component of mRNP complexes as suggested by the findings that Scp160 polysome-association is mRNA-dependent (Frey, Pool, and Seedorf 2001) and that upon EDTA treatment, which dissociates polysomes and 80S monosomes into single subunits (Nolan and Arnstein 1969; Blobel 1971), Scp160 is released from ribosomes in a complex also containing the major yeast poly(A)-binding protein Pab1(Lang and Fridovich-Keil 2000). A third protein identified in complex with Scp160 and Pab1 after yeast cell cytosolic extract treatment with EDTA was Bfr1 (Lang and Fridovich-Keil 2000). This protein was also among the enriched proteins in our mRNP affinity purification experiments. Several lines of evidence suggest that Scp160 and Bfr1 are functionally related. Firstly, both proteins bind a large and overlapping set of cellular mRNAs (Hogan et al. 2008a). Secondly, gene deletion of either *SCP160* or *BFR1* results in similar phenotypes characterized by increased cell ploidy and abnormal cell morphology (Wintersberger, Kühne, and Karwan 1995; C. L. Jackson and Képès 1994). As mentioned above, the two proteins also seem to be physically associated as components of mRNP complexes (Lang and Fridovich-Keil 2000). Similarly to Scp160, Bfr1 associates with polyribosomes in an RNase-sensitive manner (Lang et al. 2001). However, the exact molecular function of Scp160 and Bfr1 interactions with the mRNA and polyribosomes has remained elusive. The deletion of *BFR1* has been shown to largely disrupt Scp160 recruitment to polyribsomes; interestingly, the opposite was not observed (Lang et al. 2001). This finding suggests a role for Bfr1 in recruiting Scp160-containing mRNPs to ribosomes for translation (Lang et al. 2001). The role of Scp160 in translation was recently addressed in our laboratory (Hirschmann et al. 2014). Unexpectedly, the results point to the possibility that Scp160 may exert its positive effect on translation by increasing the pool of tRNAs available for the translation machinery via promoting tRNA recycling and/or preventing tRNA diffusion.

PGK1-6MS2L and *ENO2-6MS2L* have co-purified with two related proteins – the GTPase Rbg1 and its binding partner Tma46. Both proteins cosediment with 80S ribosomes and polyribosomes in sucrose density gradients, suggesting a function in translation (Francis et al. 2012; Daugeron et al. 2011). Ribosome recruitment of the Rbg1-Tma46 complex is mediated by

the latter protein, which also modulates Rbg1's GTP binding and hydrolytic activity (Francis et al. 2012). Rbg1 and Tma46 seem to perform overlapping roles with several other proteins – a severe growth defect is only observed in a triple knock-out strain where *RBG1* and its paralog *RBG2* is deleted in combination with *SLH1* (Daugeron et al. 2011), a gene encoding for a putative RNA helicase (Martegani et al. 1997; V. Pena et al. 2009). The reduced amount of polyribosomes and accumulation of 80S monosomes in Δ*rbg1*Δ*rbg2*Δ*slh1* strain hints at a possible role for Rbg1 and Tma46 in translation initiation because a similar change in polysome profile is observed also for strains defective in translation initiation (Daugeron et al. 2011).

Table 15. **MS2L-tagged RNA co-purifying proteins involved in translation regulating.** Log_2 (H/L) ratios of enriched proteins are in bold.

Protein ID	Gene	BB		RNase		ENO2		6MS2L	
		for	rev	for	rev	for	rev	for	rev
Possible role in general translation regulation									
P38011	ASC1	**0.90**	**-2.61**	**2.68**	**-3.02**	**2.39**	**-3.68**	**2.52**	**-1.88**
P06105	SCP160	**0.79**	**-1.18**	**0.87**	**-0.76**	-0.23	-0.93	**1.49**	**-1.25**
P38934	BFR1	**2.01**	**-2.61**	**1.79**	**-2.60**	**3.05**	**-4.43**	**2.51**	**-1.67**
Possible role in translation initiation									
P39729	RBG1	**3.95**	**-2.76**	**2.49**	**-3.07**	**2.87**	**-3.21**		
Q12000	TMA46	**4.05**	**-2.96**	**2.70**	**-3.25**	**1.07**	**-3.19**		
P53316	YGR250C	0.67		**1.23**	**-1.37**		-3.51	**4.02**	**-3.53**
P40561	SGN1		-1.07	**0.95**	**-1.28**		-3.59	**3.92**	**-3.27**
Role in translation repression									
P10080	SBP1	1.97	-0.39	**0.66**	**-0.91**	0.02	-2.07	**3.51**	**-2.03**
P39015	STM1	**2.50**	**-2.76**	**2.51**	**-2.97**	**2.54**	**-4.39**	**1.82**	**-1.42**
P39517	DHH1			0.48	-0.54	**1.05**	**-2.34**	0.61	-0.01
P25644	PAT1		-3.82	**3.76**	**-4.70**	**3.88**	**-6.48**	**3.19**	**-3.13**
mRNA- and ribosome-associated protein									
Q04600	TMA64			**1.21**	**-1.67**	3.33			

A putative RBP encoded by *YGR250C* (Feroli et al. 1997; Sartori et al. 2000) was enriched after *PGK1-6MS2L* and 6MS2L-RNA and possibly also after *ENO2-6MS2L* affinity purification. In case of the latter mRNA, Ygr250c was not quantified in the forward labelling experiment; however, the H/L ratio obtained in the reverse labelling experiment suggested >11-fold enrichment. An interaction between Rbg1 and Ygr250c has been identified by yeast 2-hybrid system (Wout et al. 2009; Ito et al. 2000). However, this interaction was not observed in a genome-wide analysis of yeast protein complex composition (Gavin et al. 2006). Instead, the most highly scored Ygr250c's interaction partner in this study was Sgn1 (Gavin et al. 2006) – an mRNA-binding protein proposed to modulate mRNA expression in the cytoplasm possibly by

enhancing translation initiation (Winstall et al. 2000). A role for Sgn1 in translation initiation is also supported by the findings of a genome-wide analysis of yeast protein complex composition. Namely, the two highest scoring interaction partners of Sgn1 in this study were eIF4G1/*TIF4631* and eIF4G2/*TIF4632* (Gavin et al. 2006). Furthermore, 8 out of 10 identified interaction partners of Ygr250c (including eIF4G1/*TIF4631* and eIF4G2/*TIF4632*) were also among the interaction partners of Sgn1 (Gavin et al. 2006), suggesting that the two proteins act in concert possibly as components of one protein complex.

In our experiments Sgn1 was enriched, similarly to Ygr250c, after *PGK1-6MS2L* and 6MS2L-RNA affinity purification. In case of *ENO2-6MS2L*, Sgn1 was not quantified in the forward labelling experiment but was >12-fold enriched in the reverse labelling experiment, suggesting that the protein might also associate with *ENO2-6MS2L*-containig mRNPs. The missing H/L ratio in *ENO2-6MS2L* MS data set for both Sng1 and Ygr250c in the forward labelling experiment and a similar enrichment level in the reverse labelling experiment supports the notion that the two proteins might function as part of one protein complex.

Several enriched proteins in each MS data set have been implicated in translation repression. These proteins include Sbp1, Stm1 and the mRNA decapping activators Dhh1 and Pat1. The latter protein was enriched in all three MS data sets and with >8.5-fold enrichment was also the most highly enriched of the four above-mentioned proteins. *In vitro* experiments have shown that Pat1 interferes with an early step in translation by limiting the formation of 48S PIC (Nissan et al. 2010).

The second protein among the translational repressors enriched in all three MS data sets was Stm1. This ribosome-associated protein (M. W. Van Dyke et al. 2004; N. Van Dyke, Baby, and Van Dyke 2006) has been shown by genetic analysis to promote Dhh1-meidated translational repression and mRNA decay (Balagopal and Parker 2009). *In vitro* studies have revealed that Stm1 can block translation after ribosomal subunit joining (Balagopal and Parker 2011).

The determined H/L ratios hint at the possibility that Sbp1 and Dhh1 are similarly to Pat1 and Stm1 enriched after the affinity purification of all three MS2L-tagged RNAs. However, the set threshold criteria for enriched proteins were fulfilled by Sbp1 only after *PGK1-6MS2L* and 6MS2L-RNA affinity purification and by Dhh1 only after *ENO2-6MS2L* affinity purification. Translational repression upon glucose deprivation is attenuated in Δ*sbp1* as well as in Δ*dhh1* yeast cells, revealing a role for both proteins in global translation repression of mRNAs (J. Coller and Parker 2005; Segal, Dunckley, and Parker 2006). Genetic analysis has shown that Sbp1 and Dhh1 function together to promotes translation repression (Segal, Dunckley, and Parker 2006); however, Dhh1 alone is required for mRNA decapping, revealing an important difference in the function of the two proteins (Segal, Dunckley, and Parker 2006; J. M. Coller et al. 2001). Sbp1

has been proposed to block the recruitment of 43S PIC to the mRNA because recombinant Sbp1 can directly bind eIF4G and repress the translation of a reporter mRNA (Rajyaguru, She, and Parker 2012). *In vitro*, Dhh1 has been shown to interfere with stable 48S PIC formation (J. Coller and Parker 2005), whereas *in vivo* the protein seems to interfere with the elongation step by reducing ribosome transit rate (Sweet, Kovalak, and Coller 2012).

The protein encoded by *TMA64* was enriched after *PGK1-6MS2L* and possibly also after *ENO2-6MS2L* affinity purification. In case of the latter mRNA, Tma64 was 10-fold enriched in the forward labelling experiment but was not quantified in the reverse labelling experiment, suggesting that the protein might have dissociated form the mRNP during affinity purification. The function of Tma64 is unknown. The protein contains a putative pseudouridine synthase and archaeosine transglycosylase (PUA) RNA-binding domain (Fleischer et al. 2006), which, among other proteins, is found in pseudouridine synthase enzymes and in RNA methyl transferases (reviewed in Pérez-Arellano, Gallego, and Cervera 2007). Tma64 has been found to co-purify with ribosomes (Fleischer et al. 2006) and with epitope-tagged Pab1 in an RNA-independent manner (R. Richardson et al. 2012). These findings hint at the possibility that Tma64 is a component of mRNPs engaged in translation.

MS2L-tagged RNAs seem to be largely degraded in the 5'→3' exonucleolytic decay pathway

All studied MS2L-tagged RNAs have co-purified with several mRNA decay factors (Table 16). The repertoire of these proteins suggests that the decay of *PGK1-6MS2L* and *ENO2-6MS2L* and possibly also 6MS2L-RNA is mechanistically similar and involves the same cellular mRNA decay pathways. Most of the identified mRNA decay factors were highly enriched after MS2L-tagged RNA affinity purification with $\log_2$ (H/L) ratios >2 or <-2. The high enrichment of mRNA decay factors suggests that mRNA degradation comprises a prominent step in the life cycle of the studied RNAs.

The proteome of *PGK1-6MS2L* and *ENO2-6MS2L*-containing mRNPs indicates that the mRNAs are degraded in 5'→3' exonucleolytic decay pathway. The prerequisite for mRNA degradation in 5'→3' direction is 5' cap removal. Indeed, both MS2L-tagged mRNAs have co-purified with almost the entire set of proteins known to assemble into a decapping mRNP, including the two subunits of the decapping complex and several decapping activators (see Introduction, "Decapping" and references therein). Dcp2, the catalytic core of the decapping complex, was >9-fold enriched after *PGK1-6MS2L* and *ENO2-6MS2L* affinity purification and Dcp1, which stimulates the catalytic step, was >2.5-fold enriched. Structural analysis of the yeast

decapping complex has shown that Dcp2 and Dcp1 form a 1:1 complex (She et al. 2008) with Dcp2 being the subunit that interacts with the 5' cap and the mRNA body (Deshmukh et al. 2008). Therefore, the lower enrichment level of Dcp1 likely reflects the loss of this subunit from the decapping complex during affinity purification.

Table 16. MS2L-tagged RNA co-purifying proteins involved in mRNA decay. Log_2 (H/L) ratios of enriched proteins are in bold.

Protein ID	Gene	BB		RNase		ENO2		6MS2L	
		for	rev	for	rev	for	rev	for	rev
Decapping complex									
P53550	DCP2	**4.38**	**-5.01**	**4.28**	**-4.10**	**3.24**	**-3.90**		
Q12517	DCP1			**1.91**	**-2.29**	**1.52**	**-2.71**		-0.07
Decapping activators[a]									
P39998	EDC3		-4.04	**3.54**	**-4.00**	**3.11**	**-4.60**		
P40070	LSM4			**1.59**	**-1.55**	**0.58**	**-0.82**	**3.42**	**-2.23**
P47017	LSM1			1.82		**2.46**	**-2.94**		-2.81
P38203	LSM2		-0.49	3.26		**2.87**	**-3.17**		
mRNA exoribonucleases									
P22147	XRN1	**2.99**	**-3.42**	**2.89**	**-2.98**	**3.16**	**-4.08**	**3.39**	**-2.60**
P53010	PAN2			**3.09**	**-3.88**	**3.10**	**-4.45**	**4.63**	**-4.99**
P36102	PAN3			**3.13**	**-4.30**	**3.10**	**-4.22**	**5.87**	**-4.74**
Nonsense-mediated decay									
P30771	UPF1	**6.78**	**-7.41**	**5.92**	**-6.08**	**3.18**	**-4.78**	**4.45**	**-4.08**
Q03466	EBS1	**4.99**	**-5.20**	**4.47**	**-5.49**	**4.86**	**-4.72**		
Q12129	NMD4			4.65		**1.65**	**-4.17**	**4.56**	**-3.06**

[a] The Log_2 (H/L) of decapping activators Pat1 and Dhh1 are listed in table 15.

From the well characterized decapping activators Pat1, Dhh1, Scd6, Edc3 and Lsm1-7 only Scd6 was not found among the enriched proteins after *PGK1-6MS2L* or *ENO2-6MS2L* affinity purification. Pat1 and Edc3 with >8.5-fold enrichment were the most highly enriched decapping activators in both MS data sets. Dhh1 was >2-fold enriched after *ENO2-6MS2L* affinity purification. The protein did not classified as enriched after *PGK1-6MS2L* affinity purification; however, the determined log_2 (H/L) ratios 0.48 and -0.54 suggest that Dhh1 also co-purified with *PGK1-6MS2L*. Neither *PGK1-6MS2L* nor *ENO2-6MS2L* co-purified with all seven subunits of the Lsm1-7 complex. The enriched subunits included Lsm4, Lsm1 and Lsm2 in *ENO2-6MS2L* MS data set and Lsm4 in *PGK1-6MS2L* MS data set. In case of the latter mRNA, Lsm1 and Lsm2 were enriched in the forward labelling experiment but were not quantified in the reverse labelling experiment, suggesting that the same set of Lsm1-7 subunits may have co-purified with *PGK1-6MS2L* as with *ENO2-6MS2L*. The absence of Lsm3 and Lsm5-7 among the enriched proteins, as well as Lsm1 and Lsm2 missing detection in *PGK1-6MS2L* reverse

experiment indicates that Lsm1-7 complex is prone to dissociation during mRNP affinity purification. Interestingly, the Lsm subunits enriched in our MS data sets have been shown to interact with mRNA or with Pat1 (Chowdhury, Mukhopadhyay, and Tharun 2007; Sharif and Conti 2013; D. Wu et al. 2013). These interactions likely help to maintain Lsm4, Lsm1 and Lsm2 attached to the mRNP during MS2L-tagged mRNA affinity purification.

Decapped mRNAs are rapidly degraded by the 5'→3' exoribonuclease Xrn1. Xrn1was >7-fold enriched after *PGK1-6MS2L* and *ENO2-6MS2L* affinity purification, indicating that the exorbonuclease is, at least partly, responsible for the degradation of MS2L-tagged *PGK1* and *ENO2*.

The set of mRNA decay factors co-purifying with 6MS2L-RNA had one major difference compared to *PGK1-6MS2L* and *ENO2-6MS2L* co-purifying proteins – the two subunits of the decapping complex did not co-purify with 6MS2L-RNA. However, other important components of the 5'→3' exonucleolytic decay pathway, including Pat1 and the Lsm4 subunit of the Lsm1-7 complex plus the 5'→3' exoribonuclease Xrn1, were among the enriched proteins. These results suggest that 6MS2L-RNA is similarly to *PGK1-6MS2L* and *ENO2-6MS2L* degraded in 5'→3' decay pathway. The decapping complex might have been lost from 6MS2L-RNA during affinity purification.

In the deadenylation dependent mRNA decay pathway mRNA decapping and 5'→3' exonucleolytic decay is preceded by poly(A) tail shortening to oligo(A) length. Surprisingly, none of the MS2L-tagged RNAs co-purified with components of the Ccr4-Not complex, which is believed to be the major poly(A)-specific 3' exoribonuclease in yeast (see Introduction, "Deadenylation" and references therein). Instead, the components of the heterodimeric Pan2-Pan3 complex were >8-fold enriched in all three MS data set. Pan2-Pan3 complex has been shown to mediate the shortening of the newly synthesised poly(A) tails to mRNA-specific lengths. Therefore, the enrichment of Pan2-Pan3 in all three MS data sets may reflect the trimming step of poly(A) tails of the MS2L-tagged RNAs. However, resent findings by Sun et al. (M. Sun et al. 2013) suggest that Pan2-Pan3 complex could also perform the deadenylation of the MS2L-tagged RNAs leading to mRNA decapping and decay. Namely, comparative dynamic transcriptome analysis suggested that Ccr4-Not and Pan2-Pan3 deadenylase complexes prefer different mRNA substrates (M. Sun et al. 2013). Unfortunately, *PGK1* and *ENO2* were not among the analysed mRNAs in this study and therefore it is not known if these mRNAs would be preferentially deadenylated by Pan2-Pan3 complex. It remains possible that Pan2-Pan3 complex is, in addition to trimming the initially synthesised poly(A) tails, responsible for *PGK1-6MS2L*, *ENO2-6MS2L* and 6MS2L-RNA deadenylation, ultimately leading to the 5'→3' exonucleolytic decay of these RNAs.

MS2L-tagged RNAs may be targeted by nonsense-mediated decay

The most highly enriched protein after *PGK1-6MS2L* affinity purification in the two biological replicate experiments was Upf1 – the key effector of nonsense-mediated mRNA decay pathway (see Introduction, "NMD factors and consequences of their activation" and references therein). The protein was also highly enriched after *ENO2-6MS2L* and 6MS2L-RNA affinity purification, raising the possibility that the studied MS2L-tagged RNAs may be targeted by NMD (Table 16).

The only difference between *PGK1-6MS2L* and *ENO2-6MS2L* and the respective wt mRNAs is the 6MS2L tag inserted immediately downstream of the translation termination codon, which increases the distance to poly(A) tail by 421 nt compared to wt mRNA. Positioning the normal stop codon too far upstream of the poly(A) tail has been shown to redefine the stop codon as premature and activate NMD (see Introduction, "NMD targets and the mechanism of their recognition" and references therein). The MS2L-tagging technique (Haim et al. 2007) may thus have the potential to turn wt mRNAs into NMD targets.

6MS2L-RNA may also contain NMD-activating features, which may include a large distance between the stop codon and the poly(A) tail. Proteins of the translation machinery co-purifying with 6MS2L-RNA indicate that the transcript is translated. Therefore, 6MS2L-RNA seems to be sensed as an mRNA by the yeast cells. If the first AUG codon of the predicted 6MS2L-RNA transcript (see Results, "Control RNA to determine the effect of MS2L tag on mRNP protein composition") is used to initiate translation, 500 nt would separate the translation stop codon from the poly(A) tail, possibly leading to NMD activation due to a large distance between the stop codon and the poly(A) tail.

Other enriched proteins in the three MS data sets implicated in NMD include Ebs1 and Nmd4. Ebs1 was >22-fold enriched after *PGK1-6MS2L* and *ENO2-6MS2L* affinity purification but was missing among the enriched proteins after 6MS2L-RNA purification. Nmd4 was enriched after *ENO2-6MS2L* and 6MS2L-RNA affinity purification. In case of *PGK1-6MS2L* purification, the protein was 25-fold enriched in the forward experiment but was not quantified in the reverse experiment, suggesting that Nmd4 might have dissociated from the mRNP during affinity purification. Very little is known about the role of Nmd4 in nonsense-mediated decay. The protein was originally identified in a yeast 2-hybrind screen searching for Upf1 interaction partner (F He and Jacobson 1995). Later genetic studies have shown that *NMD4* deletion suppresses growth defects in Δ*xrn1* cells, whereas the deletion of *NMD4* in Δ*lsm7* or Δ*ski2* cells leads to a stronger growth defect than in single mutants (Wilmes et al. 2008). The role of Ebs1 in NMD is also not well defined. The protein seems to perform a non-essential function in canonical NMD as suggested by the finding that the level of NMD substrates is less elevated in Δ*ebs1* cells than in Δ*upf1*, Δ*upf2* or Δ*upf3* cells (Luke et al. 2007). Upf1 co-immunoprecipitates

with Ebs1 in an RNase-insensitive manner, suggesting a physical interaction between the proteins (Luke et al. 2007).

MS2L-tagged RNA co-purifying proteins implicated in poly(A) tail-mediated interactions

Poly(A) tail provides a platform for a multitude of RNA-protein and protein-protein interactions. An important role in the poly(A) tail-mediated interactions is played by the major yeast poly(A)-binding protein Pab1. In one study the protein was shown to be involved in about 200 RNA-dependent but ribosome-independent protein-protein interactions (Klass et al. 2013). Another study identified 55 proteins that co-immunoprecipitated with Pab1 in an RNA-independent manner (R. Richardson et al. 2012). Collectively, these two studies identify a set of proteins that associate with Pab1-containing mRNPs. Table 17 shows the identified proteins in the above-mentioned studies that were also enriched in our affinity purification experiments. Altogether, 61 proteins enriched in our MS data sets have previously been found to co-purify with Pab1, indicating that the MS2L-tagged RNAs are involved in multiple protein-protein interactions through their Pab1-bound poly(A) tails. The current chapter will focus on four proteins in the SILAC protein-protein interaction networks whose association with the mRNP is mediated by the poly(A) tail (Table 18).

As expected, Pab1 was enriched after *PGK1-6MS2L* and *ENO2-6MS2L* affinity purification, indicating that these mRNAs carry a poly(A) tail that is bound by Pab1. Pab1 was enriched also after 6MS2L-RNA affinity purification, confirming our hypothesis that the *CYC1* transcriptional terminator would provide the necessary signals for poly(A) addition to the 6MS2L-RNA transcript. Surprisingly, the enrichment level of Pab1 was roughly 6-fold higher after 6MS2L-RNA affinity purification compared to the affinity purifications of the two MS2L-tagged mRNAs. This finding hints at the possibility that compared to the MS2L-tagged mRNAs, 6MS2L-RNA has on average a longer poly(A) tail that can accommodate more Pab1 molecules, thus leading to a higher enrichment level of Pab1 after 6MS2L-RNA affinity purification (Beilharz and Preiss 2007). A difference in the average poly(A) tail length can reflect differences in deadenylation and mRNA decay kinetics (reviewed in Eckmann, Rammelt, and Wahle 2011); however, it remains to be experimentally determined whether the deadenylation rate differs between 6MS2L-RNA and the MS2L-tagged mRNAs.

Table 17. **MS2L-tagged RNA co-purifying proteins previously found to co-purify with epitope-tagged Pab1 (see next page).** Protein co-purification with Pab1 was shown to be RNA-independent (reference 1) or RNA-dependent but ribosome-independent (reference 2). An MS2L-tagged RNA co-purifying protein was considered enriched if it fulfilled the threshold criteria $\log_2$ (H/L) > 0.5 or <0.5 (+).

Description	Reference[a]	Gene	BB[b]	RNase	ENO2	6MS2L
Translation initiation, elongation[c]	1, 2	eIF4G1/TIF4631	+	+	+	+
	1,2	eIF4G2/TIF4632	+	+	+	+
	2	eIF5B/FUN12			+	+
	2	eIF2α/SUI2			+	
	2	eIF2γ/GCD11			+	
	1, 2	eIF2A/YGR054w			+	
	2	eIF4E/CDC33				+
	1, 2	eIF3 p135/CLU1				+
	1, 2	eEF3/YEF3	+	+		
Translation repression	2	STM1	+	+	+	+
	2	PAT1		+	+	+
	2	SBP1		+		+
mRNA decay	1, 2	XRN1	+	+	+	+
	2	LSM4		+	+	+
	2	LSM2			+	
Nonsense-mediated decay	1	UPF1	+	+	+	+
	2	EBS1	+	+	+	
Nuclear cap-binding complex	2	CBC2				+
	1, 2	CBC1				+
Poly(A)$^+$ RNA-binding protein	1	HRB1		+	+	
	2	NAB2		+		+
	1, 2	PUB1		+		+
RNA-binding protein	2	BFR1	+	+	+	+
	2	KHD1		+	+	+
	2	SRO9		+	+	
	1, 2	YGR250C		+		+
	1, 2	SGN1		+		+
	1	LHP1		+		
	2	DED1				+
Splicing	2	MSL5			+	
	2	PSP2			+	
Ribosome biogenesis	2	ARX1	+	+	+	
	1, 2	CBF5	+		+	
	1, 2	RRP5				+
	2	DIM1	+			
	2	RRP7	+			
	2	UTP22	+			
	2	NOP4	+			
	1	KRR1	+			
	1	KRI1	+			
	1	NUG1	+			
Mitochondrial	1, 2	MIS1	+		+	+
	1, 2	YLR419W	+		+	
	2	SHM1			+	+
	2	MGM101			+	
	2	MSS116	+			
Translation machinery-associated	2	RBG1	+	+	+	
	1, 2	TMA46	+	+	+	
Ubiquitin-specific protease	1, 2	UBP3	+	+	+	+
Ubiquitin protease cofactor	2	BRE5	+			
Inosine monophosphate dehydrogenase	2	IMD4		+	+	+
Nascent peptide maturation	2	ARD1			+	
Histone acetyltransferase complex	2	TRA1	+			

[a] Reference 1 (R. Richardson et al. 2012), reference 2 (Klass et al. 2013).
[b] Abbreviations: BB – Boiled Beads sample of *PGK1-6MS2L* affinity purification, RNase – RNase eluate of *PGK1-6MS2L* affinity purification.
[c] Ribosomal proteins are omitted from the comparison.

Pab1 has been found to interact with Pbp1 in yeast 2-hybrid assay (D A Mangus, Amrani, and Jacobson 1998). Using the same method, an interaction was identified between Pbp1 and Pbp4 (David A Mangus, Smith, et al. 2004), as well as between Pbp1 and Mkt1 (Tadauchi et al. 2004). *ENO2-6MS2L* and 6MS2L-RNA co-purified with all four above-mentioned proteins. *PGK1-6MS2L* co-purified with Pab1 and Mkt1 and possibly also with Pbp1, which was among the enriched proteins in the forward labelling experiment but was not quantified in the reverse labelling experiment (Table 18). The exact molecular function of Pbp1, Mkt1 and Pbp4 is not well defined; all three proteins are encoded by non-essential genes and seem to regulate the expression of their target mRNAs at the post-transcriptional level. Pbp1 is a negative regulator of Pan2-Pan3 deadenylase activity (David A Mangus, Smith, et al. 2004); the protein cosediments with polyribosomes in sucrose density gradients (D A Mangus, Amrani, and Jacobson 1998; Tadauchi et al. 2004) and localizes to stress granules upon glucose deprivation (Buchan, Muhlrad, and Parker 2008). Stress granules are thought to store mRNPs stalled in translation initiation because the typical components of these cytoplasmic aggregates are translation initiation factors and the 40S ribosomal subunit (reviewed in Carolyn J. Decker and Parker 2012). Pbp1 also plays a role in stress granule formation as indicated by a strong reduction in stress granule formation in Δ*pbp1* cells upon glucose deprivation (Buchan, Muhlrad, and Parker 2008). Glucose deprivation also leads to the accumulation of Pbp4 in stress granules; however, unlike Δ*pbp1* cells, Δ*pbp4* cells do not show a strong defect in stress granule formation (Swisher and Parker 2010).

Table 18. MS2L-tagged RNA co-purifying proteins that either directly or indirectly bind the poly(A) tail. Log_2 (H/L) ratios of enriched proteins are in bold.

Protein ID	Gene	BB		RNase		ENO2		6MS2L	
		for	rev	for	rev	for	rev	for	rev
P04147	PAB1	**2.46**	**-3.26**	**2.58**	**-3.36**	**2.12**	**-3.66**	**5.33**	**-5.58**
P53297	PBP1	**1.55**	**-2.00**	1.30		**2.75**	**-4.12**	**3.23**	**-2.99**
Q07362	PBP4			-0.51	0.43	**3.30**	**-3.43**	**3.49**	**-2.67**
P40850	MKT1	**1.49**	**-1.67**	**1.34**	**-1.60**	**2.00**	**-2.95**	**3.43**	**-2.88**

Pbp1 has been implicated together with Mkt1 in positive regulation of *HO* endonuclease mRNA translation (Tadauchi et al. 2004). Similarly to Pbp1, Mkt1 cosediments with polyribosomes (Tadauchi et al. 2004). Mkt1 polysome-association depends on Pbp1, but not *vice versa* (Tadauchi et al. 2004). A role for Mkt1 in post-transcriptional gene expression regulation is further supported by the large number of different cellular processes that are influenced by allelic variation of *MKT1*. Namely, the gene encoding for *MKT1* has been identified in several quantitative trait locus mapping studies to provide resistance to stressful growth conditions including exposure to high temperature (L. M. Steinmetz et al. 2002; Sinha et al. 2006), high

ethanol (Swinnen et al. 2012) or low glucose (Parreiras, Kohn, and Anderson 2011). *MKT1* allelic variation also contributes to sporulation efficiency (Deutschbauer and Davis 2005), mitochondrial genome maintenance (Dimitrov et al. 2009) and drug sensitivity (Demogines et al. 2008).

A subpopulation of MS2L-tagged RNA-containing mRNPs might be sequestered to stress granules for translation repression

The more than 6-fold enrichment of two stress granule components, Pbp1 and Pub4, after *ENO2-6MS2L* and 6MS2L-RNA affinity purification (Table 18) hinted at the possibility that a subpopulation of mRNPs containing these two transcripts might be stalled in the process of translation initiation and are possibly localized to stress granules. Stress granules generally contain 40S ribosomal subunits and translation initiation factors eIF4E, eIF4G, eIF4A, eIF4B, eIF3 and eIF2. Besides these components, four additional proteins are considered to be typical stress granule constituents: Pab1 and Pbp1 discussed in the previous chapter plus Pub1 and Ded1 (reviewed in Carolyn J. Decker and Parker 2012).

Pub1 was enriched after *PGK1-6MS2L* and 6MS2L-RNA affinity purification and likely co-purified also with *ENO2-6MS2L* (Table 19). Besides Pab1, Pub1 is the major proteins that is crosslinked to poly(A)$^+$ RNA by UV light (J. T. Anderson, Paddy, and Swanson 1993; Matunis, Matunis, and Dreyfuss 1993). The protein contains three RNA recognition motifs, which have a high binding preference for U- or UA-rich sequences (Santiveri et al. 2011). Pub1 has been shown to directly bind to eIF4G1/*TIF4631* (Santiveri et al. 2011) and to the poly(A)$^+$ RNA-binding protein Nab2 (Apponi et al. 2007), both of which were among the enriched proteins in several MS data sets (Table 13 and 20). Pub1 does not co-sediment with polyribosomes on sucrose density gradients (J. T. Anderson, Paddy, and Swanson 1993; Ripmaster and Woolford 1993), which is in agreement with a role as a component of translationally inactive mRNPs (Buchan, Muhlrad, and Parker 2008). Interestingly, Pub1 was considerably more enriched after 6MS2L-RNA affinity purification (>4.5-fold enrichment) compared to *PGK1-6MS2L* affinity purification (>1.5 fold enrichment), suggesting that the subpopulation of translationally repressed mRNPs might be higher for 6MS2L-RNA.

The DEAD-box RNA helicase Ded1 (reviewed in Tarn and Chang 2009) was enriched only after 6MS2L-RNA affinity purification. The later protein seems to play an important regulatory role in stress granule formation (Hilliker et al. 2011). On the one hand, Ded1 may act as a translational repressor because the binding of Ded1 to eIF4F leads to mRNP accumulation in stress granules. On the other hand, Ded1 may activate translation in an ATP-dependent

manner. ATP hydrolysis by Ded1 leads to mRNP exit from stress granules and completion of translation initiation (Hilliker et al. 2011). The relatively low enrichment level (>1.5-fold enrichment) of Ded1 after 6MS2L-RNA affinity purification suggests that only a minor fraction 6MS2L-RNA-containing mRNPs are associated with this protein.

Table 19. MS2L-tagged RNA co-purifying proteins implicated in stress granule formation. Log_2 (H/L) ratios of enriched proteins are in bold.

Protein ID[a]	Gene	BB		RNase		ENO2		6MS2L	
		for	rev	for	rev	for	rev	for	rev
P32588	PUB1			**0.65**	**-0.73**	0.35	-1.80	**2.89**	**-2.22**
P06634	DED1	0.23	0.07	0.22	0.02	0.55	0.45	**1.09**	**-0.64**

[a] The Log_2 (H/L) of stress granule components Pab1 and Pbp1 are listed in table 18.

The nuclear history of MS2L-tagged RNAs is reflected by the enriched RBPs

All three tested MS2L-tagged RNAs have co-purified primarily with proteins whose steady state localization is cytoplasmic. The nuclear phase of mRNA life cycle of the studied transcripts is reflected by a few enriched shuttling RNA-binding proteins and nuclear RNA decay factors (Table 20).

The enriched shuttling RBPs co-purifying with MS2L-tagged RNAs include Nab2, Hrb1, Sro9 and Khd1 (also known as Hek2), all of which were enriched after *PGK1-6MS2L* affinity purification. *ENO2-6MS2L* co-purified with Hrb1 and Sro9 and possibly also with Nab2 and Khd1, which were enriched in one of the biological replicate experiments. 6MS2L-RNA co-purified with Nab2 and Khd1 but not with Sro9 or Hrb1. The latter protein is a shuttling SR-like RBP (Häcker and Krebber 2004) that is recruited to the transcribed genes via the THO complex (Häcker and Krebber 2004; E. Hurt et al. 2004). Recent findings indicated that Hrb1 is a quality control factor that ensures the export of only correctly spliced mRNAs (Hackmann et al. 2014). Hrb1 may recruit the TRAMP complex to initiate mRNA decay of incorrectly spliced transcripts (Hackmann et al. 2014). On properly spliced mRNAs, however, Hrb1 can recruit the mRNA export receptor Mex67 to enable quality controlled mRNA export (Hackmann et al. 2014). Interestingly, the binding of Hrb1 is not limited to intron-containing transcripts (E. Hurt et al. 2004; Hackmann et al. 2014), explaining why the protein co-purified with *PGK1-6MS2L* and *ENO2-6MS2L* derived from intronless genes.

Sro9, which similarly to Hrb1 co-purified with *PGK1-6MS2L* and *ENO2-6MS2L*, is associated with both transcribed genes (Röther et al. 2010) and translating ribosomes (Sobel and Wolin 1999). The protein has therefore been proposed to be loaded onto mRNA during

transcription and shuttle with the mature mRNP to cytoplasm (Röther et al. 2010). The exact molecular function of Sro9 remains unknown; however, since the protein has been shown not to be essential for the expression and stability of specific transcripts, the association of Sro9 with translating ribosomes hints at a role for Sro9 in translation regulation of bound mRNAs (Röther et al. 2010).

Table 20. Nuclear or shuttling RNA-binding proteins that co-purify with MS2L-tagged RNAs. Log_2 (H/L) ratios of enriched proteins are in bold.

Protein ID	Gene	BB		RNase		ENO2		6MS2L	
		for	rev	for	rev	for	rev	for	rev
Shuttling RNA-binding proteins									
P32505	NAB2			**1.16**	**-1.25**	1.78		**3.80**	**-3.75**
P38922	HRB1			**1.77**	**-1.87**	**3.10**	**-5.22**		
P25567	SRO9			**1.30**	**-1.69**	**1.65**	**-3.69**		
P38199	KHD1			**0.54**	**-0.76**		-2.63	**3.05**	**-2.38**
Nuclear cap-binding complex									
Q08920	CBC2				-0.12			**4.68**	**-4.74**
P34160	CBC1			0.79				**4.89**	**-5.38**
Nuclear mRNA decay factors									
P47047	MTR4	0.36		0.12	0.33	0.95		**0.99**	**-0.60**
Q02792	RAT1			0.05	0.12	**1.21**	**-1.52**		-1.52

The multifunctional poly(A)$^+$ RNA binding protein Nab2 (see Introduction, "mRNP export factors are recruited during transcription") co-immunoprecipitates with a large fraction of the yeast transcriptome, including *PGK1* (Batisse et al. 2009). This protein-mRNA interaction could be confirmed – Nab2 was moderately enriched (>2-fold enrichment) after *PGK1-6MS2L* affinity purification. Co-purification of Nab2 with *ENO2-6MS2L* was detected only in the forward labelling experiment (3.4-fold enrichment), suggesting that *ENO2-6MS2L* interaction with Nab2 might have been lost during affinity purification in the reverse labelling experiment. Remarkably, Nab2 was >13-fold enriched after 6MS2L-RNA affinity purification. This finding points to the possibility that the mRNA-like 6MS2L-RNA transcript may be differently regulated than the MS2L-tagged mRNAs. Nab2 is released from the mRNP at the cytoplasmic face of the nuclear pore complex (reviewed in Oeffinger and Zenklusen 2012). The higher level of Nab2 co-purification with 6MS2L-RNA therefore suggests that 6MS2L-RNA-containg ribonucleoprotein complex export form the nucleus to the cytoplasm and/or remodelling at the NPC cytoplasmic face may happen at a slower rate compared to MS2L-tagged mRNA-containing mRNPs.

The KH-domain protein 1, Khd1, was moderately enriched (>1.41-fold enrichment) after *PGK1-6MS2L* and relatively highly enriched (>5.2-fold enrichment) after 6MS2L-RNA affinity purification. The 6.2-fold enrichment of Khd1 in *ENO2-6MS2L* forward labelling experiment

suggests that Khd1 might also interacts with this mRNA. Even though Khd1 is associated with a large number of yeast transcripts (Hasegawa, Irie, and Gerber 2008; Hogan et al. 2008a), the interaction with *PGK1* and *ENO2* has previously not been reported (Hasegawa, Irie, and Gerber 2008), suggesting that Khd1 may have even more cellular targets than currently known. Hogan et al. have shown that immunoaffinity enrichment of mRNAs associated with Khd1 is negatively correlated with ribosome occupancy (Hogan et al. 2008a). Since Khd1 is required for the translational repression of *ASH1* mRNA expression during mRNA localization to the distal tip of the daughter cell (Paquin et al. 2007), the protein has been proposed to participate also in the translational control of its other target mRNAs (Hogan et al. 2008a).

6MS2L-RNA has co-purified with the two subunits of the nuclear cap-binding complex. Remarkably, more than 25-fold enriched, Cbc2 and Cbc1 (also known as Sto1) were among the most highly enriched proteins in 6MS2L-RNA MS data set. In contrast, the nuclear CBC subunits were not among the enriched proteins after affinity purification of the two MS2L-tagged mRNAs. Despite this, *PGK1* and *ENO2* are expected to be bound by the nuclear CBC (see Introduction, "Transcription elongation and the concomitant pre-mRNA processing events" and references therein). A 1.7-fold enrichment of Cbc1 in *PGK1-6MS2L* forward labelling experiment therefore suggests that only a minor fraction of the total cellular *PGK1-6MS2L* or *ENO2-6MS2L* pool is bound by the nuclear CBC. The steady state translation depends on the cap-binding translation initiation factor eIF4E (reviewed in Topisirovic et al. 2011). The exchange of the nuclear CBC to eIF4E is thought to take place before or during the pioneer round of translation (see Introduction, "Translation is accompanied by mRNP remodelling" and references therein). The absence of Cbc2 and Cbc1 among the enriched proteins after MS2L-tagged mRNA affinity purification indicates that the majority of cellular *PGK1-6MS2L* and *ENO2-6MS2L* transcripts have lost the nuclear CBC. Therefore, it is plausible to think that the MS2L-tagged mRNAs become rapidly engaged in translation upon export from the nucleus. The efficiency of nucleocytoplasmic export, RNP remodelling after export and/or pioneer translation initiation complex formation might be less efficient for 6MS2L-RNA-containing RNPs, leading to a prolonged association with the nuclear CBC. Collectively, the high enrichment of the nuclear CBC and Nab2 after 6MS2L-RNA affinity purification indicate that some aspects of 6MS2L-RNA regulation are different compared to MS2L-tagged mRNAs.

Besides cytoplasmic 5'→3' exonucleolytic mRNA decay, *ENO2-6MS2L* and 6MS2L-RNA transcripts seem also to be degraded in the nucleus (Table 20). The RNA helicase encoded by *MTR4* was enriched after 6MS2L-RNA and possibly also after *ENO2-6MS2L* affinity purification. Mtr4 is a component of the TRAPM complex, which acts as a co-factor for the nuclear RNA exosome (see Introduction, "Nuclear mRNP quality control" and references

therein). The gene product of *RAT1*, the nuclear 5'→3' exonuclease, was enriched after *ENO2-6MS2L* and possibly also after 6MS2L-RNA affinity purification. The relatively low enrichment level of Mtr4 and Rat1, which does not exceed 3-fold enrichment, suggests that nuclear mRNA decay of *ENO2-6MS2L* and 6MS2L-RNA is by far less prominent than cytoplasmic 5'→3' exonucleolytic decay of these RNAs (Table 16).

Ribosome biogenesis factors, tRNA-modifying enzymes, metabolic enzymes, mitochondrial proteins etc. – the studied MS2L-tagged RNA-containing mRNPs are involved in many unanticipated interactions with other cellular proteins

The functional diversity of proteins co-purifying with the MS2L-tagged RNAs is surprisingly high. The determined mRNP proteomes do not only contain proteins that participate in the various steps of the mRNA life cycle like mRNP biogenesis, translation and mRNA decay but also contain many proteins with well established roles in cellular processes that are unrelated to mRNA biology. The following chapter will introduce the MS2L-tagged RNA co-purifying proteins whose "standard" cellular function is not in mRNA regulation.

Ribosome biogenesis factors co-purifying with MS2L-tagged RNAs

Several ribosome biogenesis factors were enriched after affinity purification of the MS2L-tagged RNAs (Table 21). Most of these proteins were not highly enriched; only two proteins, Arb1 and Arx1, were >5-fold enriched. None of the ribosome biogenesis factors classified as enriched in all three MS data sets, although in one data set out of three Mrd1 and Arx1 failed only slightly to fulfil the threshold criteria, suggesting that the two proteins might have co-purified with all tested MS2L-tagged RNAs. Interestingly, the largest number of ribosome biogenesis factors classified as enriched in the BB sample MS data set, followed by *ENO2-6MS2L* MS data set.

Table 21. **MS2L-tagged RNA co-purifying proteins involved in ribosome biogenesis.** Log_2 (H/L) ratios of enriched proteins are in bold.

Protein ID	Gene	BB		RNase		ENO2		6MS2L	
		for	rev	for	rev	for	rev	for	rev
P33322	CBF5	**1.24**	**-1.12**	0.89		**1.25**	**-2.03**		
Q06106	MRD1			0.64	-0.30	**1.34**	**-2.60**	**1.27**	**-0.76**
P41819	DIM1	**0.51**	**-1.18**	1.39					
P25368	RRP7	**1.26**	**-0.52**						
P53254	UTP22	**1.07**	**-1.01**						
Q05022	RRP5	0.76	-0.36	0.62	-0.38			**1.96**	**-1.72**

Protein ID	Gene	BB		RNase		ENO2		6MS2L	
		for	rev	for	rev	for	rev	for	rev
P25586	KRR1	**0.82**	**-0.88**						
P42846	KRI1	**0.96**	**-1.10**				-3.34		
Q12499	NOP58	0.60	-0.26	**0.74**	**-0.66**				
P40024	ARB1	0.43	-0.40	**1.57**	**-1.40**	**3.26**	**-3.44**		
Q03862	ARX1	**1.28**	**-1.44**	**1.27**	**-1.50**	**2.33**	**-3.97**	1.16	-0.43
P38779	CIC1			-0.05		**0.53**	**-1.20**		
Q08208	NOP12	0.95		0.40		**1.07**	**-1.69**		-1.38
P53883	NOP13			-0.10	0.11	**0.69**	**-1.40**	1.64	-0.36
P40010	NUG1	**0.56**	**-0.75**						
P37838	NOP4	**0.73**	**-0.63**						
P38786	RPP1	**0.70**	**-2.40**						

Table 22. **Description of the enriched proteins involved in ribosome biogenesis.**

Gene[a]	Pre-ribosome	Location[b]	Description	Reference
CBF5	pre-90S	noc	Catalytic subunit of box H/ACA snoRNPs, which pseudouridylates both large and small rRNA	(D. L. Lafontaine et al. 1998; Duan et al. 2009)
MRD1	pre-90S	noc	40S biogenesis; required for initial A_0-A_2 cleavage of 35S pre-rRNA to produce 18S rRNA	(Jin et al. 2002; Segerstolpe et al. 2013)
DIM1	pre-90S	noc	40S biogenesis; rRNA dimethyladenosine tranferase; depletion leads to diminished accumulation of mature 18S rRNA	(Grandi et al. 2002; D. Lafontaine, Vandenhaute, and Tollervey 1995)
RRP7	pre-90S	noc, nuc	40S biogenesis; component of CURI complex	(Baudin-Baillieu et al. 1997; Rudra et al. 2007; Lin et al. 2013)
UTP22	pre-90S	noc, nuc	40S biogenesis; component of CURI complex	(Rudra et al. 2007; Bernstein et al. 2004; Lin et al. 2013)
RRP5	pre-90S	noc	40S and 60S biogenesis; required for cleavage of 35S pre-rRNA at sites A_0-A_3 to produce 18S rRNA and 5.8S rRNA	(Venema and Tollervey 1996; de Boer et al. 2006)
KRR1	pre-90S	noc	40S biogenesis; physically and functionally interacts with Kri1	(Gromadka and Rytka 2000; Sasaki, Toh-E, and Kikuchi 2000)
KRI1	pre-90S	noc	40S biogenesis; associates with snR30, which is a box H/ACA snoRNA	(Sasaki, Toh-E, and Kikuchi 2000; Hoareau-Aveilla et al. 2012)
NOP58	pre-90S	noc	box C/D snoRNP component, required for cleavage of 35S pre-rRNA at sites A_0 and A_2 to produce 18S rRNA	(D. L. Lafontaine and Tollervey 1999; P. Wu et al. 1998; Grandi et al. 2002)
ARB1	pre-90S, 40S, 60S	nuc, cyt	40S and 60S biogenesis; depletion leads to delayed processing of rRNA in 40S and 60S biogenesis pathways; shuttles	(Dong et al. 2005; Altvater et al. 2012)
ARX1	pre-60S	noc, nuc, cyt	pre-60S subunit export	(Bradatsch et al. 2007)
CIC1	pre-60S	noc	Cic1 co-purifies with 60S pre-ribosomes; interacts with 26S proteasome	(Oeffinger et al. 2007; Jäger et al. 2001; Harnpicharnchai et al. 2001)
NOP12	pre-60S	noc	60S biogenesis; involved in pre-25S processing; similar to Nop13 and Nsr1	(K. Wu, Wu, and Aris 2001; Granneman, Petfalski, and Tollervey 2011)
NOP13	pre-60S	noc, nuc	Nop13 contains RNA recognition motif; similar to Nop12 and Nsr1	(K. Wu, Wu, and Aris 2001)
NUG1	pre-60S	noc, nuc	60S export; putative GTPase	(Bassler et al. 2001; Oeffinger et al. 2007)
NOP4	pre-60S	noc, nuc	60S biogenesis; depletion leads to diminished accumulation of mature 25S rRNA	(C. Sun and Woolford 1997; C. Sun and Woolford 1994)
RPP1	-	noc, nuc, cyt	Component of RNase MRP and RNase P; RNase MRP required for production of 5.8S rRNA	(Houser-Scott et al. 2002; Marvin et al. 2011)

[a] Localization and function data was retrieved from *Saccharomyces* Genome Database (http://www.yeastgenome.org/; see also reference (Cherry et al. 2012)).
[b] Abbreviations: noc – nucleolus, nuc – nucleus, cyt – cytoplasm.

The repertoire of the enriched ribosome biogenesis factors covers various ribosome biogenesis steps from pre-rRNA processing to mature 18S and 25S rRNAs and pre-60S ribosomal particle export. Ribosome biogenesis starts within the nucleolus with the transcription of 35S pre-rRNA (reviewed in Kressler, Hurt, and Baβler 2010). Pre-rRNA transcription is accompanied by rRNA folding, modification and cleavage, as well as association with ribosomal proteins and *trans*-acting factors. Small nucleolar ribonucleoprotein particles (snoRNPs) perform pre-rRNA 2'-O-ribose methylation of nucleoside ribose moieties and uridine conversion into pseudouridines. 35S pre-rRNA is cleaved in 90S pre-ribosomal particle that, besides the pre-rRNA, contains around 50 non-ribosomal proteins, U3 snoRNA and several small subunit ribosomal proteins. Pre-rRNA sequential cleavage at sites A_0, A_1 and A_2 separates pre-40S and pre-60S ribosomal particles, which continue maturation along two independent assembly pathways. The maturation of pre-40S and pre-60S ribosomal particles, which includes final cleavage of rRNA, association of a few ribosomal proteins and release of *trans*-acting factors, is completed in the cytoplasm (reviewed in Panse 2011). Importantly, pre-40S and pre-60S ribosomal subunits acquire translation competence only after all the maturation steps have been successfully completed (reviewed in S. Schütz and Panse 2012). Therefore, it seems highly unlikely that ribosome biogenesis factors could have co-purified with MS2L-tagged RNAs owing to interactions with ribosomes engaged in translation of the MS2L-tagged RNAs. A more likely explanation to ribosome biogenesis factor co-purification with MS2L-tagged RNAs is that these proteins directly bind to mRNA and/or Pab1. This notion is supported by the findings from other groups, which have identified several ribosome biogenesis factors among the enriched proteins after epitope-tagged Pab1 affinity purification (Table 17) from yeast cell extracts treated with RNase A (R. Richardson et al. 2012) or supplemented with EDTA (Klass et al. 2013), which disrupts the association of ribosomal subunits. A large set of ribosome biogenesis factors was also enriched after poly(A)$^+$ RNA affinity purification form glucose deprived yeast cells; the authors present evidence that the enrichment of ribosome biogenesis factors was not due to co-purification of ribosomes (S. F. Mitchell et al. 2013). The human homologues of most of the enriched ribosome biogenesis factors (Appendix, Table 3) have been found to co-purify with poly(A)$^+$ RNA also in mammalian cells (Castello et al. 2012; Baltz et al. 2012). Collectively, our results support the notion that there may be extensive cross-talk between mRNA regulation and ribosome biogenesis (S. F. Mitchell et al. 2013).

tRNA-modifying enzymes co-purifying with MS2L-tagged RNAs

tRNA-modifying enzymes that perform uridine to pseudouridine isomerisation or methylation of ribonucleotide nucleobase or ribose comprise a small but interesting group of enriched proteins in each MS data set because several of these enzymes are highly enriched (Table 23 and 24). One of the six identified tRNA-modifying enzymes, Pus1, has previously been found to co-purify with poly(A)$^+$ RNA from glucose deprived yeast cells (S. F. Mitchell et al. 2013). Our results point to the possibility that association of tRNA-modifying enzymes with mRNA may be more common than previously known. The finding that several tRNA-modifying enzymes were enriched not only after MS2L-tagged *PGK1* and *ENO2* affinity purification but also after mRNA-like 6MS2L-RNA affinity purification suggests that tRNA-modifying enzymes might play an important role in the regulation of a wide variety of mRNAs. Remarkably, the human homologues of Pus1, Trm2, Ncl1 and Pus7 have also been found to co-purify with poly(A)$^+$ RNA (Castello et al. 2012; Baltz et al. 2012), suggesting that the role of tRNA-modifying enzymes in mRNA biology is evolutionarily conserved. The low abundance of mRNAs has hampered the analysis of mRNA modifications (reviewed in Ge and Yu 2013; Motorin and Helm 2011; Motorin, Lyko, and Helm 2010). Therefore, it remains to be determined if tRNA-modifying enzymes also catalyze the modification of mRNA and, if yes, what the exact effect of these modifications on mRNA regulation would be.

An additional protein involved in tRNA maturation, Lhp1, was enriched after *PGK1-6MS2L* affinity purification and the protein possibly also co-purified with *ENO2-6MS2L* and *6MS2L-RNA* (Table 23). In case of the latter MS2L-tagged RNAs, Lhp1 was quantified only in one of the biological replicate experiments, suggesting that Lhp1 may associate with all three MS2L-tagged RNAs. Lhp1 is not a tRNA-modifying enzyme. Instead, the protein stabilizes tRNA structure to enable proper tRNA 3' end formation (Yoo and Wolin 1997). The protein has been proposed to act as a molecular chaperone for all RNA Pol III transcripts since Lhp1 also stabilizes the newly synthesised U6 RNA to facilitate the assembly of U6 snRNP (Pannone, Xue, and Wolin 1998). Lhp1 has also been found to co-purify with epitope-tagged Pab1 (R. Richardson et al. 2012). Surprisingly, the interaction with Pab1 was RNA-independent, suggesting that protein-protein interactions contribute to Lhp1 association with Pab1-containing mRNPs. The possible role of Lhp1 in the context of an mRNP remains to be determined; however, it seems plausible that Lhp1 could act as a molecular chaperone also for RNA Pol II transcription products.

Table 23. **MS2L-tagged RNA co-purifying enzymes involved in tRNA modification.** Log_2 (H/L) ratios of enriched proteins are in bold.

Protein ID	Gene	BB		RNase		ENO2		6MS2L	
		for	rev	for	rev	for	rev	for	rev
Q02648	TRM44	3.26		**3.71**	**-4.93**	**3.76**	**-6.77**		
P33753	TRM2			**3.46**	**-3.49**	**4.22**	**-6.11**	**4.64**	**-6.24**
P38205	NCL1			0.89		1.06		**4.78**	**-4.24**
Q12211	PUS1			1.02		**1.49**	**-2.40**		-1.91
P48567	PUS4			1.82		**2.44**	**-4.71**	**3.33**	**-3.24**
Q08647	PUS7			0.51	-0.25	**0.91**	**-1.95**	**2.49**	**-1.83**
P33399	LHP1			**0.91**	**-1.05**	1.3			-2.44

Table 24. Description of the enriched tRNA-modifying enzymes.

Gene[a]	Location[b]	Description
TRM44	cyt	$tRNA^{Ser}$ Um_{44} 2'-O-methyltransferase
TRM2	UNK	tRNA(m^5U_{54}) methyltransferase
NCL1	nuc	tRNA(m^5C_{34}, m^5C_{40}, m^5C_{48}, m^5C_{49}) methyltransferase
PUS1	nuc	pseudouridine synthase; catalyzes pseudouridylation at positions 26-28, 34-36, 65, and 67 in tRNA, as well as at position 44 in U2 snRNA
PUS4	nuc, mito	tRNA pseudouridine synthase; catalyzes pseudouridylation at position 55 in cytoplasmic as well as mitochondrial tRNAs
PUS7	nuc	pseudouridine synthase; catalyzes pseudouridylation at positions 35 and 56 in U2 snRNA, position 50 in 5S rRNA, position 13 in cytoplasmic tRNAs, and position 35 in pre-$tRNA^{Tyr}$

[a] Localization and function data was retrieved from *Saccharomyces* Genome Database (http://www.yeastgenome.org/; see also reference (Cherry et al. 2012)).
[b] Abbreviations: cyt – cytoplasm, nuc – nucleus, mito – mitochondrion, UNK – unknown.

Metabolic enzymes co-purifying with MS2L-tagged RNAs

All tested MS2L-tagged RNAs have co-purified with several metabolic enzymes (Table 25). The gene products of *IMD2*, *IMD3*, *IMD4*, *ALD5* and *MIS1* were enriched in all three MS data sets, whereas the proteins encoded by *ADH3*, *MET6*, *LEU2*, *ZWF1*, *ARO1*, *HIS4* and *URA3* were enriched only in single MS data sets. The latter proteins were almost exclusively <2-fold enriched. In contrast, Imd2, Imd3, Imd4, Ald5 and Mis1 were >2-fold enriched in most affinity purification experiments. Except for Ald5, these enzymes have previously been found to co-purify with mRNPs in yeast (S. F. Mitchell et al. 2013; Klass et al. 2013), suggesting that their co-purification with MS2L-tagged RNAs is specific. *IMD2*, *IMD3* and *IMD4* are closely related genes in *S. cerevisiae*, which encode for proteins with potential inosine monophosphate dehydrogenase (IMDH) activity (Hyle, Shaw, and Reines 2003). IMDH is a key enzyme in *de novo* GTP biosynthesis, which catalyses the first committed step in the pathway. The three Imd proteins have been found to form heteromeric complexes *in vivo* (McPhillips, Hyle, and Reines 2004), suggesting that they may have co-purified with the MS2L-tagged RNAs as one complex. In mammalian cells, IMDH has been shown to be recruited to actively transcribed genes through

phosphorylated serine 2 in RNA Pol II C-terminal domain (J.-H. Park and Ahn 2010) and to bind to RNA *in vivo* (McLean et al. 2004; Mortimer and Hedstrom 2005). In *S. cerevisiae*, Imd2-4 were among the enriched proteins after poly(A)$^+$ RNA affinity purification from glucose deprived cells (S. F. Mitchell et al. 2013) and Imd4 was enriched after TAP-tagged Pab1 affinity purification from logarithmically growing cells (Klass et al. 2013). The experimental conditions used in the latter study enabled the detection of RNA-dependent but ribosome-independent RNA-protein and protein-protein interactions, suggesting that Imd4 is a component of Pab1-containing mRNPs that are devoid of ribosomes. Our results thus further confirm the existing evidence that Imd2, Imd3 and Imd4 are mRNP proteins (S. F. Mitchell et al. 2013; Klass et al. 2013). The role of Imd2-4 in mRNA metabolism remains undefined. However, this role is probably not essential since yeast cells can survive without the four genes encoding for IMDH isozymes if their growth medium is supplemented with guanine, indicating that the only essential function of Imd2-4 (*IMD1* is likely a pseudogene) is in GTP biosynthesis (Hyle, Shaw, and Reines 2003).

All tested MS2L-tagged RNAs co-purified with the gene product of *MIS1*, which encodes for the mitochondrial trifunctional C1-tetrahydrofolate synthase (Shannon and Rabinowitz 1988). The enzyme catalyses the synthesis of mitochondrial 10-formyltetrahydrofolate (Shannon and Rabinowitz 1988) that provides an active one-carbon unit for the formylation of mitochondrial initiator tRNA, Met-tRNA$_f^{Met}$ (Dickerman et al. 1967). Interestingly, two different studies have identified Mis1 among the enriched proteins after epitope-tagged Pab1 affinity purification (Klass et al. 2013; R. Richardson et al. 2012). Mis1 co-purified with Pab1 also when the lysate used for Pab1 immunoprecipitation was previously treated with RNase to eliminate Pab1 binding to poly(A) tail (R. Richardson et al. 2012), indicating that the two proteins are associated via protein-protein interactions. Our results and the previous results from others (Klass et al. 2013; R. Richardson et al. 2012) thus suggest that Mis1 is an mRNP protein. The role of Mis1 in mRNA metabolism remains to be identified; however, since the deletion of *MIS1* has no detectable impact on cell growth (Shannon and Rabinowitz 1988), Mis1 function in mRNA metabolism cannot be essential.

The last of the metabolic enzymes enriched in all three MS data sets – the minor isoform of mithocondrial aldehyde dehydrogenase encoded by *ALD5* – has previously not been reported to associate with mRNA nor Pab1 (Klass et al. 2013; R. Richardson et al. 2012; Tsvetanova et al. 2010; Scherrer et al. 2010; S. F. Mitchell et al. 2013). These results argue against a possibility that Ald5 could be an mRNP protein. However, the relatively high enrichment level of Ald5 after affinity purification of all three MS2L-tagged RNAs suggests that Ald5 co-purification has been specific. A common element in all three RNAs subjected to mRNP affinity purification is the

6MS2L-tag. This RNA sequence, which naturally does not occur in yeast transcripts, might have the potential to bind Ald5.

Table 25. Metabolic enzymes co-purifying with MS2L-tagged RNAs. Log_2 (H/L) ratios of enriched proteins are in bold.

Protein ID	Gene	BB		RNase		ENO2		6MS2L	
		for	rev	for	rev	for	rev	for	rev
P38697	IMD2			-0.03	-0.54	**1.55**	**-3.37**	**2.16**	**-1.84**
P50095	IMD3			**0.62**	**-1.10**	**1.81**	**-3.00**	**2.78**	**-1.95**
P50094	IMD4			**0.81**	**-1.36**	**2.16**	**-3.72**	**2.95**	**-2.41**
P40047	ALD5			**1.38**	**-1.26**	**2.79**	**-4.16**	**2.62**	**-2.29**
P09440	MIS1	**2.52**	**-2.96**	2.10		**1.82**	**-3.95**	**1.97**	**-1.46**
P37292	SHM1			0.56	-0.09	**3.61**	**-4.65**	**1.2**	**-1.05**
P07246	ADH3			**1.08**	**-0.71**	0.04		0.09	0.63
P05694	MET6	-0.02		**1.17**	**-0.65**	1.59	1.49	-0.42	-1.42
P04173	LEU2	2.09	-0.30	**0.83**	**-0.54**	-0.89	1.63	-0.20	0.76
P11412	ZWF1			1.42		**0.87**	**-0.75**	0.22	0.39
P08566	ARO1	0.05	-0.23	0.41	0.06	**0.63**	**-0.61**	0.25	0.35
P00815	HIS4	0.40	-0.20	1.05	0.07	0.85		**0.50**	**-0.67**
P03962	URA3			2.70		0.41		**0.60**	**-1.07**

Table 26. The function of the enriched metabolic enzymes not discussed in the text.

Gene[a]	Location[b]	Description
ADH3	mito	Mitochondrial alcohol dehydrogenase isozyme III; involved in the shuttling of mitochondrial NADH to the cytosol under anaerobic conditions and ethanol production
MET6	cyt, pl memb	Cobalamin-independent methionine synthase; involved in methionine biosynthesis and regeneration; requires a minimum of two glutamates on the methyltetrahydrofolate substrate, similar to bacterial metE homologs
LEU2	cytosol	Beta-isopropylmalate dehydrogenase (IMDH); catalyzes the third step in the leucine biosynthesis pathway; can additionally catalyze the conversion of β-ethylmalate into α-ketovalerate
ZWF1	cyt	Glucose-6-phosphate dehydrogenase (G6PD); catalyzes the first step of the pentose phosphate pathway; involved in adapting to oxidative stress; homolog of the human G6PD which is deficient in patients with hemolytic anemia; protein abundance increases in response to DNA replication stress
ARO1	cyt	Pentafunctional arom protein; catalyzes steps 2 through 6 in the biosynthesis of chorismate, which is a precursor to aromatic amino acids
HIS4	cyt	Multifunctional enzyme containing phosphoribosyl-ATP pyrophosphatase; phosphoribosyl-AMP cyclohydrolase, and histidinol dehydrogenase activities; catalyzes the second, third, ninth and tenth steps in histidine biosynthesis
URA3	cytosol	Orotidine-5'-phosphate (OMP) decarboxylase; catalyzes the sixth enzymatic step in the de novo biosynthesis of pyrimidines, converting OMP into uridine monophosphate (UMP); converts 5-FOA into 5-fluorouracil, a toxic compound

[a] Localization and function data was retrieved from *Saccharomyces* Genome Database (http://www.yeastgenome.org/; see also reference (Cherry et al. 2012)).
[b] Abbreviations: cyt – cytoplasm, ch – chromatin, pl memb – plasma membrane, mito – mitochondrion

The gene product of *SHM1* was highly enriched after *ENO2-6MS2L* affinity purification (>12-fold enrichment) and classified as an enriched protein also after 6MS2L-RNA affinity purification (>2-fold enrichment). The gene encodes for the mitochondrial isozyme of serine hydroxymethyltransferase (McNeil et al. 1994), which, depending on the yeast growth conditions, may synthesise serine form glycine and one-carbon units or provide one-carbon units for purine

synthesis (Kastanos, Woldman, and Appling 1997). Shm1 has not been identified as an RBP in yeast (Tsvetanova et al. 2010; Scherrer et al. 2010); however, the protein was found to co-purify with epitope-tagged Pab1 in an RNA-dependent manner (Klass et al. 2013). The high enrichment level of Shm1 after *ENO2-6MS2L* affinity purification further suggests that Shm1 is an mRNP protein. Interestingly, Shm1 participates in interconversion of the one-carbon units carried by tetrahydrofolate together with the above-mentioned protein Mis1, hinting at the possibility that the two proteins might have co-purified with *ENO2-6MS2L* as components of one protein complex.

Based on previously published findings, only one of the seven metabolic enzymes enriched in single MS data sets (Table 26) can be functionally related to the MS2L-tagged RNA it has co-purified with. The gene product of *MET6*, a cobalamin-independent methionine synthase, co-purified with *PGK1-6MS2L*. Interestingly, Met6 and Pgk1 can both be detected in isolated yeast plasma membrane fraction (Delom et al. 2006). Same subcellular localization of the two proteins may provide the basis for Met6 co-purification with *PGK1-6MS2L*, possibly via the ribosome-bound Pgk1 nascent peptide. The association of Pgk1 and Met6 was also observed in a large-scale screen of yeast protein complexes (Gavin et al. 2006), further suggesting that Met6 co-purification with *PGK1-6MS2L* is specific.

MS2L-tagged RNA co-purifying proteins involved in ubiquitin-mediated regulation

Two MS2L-tagged RNA co-purifying proteins suggest that mRNPs may be associated with deubiquitination activity provided by the Ubp3-Bre5 deubiquitination complex (Table 27). The ubiquitin-specific protease 3, Ubp3 (Baker, Tobias, and Varshavsky 1992), classified as enriched after MS2L-tagged *PGK1* and *ENO2* affinity purification. A relatively high (4.4-fold) enrichment in 6MS2L-RNA reverse labelling experiment suggests that the protein might also co-purify with this mRNA-like transcript. Bre5, an essential positive regulator of Ubp3-mediated deubiquitination (Cohen et al. 2003), classified as enriched only in the BB sample MS data set. However, it seems likely that Bre5 has co-purified also with *ENO2-6MS2L* since the protein was 12.8-fold enriched in *ENO2-6MS2L* forward labelling experiment. Ubp3-Bre5 complex is involved in diverse cellular processes such as transcription activation and elongation (Chew et al. 2010; Kvint et al. 2008), DNA repair by non-homologous end joining (Bilsland et al. 2007), maintenance of an efficient ER to Golgi secretory pathway (Cohen et al. 2003) and autophagy of mature ribosomes in response to starvation (Kraft et al. 2008; Ossareh-Nazari et al. 2010). So far, Ubp3-Bre5 complex has not been implicated in mRNA regulation. However, the protein complex has been found to co-purify with poly(A)$^+$ RNA (S. F. Mitchell et al. 2013; Tsvetanova

et al. 2010), as well as with epitope-tagged Pab1 (Klass et al. 2013). The evolutionarily conserved Ubp3 protein has been found to co-purify with poly(A)$^+$ RNA also in mammalian cells (Castello et al. 2012; Baltz et al. 2012). Collectively, these findings are in line with our results showing that Ubp3-Bre5 can co-purify with *in vivo*-assembled mRNPs. Since Bre5 contains an RNA-recognition motif (K. Li et al. 2005), Ubp3-Bre5 complex could be directly recruited to mRNA by Bre5. However, additional Pab1-mediated protein-protein interactions might contribute to the association with mRNA. Namely, Ubp3 was found to co-purify with Pab1 in an RNA-independent manner (R. Richardson et al. 2012). Considering that several proteins participating in mRNA maturation, export and decay are regulated by ubiquitination (reviewed in Finley et al. 2012), it seems plausible that an mRNP-associated deubiquitination activity may provide a means to proofread ubiquitination-regulated steps in mRNP life cycle.

Surprisingly, all three tested MS2L-tagged RNAs have co-purified with an RNA Pol II degradation factor encoded by *DEF1* (Woudstra et al. 2002) (Table 27). Def1 is a cytoplasmic protein that upon transcription-impeding DNA damage is processed in a ubiquitin- and proteasome-dependent manner, leading to nuclear accumulation of the activated Def1 protein (M. D. Wilson et al. 2013). In the nucleus, Def1 binds to the stalled RNA Pol II and recruits the Elongin-Cullin E3 ligase complex, which polyubiquitinates the Rpb1 subunit and thus triggers the proteasome-mediated degradation of the stalled RNA Pol II (M. D. Wilson et al. 2013). RNA Pol II seems not to be the only cellular target of Def1-mediated proteolytic decay. In the context of a stalled DNA replication fork, Def1 promotes the degradation of the catalytic subunit of DNA polymerase δ (Daraba et al. 2014). Whether Def1 participates also in mRNA regulation has not been experimentally addressed. However, since the protein has been found to co-purify with Pab1 under conditions, which preserve ribosome-association with the mRNA (Klass et al. 2013), it is possible that Def1 could participate in the regulation of translationally active mRNAs. Since Def1 has been shown to act as an adaptor for E3 ubiquitin ligase recruitment to stalled RNA Pol II (M. D. Wilson et al. 2013) it seems plausible that the protein might have a similar activity in the context of an mRNP.

Table 27. MS2L-tagged RNA co-purifying proteins involved in ubiquitin-mediated regulation. Log_2 (H/L) ratios of enriched proteins are in bold.

Protein ID	Gene	BB		RNase		ENO2		6MS2L	
		for	rev	for	rev	for	rev	for	rev
Q01477	UBP3	**2.30**	**-2.57**	**1.97**	**-2.22**	**2.27**	**-3.96**		-2.12
P53741	BRE5	**2.21**	**-2.55**	2.25		3.67			
P35732	DEF1	**1.14**	**-1.02**	**0.89**	**-0.83**	**0.66**	**-2.45**	**1.25**	**-0.62**

Mitochondrial proteins co-purifying with MS2L-tagged RNAs

In addition to the mithocondrial enzymes discussed above (Table 25 and 26), *ENO2-6MS2L* and possibly also *PGK1-6MS2L* have co-purified with a few additional mitochondrial proteins (Table 28). The gene products of *YLR419W*, *MGM101*, and *RPO41*, which co-purified with *ENO2-6MS2L*, were all highly enriched (>8.5-fold enrichment). *YLR419W* encoded protein was also relatively highly enriched (>5.3-fold enrichment) in BB sample MS data set, suggesting co-purification with *PGK1-6MS2L*. *YLR419W* encodes for a putative homolog of DEAH-box family of RNA-dependent ATPases (reviewed in de la Cruz, Kressler, and Linder 1999), whose protein product has been found to be present in isolated highly pure mitochondria (Sickmann et al. 2003). Remarkably, several studies have identified Ylr419w among the enriched proteins after mRNA or eppitope-tagged Pab1 affinity purification (S. F. Mitchell et al. 2013; Klass et al. 2013; R. Richardson et al. 2012). The protein seems to be mRNA-associated under normal yeast growth conditions (Klass et al. 2013; R. Richardson et al. 2012) as well as upon glucose deprivation (S. F. Mitchell et al. 2013). mRNA-association of Ylr419w has also been detected in human (Castello et al. 2012; S. F. Mitchell et al. 2013). Our results thus confirm the previous observations that Ylr419w is an mRNP protein. The predicted role of Ylr419w in mRNA biogenesis remains to be elucidated; however this role cannot be essential since Δ*ylr419w* yeast cells are viable (Colley et al. 2000; Shiratori et al. 1999).

RPO41 encodes for the mitochondrial RNA polymerase (Greenleaf, Kelly, and Lehman 1986) and *MGM101* a component of the mitochondrial nucleoid (Meeusen et al. 1999) that participates in recombinatorial mitochondrial DNA repair (Mbantenkhu et al. 2011). The two proteins are found in the same protein complex since TAP-tagged Rpo41 co-purifies with Mgm101 (Markov et al. 2009). Mgm101 is a ssDNA-binding protein (Mbantenkhu et al. 2011), which also seems to bind RNA – the protein was identified in a screen for RNA-binding proteins by probing a high density yeast protein microarray with different sorts of RNA (Scherrer et al. 2010). Furthermore, the protein has been found to co-purify with Pab1 in an RNA-dependent but ribosome-independent manner (Klass et al. 2013), suggesting that Mgm101 is an mRNP protein. Mgm101 co-purification with *ENO2-6MS2L* might therefore be physiologically relevant and also contribute to Rpo41 co-purification with this MS2L-tagged mRNA.

Besides Ylr419w, BB sample MS data set contained one additional enriched mitochondrial protein – the DEAD-box splicing factor Mss116 (reviewed in de la Cruz, Kressler, and Linder 1999). The protein has been found to be required for group I and II intron splicing in mitochondria and proposed to facilitate splicing by destabilizing stable but inactive RNA structures (H.-R. Huang et al. 2005). Considering the role of Mss116 in mitochondrial splicing, it is not surprising that the protein has been found to co-purify with epitope-tagged Pab1 (Klass et

al. 2013; R. Richardson et al. 2012). However, since *PGK1* is an intronless cytoplasmic mRNA, *PGK1-6MS2L* co-purification with Mss116 could represent a false-positive interaction that might have occurred due to the release of mitochondrial proteins from this compartment during cell lysis. Assuming that the interaction between *PGK1-6MS2L* and Mss116 is specific, the relatively low enrichment level of Mss116 (about 1.8-fold enrichment) suggests that only a minor fraction of cellular *PGK1-6MS2L* is associated with Mss116. The same conclusion can be drawn for Hsp60. This mitochondrial chaperonin was about 1.5-fold enriched in RNase eluate after *PGK1-6MS2L* affinity purification. The protein has not been implicated in mRNA metabolism and therefore the physiological relevance of *PGK1-6MS2L* and Hsp60 interaction remains elusive.

Table 28. Mitochondrial proteins co-purifying with *PGK1-6MS2L* and *ENO2-6MS2L*. Log_2 (H/L) ratios of enriched proteins are in bold.

Protein ID	Gene	BB		RNase		ENO2		6MS2L	
		for	rev	for	rev	for	rev	for	rev
Q06698	YLR419W	**2.41**	**-2.67**	-3.30		**3.09**	**-4.70**		
P32787	MGM101	0.41		0.93		**3.57**	**-6.27**		
P13433	RPO41			1.72		**3.19**	**-4.42**		
P15424	MSS116	**0.74**	**-0.97**						
P19882	HSP60	0.29	-0.28	**0.54**	**-0.59**	0.92	-0.12		0.27

Vacuolar and vesicular transport-involved proteins co-purifying with MS2L-tagged RNAs

The enriched proteins involved in vesicular transport or vacuolar function are listed in table 29. With one exception, these proteins showed relatively low enrichment levels. The exception, the RNaseT2 family member encoded by *RNY1* (reviewed in Luhtala and Parker 2010), was >19-fold enriched after *ENO2-6MS2L* affinity purification. The protein was not quantified in other MS2L-tagged RNA affinity purification experiments, suggesting that Rny1 might play a specific role in *ENO2-6MS2L* mRNA regulation. RNaseT2 family members are general RNases that cleave ssRNA producing mono- or oligonucleotides with a terminal 3' phosphate group (Scheer et al. 2011). In logarithmically growing yeast cells Rny1 is found in vacuoles from where it is released into the cytoplasm upon oxidative stress (Thompson and Parker 2009). Released from the vacuoles, Rny1 may cleave tRNA and rRNA, as well as promote cell death independent of its nuclease activity (Thompson and Parker 2009). Rny1-dependent tRNA cleavage has also been observed in vacuole or in vacuole-like compartment, suggesting that tRNA cleavage by Rny1 may also occur at, or inside, the vacuole (Luhtala and Parker 2012). Whether Rny1 also participates in mRNA cleavage remains unknown. However, it seems unlikely that Rny1 could have interacted with *ENO2-6MS2L* in the cytoplasm because the yeast cells used for mRNP affinity purification

were harvested in mid-log phase when Rny1 protein localization is restricted to the vacuoles (Thompson and Parker 2009). It seems more likely that *ENO2-6MS2L* has associated with Rny1 at or within the vacuole. mRNAs have been proposed to be targeted to the vacuole during autophagy of aggregated mRNPs (reviewed in Luhtala and Parker 2010). Since several P-body and stress granule components (reviewed in Carolyn J. Decker and Parker 2012) co-purified with *ENO2-6MS2L*, it is possible that *ENO2-6MS2L*-containing mRNPs aggregate and may thus be targeted by autophagy for degradation in the vacuole.

The gene products of *VPH1*, *IMH1* and *SEC16*, whose protein functions are listed in table 30, have not been implicated in mRNA metabolism. Assuming that the interaction between these proteins and the corresponding MS2L-tagged RNAs is specific, the relatively low enrichment level of Vph1, Imh1 and Sec16 suggests that they are not associated with the majority of MS2L-tagged RNA-containing RNPs.

Table 29. MS2L-tagged RNA co-purifying vacuolar proteins and proteins involved in vesicular transport. Log_2 (H/L) ratios of enriched proteins are in bold.

Protein ID	Gene	BB		RNase		ENO2		6MS2L	
		for	rev	for	rev	for	rev	for	rev
Vacuolar proteins									
Q02933	RNY1					**4.28**	**-4.88**		
P32563	VPH1	**1.32**	**-1.55**						
Vesicular transport									
Q06704	IMH1			0.32	-0.06	**0.88**	**-1.76**	**1.06**	**-0.54**
P48415	SEC16	**0.76**	**-1.13**						

Table 30. The function of the vacuolar and vesicular transport-involved proteins not discussed in the text.

Gene[a]	Location[b]	Description
VPH1	vac memb	Subunit a of vacuolar-ATPase V0 domain; vacuolar acidification
IMH1	cytosol, Golgi	Protein involved in vesicular transport; mediates transport between an endosomal compartment and the Golgi
SEC16	ER to Golgi transport vesicle membrane	COPII vesicle coat protein required for ER transport vesicle budding; essential factor in endoplasmic reticulum exit site (ERES) formation, as well as in COPII-mediated ER-to-Golgi traffic

[a] Localization and function data was retrieved from *Saccharomyces* Genome Database (http://www.yeastgenome.org/; see also reference (Cherry et al. 2012)).
[b] Abbreviations: vac memb – vacuole membrane

PGK1-6MS2L co-purifies with several glycolytic enzymes – co-translational formation of a supramolecular glycolytic enzyme complex?

Several steps of the 10 enzymatic reactions that are needed to convert one molecule of glucose into 2 molecules of pyruvate, concomitantly generating 2 ATP, are reflected in the proteome of

PGK1-6MS2L. Besides Pgk1, three proteins in the glycolytic pathway – the gene products of *HXK2*, *PGI1* and *TDH3* – classified as enriched after *PGK1-6MS2L* affinity purification (Table 31). Three additional proteins encoded by *TPI1*, *TDH2* and *ENO2* failed to meet the set threshold criteria only slightly in one of the biological replicate experiments. None of the enriched or the possibly enriched glycolytic enzymes co-purifying with *PGK1-6MS2L* has previously been identified as an RNA-binding protein in yeast (Tsvetanova et al. 2010; Scherrer et al. 2010) nor has been found to be associated with mRNPs (D. N. Richardson et al. 2011; Klass et al. 2013; S. F. Mitchell et al. 2013). Therefore, it seems unlikely that a direct binding of the glycolytic enzymes to *PGK1-6MS2L* or to *PGK1-6MS2L*-associated RBPs provides the basis for glycolytic enzyme co-purification with 6MS2L-tagged *PGK1*. An emerging view is that proteins that participate in a common metabolic or signalling pathway are organized into supramolecular complexes for optimal pathway performance (reviewed in Vonck and Schäfer 2009; Dai, Hall, and Hell 2009). Glycolysis seems to be one of such metabolic pathways whose activity can be regulated by supramolecular complex formation. Glycolytic enzyme association with membranes and cytoskeletal elements, as well as interaction between enzymes participating in sequential reactions was observed already about three decades ago (reviewed in Srere 1987). Since then a multi-enzyme glycolytic complex has been demonstrated to exist on the human erythrocyte membranes (Campanella, Chu, and Low 2005; Puchulu-Campanella et al. 2013). In *S. cerevisiae* the association of glycolytic enzymes has been shown to confer resistance against enzymatic activity inhibition by a compatible solute trehalose (Araiza-Olivera et al. 2010). Glycolytic enzymes seem to assemble in association with filamentous F-actin, which co-immunoprecipitates with several glycolytic enzymes and, in the filamentous form, increases fermentation rate and protects against trehalose inhibition in permeabilized yeast cells or yeast cell cytoplasmic extracts (Araiza-Olivera et al. 2013). Glycolytic enzyme assembly into a supramolecular complex may thus provide the answer why several proteins of this metabolic pathway were enriched after *PGK1-6MS2L* mRNA affinity purification. As discussed earlier, *PGK1-6MS2L* co-purifies with the nascent peptide likely as an mRNA-ribosome-nascent peptide complex. The emerging Pgk1 protein may co-translationally be recruited to a glycolytic enzyme complex. Pgk1 nascent peptide-mediated protein-protein interactions could therefore provide a link between *PGK1-6MS2L* and the glycolytic enzymes.

Interestingly, the gene product of *ACT1* was among the enriched proteins in BB sample MS data set (Appendix, Table 1). Even though the protein is often found as a contaminant in affinity purification experiments (Mellacheruvu et al. 2013), the role of F-actin in glycolytic enzyme complex assembly would also provide a physiologically meaningful explanation for actin co-purification with *PGK1-6MS2L* mRNA.

Large-scale analysis of yeast protein complexes has identified an almost identical set of glycolytic enzymes associated with Pgk1 and enolase 2 (Gavin et al. 2006), suggesting that similarly to Pgk1, enolase 2 is among the glycolytic enzymes that may associate into a supramolecular complex. Furthermore, both proteins also co-immunoprecipitate with actin (Araiza-Olivera et al. 2013). Therefore, it is surprising that besides enolase 2 no other glycolytic enzyme co-purified with *ENO2-6MS2L*. Since cell's metabolic state could have a profound effect on the formation of a glycolytic enzyme complex, it is important to note that yeast cells were collected in mid-log phase in all mRNP affinity purification experiments, thereby excluding the possibility that *PGK1-6MS2L*- or *ENO2-6MS2L*-containing cells would have been in different metabolic states. The lack of glycolytic enzyme co-purification with *ENO2-6MS2L* could be explained by weak protein-protein interactions between the glycolytic enzymes, which could be lost during affinity purification; this observation has also been made by others (Brandina et al. 2006).

Table 31. Several glycolytic enzymes are enriched after *PGK1-6MS2L* affinity purification. Log_2 (H/L) ratios of enriched proteins are in bold. Log_2 (H/L) ratios of proteins that did not fulfil the set threshold criteria log_2 (H/L) >0.5 or <0.5 in one of the biological replicate experiments are in gray.

Gene	Name	BB		RNase		ENO2		6MS2L	
		for	rev	for	rev	for	rev	for	rev
HXK1	Hexokinase-1			-0.37					
HXK2	Hexokinase-2			**0.94**	**-1.51**	-0.64	1.31	-0.88	-0.91
GLK1	Glucokinase-1			-0.23					
PGI1	Glucose-6-phosphate isomerase	0.06		**1.05**	**-1.84**	2.35	2.00	-1.14	-1.16
PFK1	6-phosphofructokinase subunit alpha	0.01	-0.20	0.05	0.56	-1.82	1.75	0.02	0.00
PFK2	6-phosphofructokinase subunit beta	-0.04	-0.04	0.12	0.84	-0.31	0.93	0.01	0.09
FBA1	Fructose-bisphosphate aldolase	0.66	-0.10	0.81	-0.17	1.95	0.94	-0.66	0.14
TPI1	Triosephosphate isomerase			0.27	-1.55	2.67	2.63	-1.54	-0.83
TDH1	Glyceraldehyde-3-phosphate dehydrogenase 1		-0.22	-0.18	-0.13	0.17	-0.31	-0.25	-0.59
TDH2	Glyceraldehyde-3-phosphate dehydrogenase 2	-0.67	-0.13	2.98	-0.45	2.17		0.17	
TDH3	Glyceraldehyde-3-phosphate dehydrogenase 3	-2.44	1.47	**1.13**	**-0.70**	1.87	2.49	0.15	-0.45
PGK1	Phosphoglycerate kinase	**1.68**	**-5.09**	**0.75**	**-3.58**	0.54	1.42	-0.35	-0.01
GPM1	Phosphoglycerate mutase 1			0.77	0.29	-1.29	4.83	0.14	0.80
ENO1	Enolase 1			0.18	-0.82	2.70		-0.91	-1.05
ENO2	Enolase 2	-0.17	-0.13	1.21	-0.23	**0.80**	**-2.72**	-1.16	-0.77
CDC19	Pyruvate kinase 1	-0.02	-0.14	0.26	-0.38	1.38	0.90	-0.52	-0.14

MS2L-tagged RNA co-purifying proteins that were enriched in single MS data sets

Each MS data set contains proteins that appear to have co-purified with only one of the MS2L-tagged RNAs. Such proteins include the glycolytic enzymes that co-purified with *PGK1-6MS2L*

(Table 31) and some metabolic enzymes listed in table 25. Maybe the most remarkable example of a protein that has exclusively co-purified only with one MS2L-tagged RNA is the vacuolar RNaseT2 family member Rny1, which was >19-fold enriched after *ENO2-6MS2L* affinity purification but was not quantified in other MS data sets (table 29). The following chapter will introduce additional proteins that were enriched in single MS data sets (Table 32).

The protein encoded by *WHI3* was >4-fold enriched after *PGK1-6MS2L* affinity purification. Whi3 is an RRM-containing RBP (Nash, Volpe, and Futcher 2001) that localizes to stress granules and/or P-bodies upon glucose deprivation or heat shock (Holmes et al. 2013; Cai and Futcher 2013). The protein seems to have many mRNA targets (Colomina et al. 2008; Vergés et al. 2007; Holmes et al. 2013; Cai and Futcher 2013); up to a thousand mRNAs have been identified as Whi3 targets (Holmes et al. 2013). Interestingly, *PGK1* has not been reported to be a Whi3 target mRNA, raising the possibility that the 6MS2L-tag might contribute to Whi3 co-purification with *PGK1-6MS2L* mRNA. The role of Whi3 in the regulation of its target mRNAs is not very well understood; however, the protein appears to have a mild destabilizing effect on its targets (Holmes et al. 2013; Cai and Futcher 2013).

The gene products of *TRA1* and *YNL050C* were enriched in the BB sample MS data set, suggesting that the proteins co-purify with *PGK1-6MS2L*. The later gene encodes for a putative protein with unknown function (Cherry et al. 2012). Interestingly, Ynl050c was identified as an RBP by probing a high-density yeast protein microarray with different cellular RNAs (Scherrer et al. 2010), suggesting that Ynl050c could directly bind to *PGK1-6MS2L* mRNA. Tra1 is a common subunit of SAGA and NuA4 histone acetyltransferase complexes (Grant et al. 1998; Saleh et al. 1998; C E Brown et al. 2001). Surprisingly, this nuclear protein involved in transcription activation has also been found to co-purify with epitope-tagged Pab1 in an RNA-dependent manner (Klass et al. 2013), suggesting that Tra1 might have a secondary role, possibly in the nuclear compartment, as an mRNP component.

ENO2-6MS2L mRNA co-purified with two proteins implicated in pre-mRNA splicing. The gene product of *MSL5* is a component of the commitment complex, which defines the first step in splicing. *PSP2* encodes for a suppressor of group II intron-splicing defects with a possible role in mitochondrial mRNA splicing. *ENO2-6MS2L* does not contain introns and should not be subjected to mRNA splicing. Therefore the biological role of *ENO2-6MS2L* association with Msl5 and Psp2 remains unclear. The low enrichment level (<2.2-fold enrichment) of the two proteins suggests that only a minor fraction of total cellular *ENO2-6MS2L* is associated with Msl5 and Psp2.

6MS2L-RNA co-purifying proteins encoded by *ARC1*, *GUS1* and *THS1* participate in tRNA aminoacylation. *GUS1* encodes for cytosolic glutamyl-tRNA synthetase (cERS) and *THS1*

encodes for cytosolic threonyl-tRNA synthetase (cTRS). The gene product of *ARC1* is a non-enzymatic protein that together with cERS and cytosolic methionyl-tRNA synthetase forms the so called multiaminoacyl-tRNA synthetase (MARS) complex in yeast (reviewed in Frechin et al. 2010). The MARS subunits Arc1 and Gus1 might have co-purified with 6MS2L-RNA as components of one protein complex; however, the third subunit of MARS complex might have been lost during affinity purification. The <2-fold enrichment of Arc1, Gus1 and Ths1 indicates that the interaction between the three proteins and the 6MS2L-RNA-containing RNPs is either transient or weak, which, in both cases, could lead to low enrichment levels after mRNP affinity purification.

Recent findings in human cells and archaea point to the possibility that Arc1, Gus1 and Ths1 might have co-purified with 6MS2L-RNA in complex with the translating ribosome (Kaminska et al. 2009; David et al. 2011; Raina et al. 2012; Godinic-Mikulcic et al. 2014). In both organisms several aminoacyl-tRNA synthetases (aaRSs) have been found to co-purify with ribosomal proteins (Kaminska et al. 2009; Raina et al. 2012) or to co-fractionate with polyribosomes on sucrose density gradients (David et al. 2011; Kaminska et al. 2009). Further biochemical studies in archaeon *Methanothermobacter thermautotrophicus* have identified an interaction surface for two archaeal aaRSs on the large ribosomal subunit in the vicinity of the flexible L7/L12 stalk (Godinic-Mikulcic et al. 2014). Since aaRS-ribosome association is observed in distantly related species, such as humans and archaea, it seems plausible that aaRSs can also associate with ribosomes in *S. cerevisiae*. This notion is supported by the finding that in yeast, as well as in most other organisms examined, tRNA diffusion away from the ribosome is slower than translation rate, suggesting that out-going tRNAs remain bound to the ribosome (Cannarozzi et al. 2010). Ribosome-associated aaRSs are the likely candidates to prevent tRNA diffusion from the translating ribosome (Raina et al. 2012). These proteins might capture and aminoacylate the out-going tRNAs, thereby keeping a constant supply of aminoacylated tRNAs at the translating ribosome. The co-purification of Arc1, Gus1 and Ths1 with 6MS2L-RNA might therefore reflect the process of tRNA channelling to the ribosome during translation.

The 6MS2L-RNA co-purifying proteins encoded by *SPC110*, *SKN7* and *BMH2* have previously not been implicated in mRNA biology (Tsvetanova et al. 2010; Scherrer et al. 2010; S. F. Mitchell et al. 2013; R. Richardson et al. 2012). With about 1.5-fold enrichment, Bmh2 was the least enriched of the four proteins. Bmh2 belongs to the family of 14-3-3 proteins, whose members participate in multiple cellular processes through binding to other proteins. 14-3-3 proteins may regulate the activity or subcellular location of their interaction partners or connect two binding partners with each other (reviewed in van Heusden 2009). Considering the large number of protein-protein interactions the two yeast 14-3-3 proteins are involved in (Kakiuchi et

al. 2007), it is likely that Bmh2 has co-purified with 6MS2L-RNA in complex with some other 6MS2L-RNA-bound protein(s). Indeed, a large-scale analysis in yeast found two 6MS2L-RNA co-purifying proteins – Upf1 and Spc110 – to be associated with Bmh1 and Bmh2 (Kakiuchi et al. 2007). Spc110 is a core component of the spindle pole body, which is the yeast microtubule organizing centre (reviewed in Jaspersen and Winey 2004). The >2-fold enrichment of Spc110 in both biological replicate experiments suggests that the interaction between 6MS2L-RNA and Spc110 is specific. Since 6MS2L-RNA is not a normal cellular mRNA, it may be involved in interactions that normally do not exist between mRNA and proteins. Alternatively, Spc110 may co-purify with 6MS2L-RNA due to protein-protein interactions between common interaction partners; the latter possibility may involve 6MS2L-RNA and Spc110 co-localization to the same cellular compartment.

6MS2L-RNA co-purified with the gene product of *SKN7*, a protein that regulates the nuclear response to oxidative and osmotic stress (Morgan et al. 1997; Brombacher et al. 2006; Ketela et al. 1998). Skn7 has not been implicated in mRNA regulation and the role of the protein in 6MS2L-RNA biogenesis remains unknown. However, since Skn7 acts as a transcription factor (Morgan et al. 1997; Raitt et al. 2000) the interaction between Skn7 and 6MS2L-RNA may involve the nuclear compartment.

Table 32. MS2L-tagged RNA co-purifying proteins that were enriched in single MS data sets. Log_2 (H/L) ratios of enriched proteins are in bold.

Protein ID	Gene	BB		RNase		ENO2		6MS2L	
		for	rev	for	rev	for	rev	for	rev
P34761	WHI3			**2.01**	**-2.37**				
P38811	TRA1	**1.41**	**-1.07**						
P53952	YNL050C	**2.07**	**-0.91**						
Q12186	MSL5	-0.02	0.07	0.00	-0.08	**1.12**	**-0.58**		
P50109	PSP2	0.23	0.21	0.48	0.22	**0.66**	**-0.61**		-0.28
P46672	ARC1			-0.27	0.51	-0.26	-0.27	**0.54**	**-0.96**
P46655	GUS1	0.06	-0.48	-0.10	0.45	0.17	-0.31	**0.75**	**-0.80**
P04801	THS1	-0.22	-0.26	-0.29	0.73	-0.76	0.06	**0.59**	**-0.89**
P32380	SPC110	-0.06	1.00	-0.28	1.27			**3.84**	**-1.56**
P38889	SKN7	0.05	-1.00	0.41	-0.64		-1.84	**1.91**	**-1.36**
P34730	BMH2		0.61	-0.17	-0.15	1.14		**0.56**	**-0.67**
P0CX63	YGR161W-B	0.42	-0.12	0.42	0.10	0.21	-0.44	**1.62**	**-1.26**
Q04215	YMR046C	0.03		0.01	0.18	0.01	0.12	**1.16**	**-0.71**

Two 6MS2L-RNA co-purifying proteins reveal an interesting difference in the metabolism of this mRNA-like transcript and the MS2L-tagged *PGK1* and *ENO2*. The Gag-Pol polypeptide (Ygr161w-B) and Gag polypeptide (Ymr046c) of yeast retrotransposons Ty2 and

Ty1, respectively, were enriched after 6MS2L-RNA affinity purification but not after MS2L-tagged mRNA affinity purification. Retrotransposons are mobile DNA elements, which replicate over an RNA intermediate that is transported to the cytoplasm and translated (reviewed in Perlman and Boeke 2004). Besides serving as mRNA, the RNA intermediate also serve as genomic RNA and is packaged into virus-like particle (VLP) in the cytoplasm. In VLP the RNA is reverse transcription and a double-stranded cDNA copy is synthesized that integrates into a new site in the nuclear DNA. Interestingly, different cellular mRNAs have been found to associate with VPLs (H. Xu and Boeke 1990). This finding suggests that non-Ty RNA may be packaged into VLPs, revealing that the packaging process is not highly specific (reviewed in Roth 2000). VLP formation is a multistep process that involves the association of Ty genomic RNA plus Gag and Gag-Pol polypeptides into an immature particle that does not completely encapsulate the RNA (Burns et al. 1992). Collectively, these findings point to the possibility that the abundant 6MS2L-RNA transcript (see Results, "Control RNA to determine the effect of MS2L tag on mRNP protein composition") might be packaged into VLPs, which, in their immature form, presumably provide access to the 6MS2L-tag sequence for MS2CP-PrAx2::IgG interaction.

Part 2

Don't mess with 3' UTR – integration of MS2 stem-loops affects normal regulation of at least some cellular mRNAs

The steady state level of *PGK1-6MS2L* was about 50% of the wt untagged *PGK1* level (Fig. 14A). This finding indicates that the integration of the 6MS2L tag has altered *PGK1* mRNA regulation and thus raises several questions. What is the mechanism behind the reduction of *PGK1* steady state levels upon integration of MS2 stem-loops? Is Pgk1 protein function affected by the integration of the MS2L tag? Would other cellular mRNAs be affected in as similar way as *PGK1*? Is the altered mRNA regulation upon MS2-tag integration perhaps a yeast-specific effect or would the MS2L tag have a similar effect also in other model organisms? Would it be possible to modify the MS2 system so that the integration of the MS2L tag would not affect mRNA regulation? The following chapter will try to find answers to these questions.

Integration of MS2 stem-loops likely activates nonsense-mediated mRNA decay of *PGK1* and *ENO2*

The steady state mRNA expression level is proportional to the rate of mRNA transcription and degradation. A decrease in *PGK1* mRNA steady state level upon 6MS2L tag integration (Fig. 14A) can thus be a consequence of decreased mRNA transcription or increased mRNA decay (or both). The 6 MS2 stem-loops are integrated between *PGK1*'s translation termination codon and the 3' UTR. This insertion should not affect signals in the 3' UTR important for proper 3' end formation, which, if compromised, might lead to defects in pre-mRNA cleavage and poly(A) tail addition, ultimately reducing the efficiency of export-competent mRNP assembly (see Introduction, "3' end formation and mRNA export are coupled"). Interestingly, the nuclear basket-associated proteins Mlp1 and Mlp2 have been shown to reduce reporter mRNA transcription in response to inefficient mRNP assembly in yeast cells containing a defective RNA export adapter Yra1 (Vinciguerra et al. 2005). This finding reveals a feedback mechanism between mRNP assembly and transcription (reviewed in Schmid and Jensen 2008) and points to the possibility that suboptimal mRNP assembly caused by other defects, such as inefficient 3' end processing, could result in transcriptional downregulation. However, since the integration of the 6MS2L-tag should not interfere with proper 3' end formation, it seems unlikely that the reason behind the decrease in *PGK1-6MS2L* steady state level is a reduction in mRNA transcription.

A large body of evidence indicate that an increase in the physical distance between the normal stop codon and the 3' UTR can lead to rapid mRNA decay due to the activation of post-transcriptional mRNA surveillance mechanism termed nonsense-mediate mRNA decay (see Introduction, "Nonsense-mediated mRNA decay" and references therein). The integration of the 6MS2L tag adds an extra 421 nt between the stop codon and the 3' UTR in *PGK1-6MS2L* and may thereby introduce an NMD-activating feature. Indeed, Upf1, the central regulator of NMD pathway, was the most abundant protein co-purifying with *PGK1-6MS2L* (>60-fold enrichment). Furthermore, an additional NMD factor, Ebs1, and several proteins involved in 5'→3' decay (Table 16) were also highly enriched after *PGK1-6MS2L* affinity purification. These results strongly suggest that the major mechanism behind the decrease in the steady state level of *PGK1-6MS2L* is an increase in the rate of mRNA decay due to the activation of NMD. Since *ENO2-6MS2L* has co-purified with a similar set of NMD and 5'→3' decay factors (Table 16), it seems highly likely that the integration of the 6MS2L tag has also compromised the normal regulation of this mRNA.

PGK1-6MS2L encodes for a functional protein whose expression might be promoted by the presence of MS2CP-PrAx2

The growth rate of yeast cells carrying the untagged or the 6MS2L-tagged *PGK1* allele was comparable; the doubling time of both strains at 26°C in SC -ura supplemented with 2% glucose was around 3 h (data not shown, see Materials and Methods, "Culturing cells for mRNP affinity purification optimization experiments" for yeast cell growth conditions). Since the function of Pgk1 protein is essential, this result indicates that the Pgk1 protein expressed from *PGK1-6MS2L* allele is functional.

Western blot analysis showed that Pgk1 protein levels were almost identical in yeast strains carrying the untagged or the 6MS2L-tagged *PGK1* allele (Fig. 14B). This result suggests that despite reduced steady state levels of *PGK1-6MS2L* mRNA, the yeast cells can produce a similar amount of Pgk1 protein compared to the strain carrying the wt *PGK1* allele. Importantly, the tested strains also expressed MS2CP-PrAx2. Further analysis revealed that in the absence of MS2CP-PrAx2 the Pgk1 protein level was reduced proportionally to the reduction in *PGK1-6MS2L* mRNA level (the tested yeast strains were isogenic to the strains used in mRNP affinity purification experiments except that they contained C-terminal 3myc epitope-tagged Upf1 or Stm1 and, as mentioned, did not express MS2CP-PrAx2; Ulrike Thieβ, unpublished data). This finding raises the possibility that binding of MS2CP-PrAx2 to the MS2 stem-loops may promote better translation of *PGK1-6MS2L* transcripts. Interestingly, a similar observation was made by Tsai et al., who have established an MS2L-MS2CP interaction-based *in vivo*-assembled mRNP affinity purification method from human cells (Tsai et al. 2011). They found that in the presence of the tag-binding protein the luciferase activity of a reporter mRNA containing 4 MS2L stem-loops downstream of the firefly luciferase stop codon was slightly elevated (Tsai et al. 2011). Since the effect of the integration of the MS2 stem-loops on mRNA stability was not addressed in this study, it remains unknown whether in mammalian system the integration of the 4MS2L tag had an mRNA destabilizing effect as observed by us for 6MS2L tag integration in *S. cerevisiae*.

Would the stability of all cellular mRNAs be affected by the integration of the MS2L tag?

For creating yeast strains carrying 6MS2L-tagged *PGK1* and *ENO2* we used a PCR-based chromosomal gene tagging method (Haim et al. 2007). This method was initially developed for *in vivo* visualization of specific endogenously expressed mRNAs in *S. cerevisiae* and has since been used in several studies to analyse mRNA localization (as a rule, 12 MS2 stem-loops are integrated for mRNA visualization purposes) (Zipor et al. 2009; Kilchert and Spang 2011; Casolari et al. 2012; Fundakowski, Hermesh, and Jansen 2012). The effect of the integration of MS2 stem-loops

on mRNA stability has been addressed in case of one localized mRNA, *ASH1*, which is targeted to the distal tip of yeast daughter cells during anaphase (Long et al. 1997; Takizawa et al. 1997). Remarkably, the cell-cycle dependent relative level of the MS2L-tagged *ASH1* was found to be unaltered compared to wt *ASH1* mRNA (Haim et al. 2007), demonstrating that the effect of the MS2L tag on mRNA stability is mRNA-specific. This raises the question why *ASH1-12MS2L* is immune to the mRNA destabilizing effect of the MS2L tag and *PGK1-6MS2L* is not? The answer to this question likely lies in the different mode of translational control of the two mRNAs. *PGK1* is expected to be efficiently recruited to the translating mRNA pool upon export from the nucleus (Arava et al. 2003). In contrast, *ASH1* mRNA is transported in a translationally repressed state to the but tip (Irie et al. 2002; Gu et al. 2004) where it is translated only during a short period in late anaphase (Bobola et al. 1996). Since mRNA translation is a prerequisite for NMD (reviewed in Maquat 2004), *PGK1-6MS2L* likely becomes susceptible to NMD immediately after export to the cytoplasm. *ASH1-12MS2L*, if targeted by NMD at all, should be immune to NMD during mRNA localization and become sensitive to NMD only during mRNA translation. This notion is supported by the finding that in a translationally repressed state a PTC-containing *ASH1* allele is insensitive to NMD but becomes sensitive once protein synthesis is initiated (Zheng et al. 2008).

It remains to be experimentally determined whether *ASH1-12MS2L* is subjected to NMD during mRNA translation. The finding that the cell-cycle dependent relative level of *ASH1-12MS2L* is unchanged compared to *ASH1* (Haim et al. 2007) suggests that unlike *PGK1*, *ASH1* mRNA is not destabilized by an increased distance between the normal stop codon and the poly(A) tail. Collectively, the analysis of *PGK1-6MS2L* (Fig. 14A) and *ASH1-12MS2L* (Haim et al. 2007, see Fig. 2C) steady state levels indicate that the MS2L tag affects the stability of different cellular mRNAs to a different extent. Assuming that the integration of the MS2 stem-loops introduces an NMD-activating feature, the mRNAs that are efficiently engaged in translation upon mRNA export should be destabilized to a greater extent than mRNAs whose translation is repressed or inefficient.

The MS2 system provides an attractive approach to capture *in vivo* assembled mRNPs also from mammalian cells – what about MS2L-tagged mRNA stability?

As mentioned above, Tsai et al. have developed a method based on the MS2 system for affinity purification of specific *in vivo*-assembled mRNPs from mammalian cells (Tsai et al. 2011). Similarly to us, Tsai et al. analysed the proteome of the captured mRNPs by SILAC-based quantitative proteomics. To our knowledge this is the only study published to date that combines

affinity purification of specific *in vivo*-assembled mRNPs with mRNP proteome analysis by SILAC-based quantitative proteomics. The analysis indicated that mRNPs undergoing processing in the nucleus or translation in the cytoplasm could be efficiently captured (Tsai et al. 2011). In contrast to our results, however, Tsai et al. did not identify mRNA decay factors among the proteins co-purifying with the tested MS2L-tagged mRNAs. From the NMD factors only UPF1 was identified, which, according to the recent observations in mammalian cells, seems to associate with mRNAs before NMD targets are selected (Zünd et al. 2013; J. A. Hurt, Robertson, and Burge 2013). Therefore, UPF1 co-purification alone, without other NMD factors, cannot be considered as an indicative of mRNA targeting by NMD. The findings by Tsai et al. thus raise the question whether in mammalian cells MS2 stem-loops integrated downstream of the normal translation termination codon have the potential to destabilize mRNA. Considering that an increased distance between the stop codon and the poly(A) tail is an efficient trigger of NMD not only in yeast (Zaborske, Zeitler, and Culbertson 2013) but also in mammalian cells (Eberle et al. 2008), it seems unlikely that in mammalian cells MS2L-tagged transcripts *per se* would be insensitive to NMD. A possible explanation therefore for the absence of mRNA decay and NMD factors among the identified mRNP proteins in the study by Tsai et al. is that the integration of 4 MS2 stem-loops did not increase the distance between the stop codon and the poly(A) tail sufficiently to trigger NMD of the tested mRNAs. However, even if NMD was not triggered, the cells should contain mRNPs engaged in constitutive cytoplasmic deadenylation-dependent mRNA decay (reviewed in C.-Y. A. Chen and Shyu 2011). A possible reason for not detecting this pool of mRNPs in the study by Tsai et al. could be that the subpopulation of 4MS2L-tagged mRNA-containing mRNPs undergoing deadenylation-dependent mRNA decay is very small, which might lead to mRNA decay factors escaping quantification. Alternatively, some technical reasons could be responsible in this study for not detecting mRNA decay factors. It therefore remains an open question whether integration of 4 MS2 stem-loops would have an mRNA-destabilizing effect also in mammalian cells.

In order to visualize single mRNAs in living mammalian cells, the integration of 24 MS2 stem-loops has routinely been used (Fusco et al. 2003; Shav-Tal et al. 2004; Mili, Moissoglu, and Macara 2008; Grünwald and Singer 2010; Darzacq et al. 2007; Mor et al. 2010). The experiments have largely relied on ectopic expression of artificial reporter mRNAs to analyse RNA Pol II transcription kinetics (Darzacq et al. 2007) and mRNP movement (Fusco et al. 2003; Shav-Tal et al. 2004), export (Grünwald and Singer 2010; Mor et al. 2010) or localization (Mili, Moissoglu, and Macara 2008). A more natural context for mRNA visualization in mammalian cells has only recently become available through the generation of a transgenic mouse line carrying 24 MS2 stem-loops in the 3' UTR of both β-actin alleles (Lionnet et al. 2011). For the first time the effect

of the 24MS2L-tag on mRNA stability could be directly compared; northern blot analysis indicated that in mouse embryonic fibroblasts (MEFs) derived from a heterozygous knock-in mouse line the steady state level of MS2L-tagged β-actin mRNA was about 50% compared to the untagged mRNA level (Lionnet et al. 2011, see supplementary Fig. 3). This result suggests that the stability of the endogenous β-actin mRNA is affected by the integration of the 24 MS2 stem-loops, thus hinting at the possibility that 24MS2L-tagged endogenous mRNAs in mammalian cells might not behave completely in the same way as their wt counterpart. Considering the emerging paradigm that mRNA levels are buffered by coupling between transcription and mRNA decay (Trcek et al. 2011; Bregman et al. 2011; Shalem et al. 2011; M. Sun et al. 2013; Haimovich et al. 2013; Goler-Baron et al. 2008), the presumed faster decay kinetics of 24MS2L-tagged β-actin should result in enhanced mRNA transcription, which might lead to inaccurate estimations when such an mRNA is used for the analysis of mRNA transcription regulation on a single-cell level (Lionnet et al. 2011). Another proposed application for the 24MS2L-tagged β-actin is mRNP isolation via the MS2L-tag (Lionnet et al. 2011). Our quantitative mRNA-bound proteome analysis results suggest that a 50% reduction in the steady state level of an MS2L-tagged mRNA is caused by enhanced mRNA decay. The possible destabilizing effect of the MS2L tag on mammalian mRNAs should therefore be considered when establishing an mRNP affinity purification method based on MS2L-MS2CP interaction.

Possible strategies to prevent NMD activation upon integration of the MS2L tag

Even though our results indicate that the integration of the 6MS2L tag can hamper the normal regulation of at least some cellular mRNAs, an mRNP affinity purification method based on the capture of the mRNA component of the mRNP remains an attractive approach for determining the composition of specific *in vivo*-assembled mRNPs. To date, a simple and reliable method for the protein composition analysis of specific mRNPs, which would have met wide use, is missing (see Introduction, "RNA-based RNP affinity purification"). Consequently, on the level of a specific mRNA, our understanding of the spectrum, functional importance and spatio-temporal dynamics of mRNA-protein interactions is very limited (reviewed in Müller-McNicoll and Neugebauer 2013). Currently, only two alternatives exist for "marking" specific endogenous RNAs for affinity capture: (1) antisense 2'-O-methyl RNA oligonucleotides complementary to single-stranded regions; and (2) chromosomal integration of an RNA affinity tag (see Introduction, "RNA-based RNP affinity purification" and references therein). Due to the ease of use and reliability the second approach is by far the more popular one. An RNA affinity tag that could be integrated without affecting the regulation of the mRNA therefore seems to be the

optimal solution for marking specific endogenous mRNAs for affinity purification. Since our mRNP affinity purification method is based on the MS2L-MS2CP interaction, in the following section we consider different possibilities for reducing the destabilizing effect of the 6MS2L tag on mRNA stability.

Several studies have found that NMD activation by a premature termination codon can be reduced by decreasing the distance between the PTC and the poly(A) tail (Peltz, Brown, and Jacobson 1993; Hagan et al. 1995; Eberle et al. 2008). Therefore, reducing the number of MS2 stem-loops should decrease the potential of the MS2L tag to elicit NMD. However, reducing the number of MS2 stem-loops might also affect the capture efficiency of the MS2L-tagged RNA. Even though the capture efficiency of 6MS2L-tagged *PGK1* could not be precisely determined, it seems likely that not more than a few per cent of the total cellular *PGK1-6MS2L* could be captured onto IgG-coupled beads (Fig. 13B). One possible explanation for the low capture efficiency of *PGK1-6MS2L* is that the amount of used IgG-coupled beads per affinity purification was too low to enable the capture of more *PGK1-6MS2L*-coniaining mRNPs. Alternatively, not all 6MS2L-tagged *PGK1* transcripts might be bound by MS2 coat protein and consequently would not be captured onto IgG-coupled beads. The local mRNP structure might "hide" the MS2 stem-loops so that they would not be accessible for the interaction with MS2 coat protein. Indeed, the mRNP structure of *PGK1-6MS2L*-containing mRNPs seems to be relatively compact as suggested by the finding that in affinity purified mRNPs the TEV protease cleavage site between MS2CP and PrAx2 became accessible for cleavage only after RNase treatment, which disassembles the mRNPs by digesting the MS2L-tagged mRNA (Fig. 16 and data not shown). It remains to be experimentally determined whether the number of integrated MS2 stem-loops influences the capture efficiency of the tagged mRNA. However, if this is the case then the trade-off of increased mRNA stability due to the integration of less MS2 stem-loops would likely be a reduction in mRNP capture efficiency.

Besides reducing the distance between the PTC and the poly(A) tail, NMD targets can be stabilized by tethering poly(A)-binding protein close to the PTC (Amrani et al. 2004; Behm-Ansmant et al. 2007; Kerényi et al. 2008; Eberle et al. 2008; G. Singh, Rebbapragada, and Lykke-Andersen 2008). These findings suggest that MS2L-tagged mRNAs could be stabilized by localizing Pab1 to the MS2 stem-loops. Instead of using MS2CP-PrAx2 to capture the mRNPs of interest onto IgG-coupled beads, a fusion protein of MS2CP-Pab1-PrAx2 could be used. Alternatively, Pab1 could be tethered downstream of the MS2L tag by using the PP7 system (see Introduction, "Naturally occurring RNA secondary structure elements as RNA affinity tags" and references therein). Since the stabilizing effect of Pab1 likely depends on the number of Pab1 molecules tethered, this approach would enable to control the magnitude of mRNA stabilization.

The integration of the RNA stability element (RSE) from Rous sarcoma virus (RSV) downstream of the MS2 stem-loops might provide an additional possibility to stabilize MS2L-tagged mRNAs. RSV is an avian retrovirus whose unspliced mRNA is subjected to NMD if it contains a PTC in the *gag* gene (Weil and Beemon 2006). However, the PTC-free full-length RSV mRNA is protected from NMD despite the fact that the normal *gag* translation termination codon is located 7 kb from the 3' poly(A) tail (Weil and Beemon 2006). The stability of the full-length RSV mRNA depends on a 401-nt long sequence element immediately downstream of *gag* stop codon (Weil and Beemon 2006). Currently, it is not known if this RNA element prevents NMD by preventing Upf1 recruitment or by increasing translation termination efficiency (reviewed in Quek and Beemon 2014). Since NMD is an evolutionarily conserved cellular mechanism (see Introduction, "NMD factors and consequences of their activation" and references therein), it seems plausible that RSV RNA stability element could provide protection against NMD also in yeast cells.

The mRNA-bound proteome – how much of it could we actually capture?

The three main classes of proteins co-purifying with the MS2L-tagged *PGK1* and *ENO2* mRNAs as well as with the mRNA-like 6MS2L-RNA transcript were: (1) proteins involved in mRNA translation; (2) mRNA 5'→3' decay factors; and (3) proteins associated with the poly(A) tail. The latter class includes proteins involved in mRNA poly(A) tail trimming in the cytoplasm and stress granule-associated proteins. Importantly, the three classes of enriched proteins participate in cytoplasmic processes, raising the question why nuclear events of the mRNA life cycle, such as transcription, 3' end processing and mRNP export, should be underrepresented in the mRNA-bound proteome. This is one of the issues that will be discussed in the following chapter, which focuses on the question why some mRNA-protein and protein-protein interactions might miss detection using our mRNP affinity purification strategy.

The analysis of mRNA-bound proteome is likely influenced by mRNP abundance in different cellular compartments

In comparison to cytoplasmic mRNP proteins, the number of enriched nuclear proteins in each MS data set was much lower (Table 20). The enriched proteins that are known to associate with mRNPs in the nucleus include, for instance, the two subunits of the nuclear cap-binding complex that co-purified with 6MS2L-RNA and the shuttling RNA-binding proteins Hrb1 and Sro9 that co-purified with 6MS2L-tagged *PGK1* and *ENO2*. We could not detect proteins involved in

mRNA export such as the mRNA export receptor Mex67/Mtr2 or the mRNA export adapters Sub2 and Yra1 even though these proteins are expected to interact with the MS2L-tagged RNAs (see Introduction, "mRNP export factors are recruited during transcription"). Likewise, we could not detect proteins involved in mRNA transcription and 3' end processing. These results suggest that nuclear mRNPs might be poorly accessible for affinity purification possibly due to their low abundance compared to cytoplasmic mRNPs. Besides physiological reasons such as the relatively short time it takes to assemble export-competent mRNPs upon transcription induction, which occurs within 5-40 min in mammalian cells (Mor et al. 2010), or the fast decay of pre-mRNAs if the mRNP assembly is delayed (Rougemaille et al. 2007; Saguez et al. 2008), inefficient breakage of nuclei during cryogenic grinding might contribute to low abundance of nuclear mRNPs in the cell lysate. However, another experiment done in our laboratory speaks against this latter possibility. Namely, SILAC-based quantitative proteomic analysis of mRNPs containing brome mosaic virus *RNA3* (reviewed in Noueiry and Ahlquist 2003) identified many nuclear proteins (Hanna Tumin and Ulrike Thieβ, unpublished data). This result shows that nuclear mRNPs are not, *per se*, less accessible for mRNP affinity purification than the cytoplasmic mRNPs. The efficiency of mRNP capture from certain subcellular compartments therefore rather seems to depend on mRNP abundance in these compartments. Transcription in the yeast nucleus is not a natural part of brome mosaic virus's life cycle; this positive-strand RNA virus replicates on the perinuclear ER membranes in plant cells (reviewed in den Boon, Diaz, and Ahlquist 2010). It seems plausible to think that the nucleocytoplasmic export of *RNA3*-conaining mRNPs is less efficient compared to normal cellular mRNAs, which likely contributes to the co-purification of MS2L-tagged *RNA3* with multiple nuclear proteins. In case of MS2L-tagged *PGK1* and *ENO2* and the mRNA-like 6MS2L-RNA transcript, however, the number of mRNPs on the nuclear assembly line at any given moment is presumably much lower than the number of cytoplasmic mRNPs engaged in processes such as mRNA translation or decay (Arava et al. 2003). Detection of low abundance proteins by LC-MS/MS is challenging due to dynamic range limitations (reviewed in Bantscheff et al. 2007). At very low peptide signals, *bona fide* interaction partners are difficult to distinguish from background noise and therefore such proteins may escape detection. Even though we did not observe non-specific binding of untagged mRNAs to IgG-coupled beads (Fig. 9A, 12B, 21B and data not shown), our mRNP affinity purification experiments may suffer from relatively high background of non-specifically captured proteins. Namely, northern blot (Fig. 15A) and qRT-PCR (Fig. 15B) analysis revealed non-specific ribosome binding to the IgG-coupled beads; roughly every third ribosome seems to have co-purified non-specifically (Fig. 15B, *Inada* harvesting protocol). Since RNase treatment releases ribosomal proteins not only from ribosomes specifically co-purifying with MS2L-tagged RNAs but also from non-specifically

captured ribosomes, ribosomal proteins likely comprise abundant contaminants in our mRNP affinity purification experiments. The level of contaminating ribosomal proteins may be similar to or even exceed the level of some specific mRNA interactors and therefore detection of low abundance proteins poses a key challenge for the established mRNP affinity purification method.

The position of the MS2L tag may influence the affinity purification efficiency of some mRNP proteins – is this the case in our experiments?

All tested MS2L-tagged RNAs co-purified with factors involved in mRNA 5'→3' exoribonucleolytic decay (Table 16), indicating that the 5'→3' decay pathway plays an important role in the turnover of the three MS2L-tagged RNAs (see Discussion, Part 1, "MS2L-tagged RNAs seem to be largely degraded in the 5'→3' exonucleolytic decay pathway" for further discussion). In contrast, the number of proteins involved in mRNA decay from the 3' end was limited to the two subunits of the Pan poly(A)-specific 3' exoribonuclease that trims the newly synthesised poly(A) tails to mRNA specific lengths (see Introduction, "Deadenylation" and references therein). Subunits of other protein complexes involved in the 3'→5' decay pathway – the Ccr4-Not complex, the exosome and the Ski complex – could not be detected (see Introduction, "Deadenylation" and "3'→5' mRNA decay" and references therein). This is surprising since normal, deadenylation-dependent mRNA decay, as well as fast mRNA decay promoted by NMD involve mRNA exonucleolytic degradation from both the 5'- and 3' end (see Introduction, "NMD factors and consequences of their activation" and references therein).

The reason for the absence of mRNA 3'→5' decay factors among the enriched proteins might be related to the position of the 6MS2L tag close to 3' end of the mRNA (Haim et al. 2007). Once the MS2 stem-loops are degraded by the concerted action of the cytoplasmic exosome-Ski assembly (Halbach et al. 2013), the mRNA cannot be bound by MS2CP-PrAx2 and thus such mRNPs would be excluded from the proteomics analysis. For similar reasons, the capture of mRNPs involved in nuclear 3'→5' decay by the exosome-TRAMP complex assembly might be problematic. Since, Ccr4-Not complex mediates mRNA deadenylation but not the 3'→5' decay of the mRNA body (see Introduction, "Deadenylation" and references therein), it should in principle be possible to capture Ccr4-Not-containing mRNPs. The failure to detect components of the Ccr4-Not complex among the enriched proteins might therefore be caused by dissociation of these proteins from mRNA during mRNP affinity purification. Alternatively, instead of the Ccr4-Not complex, poly(A) tail removal from the studied MS2L-tagged RNAs might be carried out by the Pan2-Pan3 heterodimer (see Discussion, Part1, "MS2L-tagged RNAs seem to be largely degraded in the 5'→3' exonucleolytic decay pathway" for further discussion).

Even though it remains an open question what proteins are responsible for the deadenylation of the MS2L-tagged RNAs, co-purification of some subunits of the Lsm1-7 complex with all three MS2L-tagged RNAs suggests that a subpopulation of these transcripts is deadenylated. Namely, Lsm1-7 complex has been found to preferentially associate with deadenylated mRNAs (S Tharun and Parker 2001).

Interestingly, northern blot analysis of the bead-captured *PGK1-6MS2L* mRNA demonstrated the accumulation of shortened *PGK1-6MS2L* RNA species (Figure 12B, compare upper and lower panel, lanes 12-13 and 18-19) that could be detected only with MS2L hybridization probe but not with *PGK1*-ORF probe (Figure 12A). This result suggests that the detected mRNA decay intermediates have lost the 5' part of the mRNA with the annealing site of *PGK1*-ORF probe; however, the 3' part of the mRNA, which contains MS2L probe annealing site, has not been degraded. Further analysis would be necessary to determine if the 5'→3' shortened *PGK1-6MS2L* RNA species also contain the 3' UTR. The presence of the 3' UTR would support the notion that the 3'→5' mRNA decay by the exosome-Ski assembly plays a minor role in the degradation of the analysed MS2L-tagged mRNAs. Since the smear below the signal corresponding to full-length *PGK1-6MS2L* forms a long "tail" (Figure 12B, lower panel, lane 13 and 19), it seems plausible that these mRNA decay intermediates indeed contain sequences downstream of the MS2L tag. Therefore, the main reason for not detecting subunits of the exosome or the Ski complex among the enriched proteins might not be related to the position of the 6MS2L tag or the instability of 3'→5' mRNA decay factor interaction with the mRNA. Instead, northern blot results hint at the possibility that the subpopulation of MS2L-tagged RNAs degraded in 3'→5' direction is very small and therefore could escape detection.

Many proteins are likely lost during mRNP affinity purification due to a weak association with the mRNP

Our mRNP affinity purification strategy does not include a covalent cross-linking step to stabilize mRNA-protein and protein-protein interactions. Consequently, the mRNPs are prone to lose weak or transient interaction partners during mRNP affinity purification. This problem is illustrated, for example, by the enrichment of most translation initiation factors in single MS data sets or by the enrichment of only certain subunits of the heptameric Lsm1-7 complex. In addition, many proteins were quantified in only one of the biological replicate experiments and therefore the number of enriched proteins in each MS data set is likely underestimated (Appendix, Table 1).

The only translation initiation factor enriched in all MS data sets was eIF4G (Table 13). The protein makes multiple contacts with the mRNA body (Berset et al. 2003; E.-H. Park et al. 2011; Yanagiya et al. 2009) and also interacts with the poly(A) tail-bound Pab1 (E.-H. Park et al. 2011; Svitkin et al. 2009; Tarun et al. 1997). Unlike most other translation factors, which dissociate from the mRNA by the end of the translation initiation step, eIF4G remains associated with the mRNA throughout the whole translation cycle (reviewed in Hinnebusch 2011). Therefore, proteins whose association with the mRNA is stable in terms of interaction strength and duration can likely be efficiently captured using our mRNP affinity purification strategy. The enrichment of most other translation initiation factors only in single MS data sets suggest that the interaction of these initiation factors with the mRNA, rRNA or ribosomal proteins and other initiation factors (Herrmannová et al. 2012; W.-L. Chiu et al. 2010; Lebaron et al. 2012; Shin et al. 2011; Hashem et al. 2013) is not strong enough to be efficiently retained throughout the affinity purification procedure.

The only subunit of the Lsm1-7 complex enriched in all three MS data sets was Lsm4 (Table 16). Two additional subunits, Lsm1 and Lsm2, were enriched after *ENO2-6MS2L* affinity purification. In one of the biological replicate experiments these two subunits co-purified also with *PGK1-6MS2L*. Likewise, Lsm1 co-purified with 6MS2L-RNA in one of the biological replicate experiment. These results indicate that the interaction between the subunits of the heptameric Lsm1-7 complex is not very stable and raise the question why the Lsm1, Lsm2 and Lsm4 subunits but not the Lsm3 and Lsm5-7 were detected? UV cross-linking experiments indicate that Lsm1 and possibly also Lsm4 directly contact the mRNA (Chowdhury, Mukhopadhyay, and Tharun 2007). Since Lsm4 was the only subunit co-purifying with all tested MS2L-tagged RNAs, our results hint at the possibility that the major RNA-binding subunit of Lsm1-7 complex may be Lsm4. Structural studies indicate that the order of the subunits in the heptameric ring is Lsm1-2-3-6-5-7-4 (Sharif and Conti 2013). The interaction between the subunits 1 and 2 may help to stabilize Lsm2 interaction with the mRNP. The decapping activator Pat1, which was enriched in all MS data sets (Table 15), has been found to bind a composite surface of Lsm2 and Lsm3 (Sharif and Conti 2013). This interaction might also contribute to the stability of Lsm2 interaction with the mRNP during affinity purification.

Collectively, the mRNA-bound proteome analysis indicates that the efficiency of detection of certain mRNP-associated mRNA-protein and protein-protein interactions is influenced by the stability and cellular abundance of these interactions. Considering these limitations, it is remarkable that many proteins with no previously identified role in mRNA regulation have co-purified with the MS2L-tagged RNAs, suggesting that such proteins indeed represent true mRNP-associated proteins (see Discussion, Part 3, "mRNA-bound proteome

analysis revealed many unexpected proteins – a hint to novel RNA-binding proteins and previously uncharacterized cellular mechanisms?"). However, the inefficient detection of low-abundance, weak or transient interactions also implies that the determined mRNA-associated proteomes do not reflect all the cellular events the MS2L-tagged RNAs are engaged in. For instance, we consistently could not detect Upf2 and Upf3 among the enriched proteins. Besides Upf1, these two proteins are essential for NMD, which is triggered upon interaction of the three Upf proteins on NMD target mRNAs (see Introduction, "NMD factors and consequences of their activation" and references therein). The absence of Upf2 and Upf3 among the enriched proteins raises the possibility that the MS2L-tagged mRNAs might not be targeted by NMD. This problem points to the need to use several parallel methods to study mRNA-protein interactions and the biological mechanisms underlying these interactions. The involvement of NMD in the turnover of MS2L-tagged RNAs, for instance, can be determined by monitoring MS2L-tagged mRNA decay upon transcription shutoff in wt, Δ*upf1* and Δ*upf2* strain background; NMD targets should be stabilized to a comparable extent in Δ*upf1* and Δ*upf2* cells (F He, Brown, and Jacobson 1997).

Part 3

mRNA-bound proteome analysis opens up a host of new questions

What are the biological mechanisms behind the co-purification of a certain set of proteins with MS2L-tagged *PGK1*, *ENO2* or the mRNA-like 6MS2L-RNA transcript? This is not an easy question to answer especially since the three analysed mRNA-bound proteomes did not only contain proteins with well established roles in the different steps of mRNA life cycle but also contained many proteins that have previously not been implicated in mRNA regulation. The final chapter of the thesis will highlight the proteins that possibly represent novel mRNP proteins. We will also compare the mRNA-bound proteomes of 6MS2L-RNA transcript to MS2L-tagged *PGK1* and *ENO2* to analyse how the MS2L tag might influence mRNP protein composition. Finally, the intriguing possibility of co-translational assembly of a supramolecular glycolytic enzyme complex will be discussed.

6MS2L-RNA – not quite an mRNA

We set out to study what proteins co-purify with a heterologously expressed RNA containing the 6 MS2 stem-loops (see Results, "Control RNA to determine the effect of MS2L tag on mRNP protein composition") to identify cellular proteins that have the potential to bind to the MS2L tag. Interestingly, the majority of proteins co-purifying with 6MS2L-RNA were also enriched after *PGK1-6MS2L* or *ENO2-6MS2L* affinity purification, indicating that during its life cycle the 6MS2L-RNA transcript is engaged in a similar network of cellular interactions as the MS2L-tagged *PGK1* and *ENO2*. It seems plausible that 6MS2L-RNA is sensed as an mRNA by the yeast cells. We assume that the *PGK1*-derived 5' UTR and the *CYC1*-derived 3' UTR that are necessary regions for *in vivo* expression of 6MS2L-RNA are responsible for this. As a consequence, the 6MS2L-RNA-bound proteome is comprised not only of proteins that bind to the MS2 stem-loops but also contains proteins that associate with the 5' and 3' UTRs. The high degree of overlap between the proteomes of 6MS2L-RNA and the MS2L-tagged mRNAs suggests that many cellular proteins have the potential to bind to the MS2 stem-loops; however, this binding likely depends on the mRNA context provided by 5' and 3' UTRs of the 6MS2L-RNA. It seems plausible that not many cellular proteins bind to the MS2 stem-loops *per se*.

The small differences in the repertoire or enrichment level of 6MS2L-RNA co-purifying proteins hint at interesting differences in the regulation of this mRNA-like transcript compared to the two MS2L-tagged mRNAs. The analysis of these differences helped to understand better the mechanisms responsible for co-purification of a certain set of proteins with the studied MS2L-tagged RNAs. For instance, the two subunits of the nuclear cap-binding complex, Cbc2 and Cbc1, were among the most highly enriched proteins in 6MS2L-RNA MS data set (Table 20). In contrast, these proteins did not classify as enriched after *PGK1-6MS2L* or *ENO2-6MS2L* affinity purification. Since the nuclear cap-binding complex is replaced by eIF4E before or during the pioneer round of translation, these results indicate that the association of eIF4E with 6MS2L-RNA is perturbed (see Discussion, Part1, "The nuclear history of MS2L-tagged RNAs is reflected by the enriched RNA-binding proteins" for further discussion). A possible reason for that could be slower remodelling kinetics of 6MS2L-RNA-containing mRNPs at the cytoplasmic side of the nuclear pores. This notion is supported by the finding that the shuttling poly(A)$^+$ RNA-binding protein Nab2, which is removed after mRNP export to the cytoplasm, was >13-fold enriched after 6MS2L-RNA affinity purification compared to the about 2.3-fold enrichment after *PGK1-6MS2L* affinity purification (Table 20).

Ribosomal proteins of both the large and small subunit were among the enriched proteins after 6MS2L-RNA affinity purification (Figure 25), demonstrating that 80S ribosomes can form on this mRNA-like transcript. Since 80S ribosome formation is paralleled by initiation of

translation elongation (see Introduction, "mRNP interactions in cap-dependent translation" and references therein), this finding strongly suggests that 6MS2L-RNA can be translated. mRNA translation, however, is a prerequisite for nonsense-mediated mRNA decay (see Introduction, "Nonsense-mediated mRNA decay" and references therein). The predicted NMD-activating feature in 6MS2L-RNA as in MS2L-tagged *PGK1* and *ENO2* is a long distance between the translation termination codon and the poly(A) tail (see Discussion, Part 1, "MS2L-tagged RNAs may be targeted by nonsense-mediated decay" for further discussion). The comparable enrichment level of Upf1 with all three studied MS2L-tagged RNAs as well as co-purification of Nmd4 with 6MS2L-RNA suggests that this mRNA-like transcript is subjected to NMD (Table 16). However, RT-PCR analysis of the steady state level of 6MS2L-RNA compared to *PGK1-6MS2L* (Fig. 18B) hints at the possibility that the decay kinetics and thus the decay mechanism of the two RNAs might be different. The transcription of 6MS2L-RNA is under the control of *PGK1* promoter. We reasoned that *PGK1* promoter-controlled expression of 6MS2L-RNA would result in a similar expression level of 6MS2L-RNA compared to untagged *PGK1*. However, this hypothesis could not be experimentally tested because the two transcripts to be compared cannot be amplified with the same set of primers. Therefore, we have compared the steady state levels of 6MS2L-RNA and *PGK1-6MS2L*. The level of the latter mRNA is about 50% of the level of untagged *PGK1* (Fig. 14A). The 2.3-fold higher level of 6MS2L-RNA compared to *PGK1-6MS2L* suggests that 6MS2L-RNA transcript levels are comparable to *PGK1* as hypothesised. This finding thus suggests that 6MS2L-RNA is not subjected to accelerated mRNA decay due to NMD. However, we cannot rule out the possibility that despite the *PGK1* promoter-controlled expression, the expression level of 6MS2L-RNA is actually higher than that of *PGK1*. Therefore, in order to unambiguously determine whether 6MS2L-RNA is subjected to NMD the decay of this transcript should be examined in wt and Δ*upf1* strain backgrounds.

mRNA-bound proteome analysis revealed many unexpected proteins – a hint to novel mRNP proteins and previously uncharacterized cellular mechanisms?

The mRNA-bound proteome analysis revealed several proteins that might, in addition to other cellular functions, participate in mRNA regulation. This class of proteins contains metabolic enzymes Imd2, Imd3, Imd4, Mis1 and Shm1 (Table 25), tRNA modification enzymes Trm44, Trm2, Pus4 and Pus7 (Table 23), ribosome biogenesis factors Cbf5, Mrd1, Arb1 and Arx1 (Table 21) and proteins involved in ubiquitin-mediated regulation (Ubp3 and Def1, Table 27). All the above-mentioned proteins co-purified with at least two of the tested MS2L-tagged RNAs. Importantly, most of these proteins have previously been found to co-purify with poly(A)$^+$ RNA

(Imd2, Imd3, Imd4, Cbf5, Mrd1 and Ubp3) or with Pab1 (Imd4, Mis1, Shm1, Cbf5, Arx1 and Ubp3) in yeast (S. F. Mitchell et al. 2013; Klass et al. 2013; R. Richardson et al. 2012). Further evidence for mRNA-association has been obtained for the ubiquitin-specific protease Ubp3 (Baker, Tobias, and Varshavsky 1992), which has been found to associate with >1000 mRNAs in *S. cerevisiae* (Tsvetanova et al. 2010). However, for all of these proteins the possible function in context of an mRNP remains unknown.

The only proteins whose mRNA-related function has been studied to some extent are the inosine monophosphate dehydrogenase isozymes encoded by *IMD2*, *IMD3* and *IMD4*. Genetic studies in yeast have demonstrated that the only essential function of the three proteins is *de novo* synthesis of GTP (Hyle, Shaw, and Reines 2003). However, observations in human cells suggest that IMPDH has a "moonlighting" function in translation regulation (reviewed in Hedstrom 2009). Several mutations in human inosine monophosphate dehydrogenase isozyme type 1 (IMPDH1) are associated with autosomal dominant form of retinitis pigmentosa (adRP) (Bowne et al. 2002; Kennan et al. 2002; Bowne et al. 2006). Surprisingly, the analysed adRP-associated mutations in IMPDH1 gene do not affect the activity of the enzyme (Mortimer and Hedstrom 2005). Instead, the mutations reduce the level of RNA co-immunoprecipitation with IMPDH1 (Mortimer and Hedstrom 2005) and disrupt polyribosome-association of the tested retinal IMPDH1 isoform (Mortimer et al. 2008). The latter finding suggests a role for mammalian IMPDH in regulation of translation (Mortimer et al. 2008). It should be noted, however, that IMPDH isoenzymes were not among the enriched proteins after poly(A)$^+$ RNA affinity purification from immortalized human cell lines (Castello et al. 2012; Baltz et al. 2012), suggesting that ribosome-association might be specific for IMPDH1 retinal isoform. It remains to be determined whether the yeast Imd2, Imd3 and Imd4 are also associated with polyribosomes and what the functional role of this interaction could be. Imd2, Imd3 and Imd4 co-purification with mRNA under normal yeast growth conditions (our data) as well as upon glucose deprivation (S. F. Mitchell et al. 2013) suggests that the proteins may associate with both translationally active and inactive pools of mRNPs.

Besides the possible role for Imd2, Imd3 and Imd4 in translation regulation (Mortimer et al. 2008), the secondary functions of the above-mentioned proteins have not been studied to our knowledge. However, the identification of 21 ribosome biogenesis factors among the poly(A)$^+$ RNA co-purifying proteins in glucose deprived yeast cells (S. F. Mitchell et al. 2013) or the co-purification of Ubp3 with >1000 transcripts (Tsvetanova et al. 2010) strongly suggests that some ribosomal biogenesis factors and Ubp3 have a jet unidentified role in mRNA life cycle. Importantly, this function seems to be evolutionarily conserved since most of the MS2L-tagged

RNA co-purifying ribosome biogenesis factors as well as Ubp3 have been found to co-purify with poly(A)$^+$ RNA also in mammalian cells (Castello et al. 2012; Baltz et al. 2012).

In addition to proteins with no well established role in mRNA regulation that co-purified with at least two of the analysed MS2L-tagged RNAs, many proteins previously not implicated in mRNA regulation were enriched also in single MS data sets. For instance, *ENO2-6MS2L* co-purified with the mitochondrial RNA polymerase Rpo41 (Greenleaf, Kelly, and Lehman 1986) and with a component of mitochondrial nucleoid Mgm101 (Mbantenkhu et al. 2011). Both proteins were >9-fold enriched, suggesting a specific interaction with *ENO2-6MS2L*. Since Rpo41 and Mgm101 are mitochondrial proteins, this finding raises the questions of where and why the cytoplasmic mRNPs containing *ENO2-6MS2L* mRNA interact with these mitochondrial proteins. The functional role of *ENO2-6MS2L* interaction with Rpo41 and Mgm101 remains unknown. However, a possible cellular site of interaction could be the mitochondrial surface. A part of cellular enolase pool is associated with mitochondrial surface where the protein is a component of a large macromolecular complex containing additional glycolytic enzymes, mitochondrial membrane carriers and enzymes of the citric acid cycle (Entelis et al. 2006; Brandina et al. 2006). The formation of such a macromolecular complex might involve *ENO2* mRNA translation in the vicinity of mitochondria, where the mRNA or the nascent enolase peptide could come into contact with mitochondrial proteins awaiting import. Alternatively, the co-purification of Rpo41 and Mgm101 with *ENO2-6MS2L* might represent false-positive interactions that occur upon the release of mitochondrial proteins due to cell breakage. Considering the high enrichment level of Rpo41 and Mgm101 (>9-fold enrichment) the latter scenario seems unlikely. However, we cannot rule out that some non-specifically bound proteins have been classified as enriched. The low enrichment level (<2-fold enrichment) and classification as enriched only in a single MS data set raises the possibility that some cytoplasmic metabolic enzymes (Leu2, Zwf1, Aro1, His4, Ura3, Met6), mitochondrial proteins (Adh3, Mss116, Hsp60) and additional proteins with various functions (Sec16, Psp2, Bmh2) represent non-specific interactors.

PGK1-6MS2L co-purified with four glycolytic enzymes – Pgk1, Hxk2, Pgi1 and Tdh3 (Table 31). In addition, three glycolytic enzymes (Tpi1, Tdh2 and Eno2) only slightly failed to meet the set threshold criteria for enriched proteins. Co-purification of Pgk1 protein with *PGK1-6MS2L* mRNA is easily explained by association between the *PGK1-6MS2L* mRNA, the ribosome and the Pgk1 nascent peptide. This chain of interactions might also contribute to the co-purification of the other glycolytic enzymes with *PGK1-6MS2L*. Namely, several lines of evidence suggest that glycolytic enzymes form a supramolecular complex (see Discussion, Part 1, "*PGK1-6MS2L* co-purifies with several glycolytic enzymes – co-translational formation of a

supramolecular glycolytic enzyme complex?" for references). Pgk1 nascent peptide might be co-translationally recruited to such a complex, resulting in *PGK1-6MS2L* co-purification with not only Pgk1 nascent peptide but also with other glycolytic enzymes. It remains to be experimentally determined if the co-purification of glycolytic enzymes with *PGK1-6MS2L* represents biologically meaningful interactions or is a result of non-specific association of abundant cytoplasmic proteins with mRNPs upon cell breakage. The fact that 6MS2L-RNA did not co-purify with any of the glycolytic enzymes supports the notion that *PGK1-6MS2L* co-purification with glycolytic enzymes is biologically meaningful and represents the association of glycolytic enzymes into a supramolecular complex.

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APPENDIX

Table 1. The list of MS2L-tagged mRNA co-purifying proteins, which fulfilled the set threshold criteria to be classified as "enriched" (forward labelling experiment log_2 (H/L) >0.5 and reverse labelling experiment log_2 (H/L) <–0.5). Each single row contains the group of proteins (proteinGroup) that could be assigned to a set of peptides that were identified by SILAC-based quantitative mass spectrometry. P – number of identified peptides; H/L – normalized SILAC ratio; log_2 – log_2 transformed normalized SILAC ratio; significance – significance B (if < 0.01, the corresponding proteinGroup is significantly up or down regulated).

Boiled Beads unique (*PGK1-6MS2L*)		**Forward experiment**				**Reverse experiment**			
Protein ID	Gene	P	H/L	log_2	Significance	P	H/L	log_2	Significance
P09440	MIS1	27	5.733	2.52	0.001493	19	0.128	-2.96	0.058185
Q06698	YLR419W	15	5.309	2.41	0.00229	7	0.158	-2.67	0.079027
P0CX47; P0CX48	RPS11A; RPS11B	9	4.952	2.31	0.003327	7	0.120	-3.06	0.052032
P53741	BRE5	12	4.640	2.21	0.004657	8	0.171	-2.55	0.088563
P0CX82; P0CX83	RPL19A; RPL19B	5	4.589	2.20	0.004922	5	0.216	-2.21	0.1216
P05748; P54780	RPL15A; RPL15B	3	4.381	2.13	0.006205	4	0.267	-1.91	0.1581
P53952	YNL050C	2	4.191	2.07	0.007696	2	0.531	-0.91	0.3201
P14120	RPL30	4	4.013	2.00	0.009449	5	0.354	-1.50	0.21699
P40212; Q12690	RPL13B; RPL13A	4	3.529	1.82	0.01687	2	0.255	-1.97	0.14966
P26321	RPL5	6	3.298	1.72	0.022462	4	0.541	-0.89	0.32554
P05738	RPL9A	9	3.059	1.61	0.030435	5	0.240	-2.06	0.13936
P53297	PBP1	9	2.935	1.55	0.035751	7	0.249	-2.00	0.14565
P0CX43; P0CX44	RPL1A; RPL1B	7	2.904	1.54	0.037233	5	0.233	-2.10	0.13403
P38811	TRA1	12	2.655	1.41	0.05182	2	0.475	-1.07	0.28984
P32563	VPH1	6	2.490	1.32	0.064808	3	0.343	-1.55	0.20958
P49626	RPL4B	9	2.488	1.32	0.064998	9	0.257	-1.96	0.15112
P25368	RRP7	5	2.391	1.26	0.074247	2	0.699	-0.52	0.40006
P41805	RPL10	2	2.391	1.26	0.074249	4	0.275	-1.86	0.16409
P33322	CBF5	4	2.356	1.24	0.077964	7	0.459	-1.12	0.28084
P53254	UTP22	12	2.098	1.07	0.11182	4	0.495	-1.01	0.30096
P42846	KRI1	3	1.949	0.96	0.13818	4	0.465	-1.10	0.28443
P60010	ACT1	6	1.820	0.86	0.16624	4	0.183	-2.45	0.097472
P25586	KRR1	7	1.770	0.82	0.17855	3	0.542	-0.88	0.32597
P0C0T4	RPS25B	6	1.718	0.78	0.19254	7	0.691	-0.53	0.39652
P48415	SEC16	13	1.697	0.76	0.19855	3	0.457	-1.13	0.27988
P15424	MSS116	9	1.676	0.74	0.20465	7	0.510	-0.97	0.30894
P37838	NOP4	7	1.661	0.73	0.20904	2	0.646	-0.63	0.37647
P02407; P14127	RPS17A; RPS17B	6	1.636	0.71	0.21669	8	0.188	-2.41	0.10163
P38786	RPP1	2	1.624	0.70	0.22043	2	0.189	-2.40	0.10192
P0CX29; P0CX30	RPS23A; RPS23B	8	1.609	0.69	0.22521	7	0.129	-2.95	0.058713
P0CX35; P0CX36	RPS4B	12	1.576	0.66	0.2362	8	0.109	-3.20	0.044872
P40010	NUG1	7	1.474	0.56	0.2738	5	0.593	-0.75	0.35141
P05756	RPS13	5	1.466	0.55	0.27689	4	0.583	-0.78	0.34648
P41819	DIM1	6	1.429	0.51	0.29207	2	0.441	-1.18	0.2705

RNase unique (*PGK1-6MS2L*)		**Forward experiment**				**Reverse experiment**			
Protein ID	Gene	P	H/L	log_2	Significance	P	H/L	log_2	Significance
P02400	RPP2B	2	14.164	3.82	6.41E-06	2	0.342	-1.55	0.11457
P25644	PAT1	10	13.558	3.76	8.92E-06	6	0.039	-4.70	7.19E-05
Q02648	TRM44	14	13.080	3.71	1.17E-05	6	0.033	-4.93	3.30E-05
P39998	EDC3	6	11.665	3.54	2.67E-05	3	0.062	-4.00	0.000622
P33753	TRM2	9	11.031	3.46	3.95E-05	5	0.089	-3.49	0.002543
P36102	PAN3	14	8.756	3.13	0.000183	8	0.051	-4.30	0.000255
P53010	PAN2	4	8.492	3.09	0.000222	6	0.068	-3.88	0.000892
P05750	RPS3	6	5.776	2.53	0.002044	5	0.219	-2.19	0.041735
P0CX51; P0CX52	RPS16A; RPS16B	6	5.179	2.37	0.003576	1	0.512	-0.97	0.23489
P26783	RPS5	7	4.096	2.03	0.010735	5	0.443	-1.17	0.18581
P34761	WHI3	4	4.040	2.01	0.011403	2	0.194	-2.37	0.030213
P26786	RPS7A	15	3.986	2.00	0.012082	9	0.406	-1.30	0.15866
Q12517	DCP1	5	3.759	1.91	0.015499	2	0.204	-2.29	0.034501
P38922	HRB1	6	3.407	1.77	0.023099	5	0.274	-1.87	0.071114
P40070	LSM4	4	3.016	1.59	0.036651	3	0.341	-1.55	0.11402
P40024	ARB1	9	2.963	1.57	0.039086	7	0.378	-1.40	0.13915
P10081	TIF1	4	2.632	1.40	0.058868	5	0.688	-0.54	0.35518
P40047	ALD5	19	2.604	1.38	0.060995	16	0.418	-1.26	0.16763
P25567	SRO9	9	2.455	1.30	0.073711	5	0.309	-1.69	0.092826
P0CX37; P0CX38	RPS6A; RPS6B	7	2.439	1.29	0.075259	8	0.645	-0.63	0.32743
P05317	RPP0	6	2.426	1.28	0.076522	5	0.355	-1.50	0.12316
P53316	YGR250C	6	2.342	1.23	0.085284	2	0.387	-1.37	0.14554
Q04600	TMA64	5	2.311	1.21	0.088839	3	0.315	-1.67	0.096408
P05694	MET6	11	2.253	1.17	0.095843	6	0.637	-0.65	0.32177
P32505	NAB2	10	2.240	1.16	0.097537	5	0.420	-1.25	0.16908
P00359	TDH3	14	2.187	1.13	0.10452	15	0.616	-0.70	0.30776
P35997;P38711	RPS27A; RPS27B	3	2.164	1.11	0.10777	2	0.457	-1.13	0.19553
P07246	ADH3	3	2.117	1.08	0.11472	2	0.613	-0.71	0.30549
P12709	PGI1	7	2.076	1.05	0.12103	4	0.280	-1.84	0.074752
P0CX84; P0CX85	RPL35A; RPL35B	3	1.986	0.99	0.13664	3	0.511	-0.97	0.23437
P48164	RPS7B	13	1.947	0.96	0.14396	11	0.398	-1.33	0.15346
P40561	SGN1	5	1.937	0.95	0.14587	2	0.412	-1.28	0.16312
P04807	HXK2	8	1.915	0.94	0.15024	3	0.352	-1.51	0.12107
P33399	LHP1	5	1.883	0.91	0.15697	3	0.484	-1.05	0.21506
P04173	LEU2	8	1.773	0.83	0.18218	4	0.690	-0.54	0.35655
P0C0V8; Q3E754	RPS21A; RPS21B	2	1.754	0.81	0.18699	2	0.419	-1.25	0.16833
P50094; O42831	IMD4; YAR075W	9	1.753	0.81	0.18722	5	0.390	-1.36	0.14759
Q3E7Y3; P0C0W1	RPS22B; RPS22A	7	1.749	0.81	0.18814	3	0.480	-1.06	0.21212
P00330	ADH1	10	1.702	0.77	0.2006	14	0.686	-0.54	0.35401
Q12499	NOP58	6	1.674	0.74	0.20858	3	0.631	-0.66	0.31791
P05759; P0CG63	RPS31; UBI4	5	1.671	0.74	0.20942	4	0.439	-1.19	0.1829
P26785	RPL16B	4	1.666	0.74	0.21072	1	0.477	-1.07	0.21002
P10080	SBP1	6	1.585	0.66	0.23558	3	0.531	-0.91	0.24857
P32588	PUB1	4	1.572	0.65	0.23962	3	0.605	-0.73	0.30011

Boiled Beads unique (*PGK1-6MS2L*)		**Forward experiment**				**Reverse experiment**			
Protein ID	Gene	P	H/L	log_2	Significance	P	H/L	log_2	Significance
P50095	IMD3	13	1.539	0.62	0.25081	11	0.466	-1.10	0.20208
P38701	RPS20	11	1.486	0.57	0.26979	9	0.665	-0.59	0.3407
P19882	HSP60	19	1.458	0.54	0.2804	9	0.666	-0.59	0.34132
P38199	HEK2	5	1.455	0.54	0.28139	2	0.589	-0.76	0.2889

Shared (*PGK1-6MS2L*)		**Forward experiment**				**Reverse experiment**			
		P	H/L	log_2	Significance	P	H/L	log_2	Significance
P30771	UPF1	16	60.681	5.92	5.90E-12	9	0.015	-6.08	3.88E-07
Q03466	EBS1	12	22.113	4.47	1.66E-07	5	0.022	-5.49	4.19E-06
P53550	DCP2	15	19.375	4.28	5.20E-07	6	0.058	-4.10	0.000465
P22147	XRN1	15	7.407	2.89	0.00051	26	0.127	-2.98	0.00852
Q12000	TMA46	8	6.489	2.70	0.001089	3	0.105	-3.25	0.004608
P38011	ASC1	11	6.393	2.68	0.001183	11	0.123	-3.02	0.007778
P04147	PAB1	29	5.960	2.58	0.00173	28	0.097	-3.36	0.003471
P39015	STM1	16	5.709	2.51	0.002173	17	0.127	-2.97	0.008689
P39729	RBG1	6	5.636	2.49	0.002325	4	0.119	-3.07	0.007029
Q01477	UBP3	7	3.914	1.97	0.013067	5	0.214	-2.22	0.039179
P40150	SSB2	21	3.711	1.89	0.016363	19	0.300	-1.74	0.087279
P38934	BFR1	22	3.463	1.79	0.02166	15	0.165	-2.60	0.019108
P14126	RPL3	2	3.415	1.77	0.022893	3	0.297	-1.75	0.084941
P39936	TIF4632	18	3.385	1.76	0.023695	10	0.193	-2.37	0.02986
P16521; P53978	YEF3; HEF3	22	2.864	1.52	0.044104	17	0.384	-1.38	0.1438
P10664	RPL4A	5	2.853	1.51	0.044713	5	0.431	-1.21	0.17674
P25443	RPS2	5	2.647	1.40	0.057788	3	0.549	-0.86	0.2614
P40850	MKT1	9	2.526	1.34	0.067367	3	0.330	-1.60	0.10682
Q03862	ARX1	10	2.411	1.27	0.078029	6	0.355	-1.50	0.12305
P39935	TIF4631	18	2.348	1.23	0.084665	17	0.296	-1.76	0.084346
P32527	ZUO1	15	2.150	1.10	0.10984	10	0.387	-1.37	0.14529
P05755	RPS9B	7	2.111	1.08	0.11553	5	0.449	-1.16	0.18956
P38788	SSZ1	18	2.110	1.08	0.11573	11	0.372	-1.42	0.13543
P0CX39; P0CX40	RPS8A; RPS8B	2	1.855	0.89	0.16287	1	0.353	-1.50	0.12181
P35732	DEF1	17	1.853	0.89	0.1635	7	0.563	-0.83	0.27131
P06105	SCP160	40	1.831	0.87	0.16843	35	0.591	-0.76	0.29042
P33442	RPS1A	6	1.704	0.77	0.20005	7	0.511	-0.97	0.23405
P00560	PGK1	19	1.686	0.75	0.20519	16	0.084	-3.58	0.002011
P07281	RPS19B	6	1.676	0.75	0.20791	5	0.659	-0.60	0.33636
P23248	RPS1B	7	1.521	0.60	0.25725	7	0.418	-1.26	0.1674

***ENO2-6MS2L* (RNase eluate)**		**Forward experiment**				**Reverse experiment**			
Protein ID	Gene	P	H/L	log_2	Significance	P	H/L	log_2	Significance
Q03466	EBS1	11	29.074	4.86	0.0085327	10	0.038	-4.72	0.087438
Q02933	RNY1	2	19.370	4.28	0.020706	2	0.034	-4.88	0.080276
P33753	TRM2	7	18.617	4.22	0.02245	8	0.015	-6.11	0.03748
P0CX55; P0CX56	RPS18A; RPS18B	3	18.299	4.19	0.023246	3	0.088	-3.50	0.16303
P25644	PAT1	11	14.727	3.88	0.035463	9	0.011	-6.48	0.02903
Q02648	TRM44	5	13.519	3.76	0.041542	8	0.009	-6.77	0.023716
P37292	SHM1	14	12.245	3.61	0.049604	10	0.040	-4.65	0.090891
P32787	MGM101	2	11.898	3.57	0.052165	2	0.013	-6.27	0.033636
P0CX82; P0CX83	RPL19A; RPL19B	4	11.636	3.54	0.05422	5	0.592	-0.76	0.44422
P0CX35; P0CX36	RPS4A; RPS4B	4	9.977	3.32	0.07021	7	0.527	-0.92	0.42409
Q07362	PBP4	2	9.872	3.30	0.071427	5	0.093	-3.43	0.16885
P40024	ARB1	8	9.581	3.26	0.074966	5	0.092	-3.44	0.16793
P53550	DCP2	5	9.420	3.24	0.077032	10	0.067	-3.90	0.13481
P13433	RPO41	9	9.110	3.19	0.081243	4	0.047	-4.42	0.10319
P30771	NAM7	12	9.046	3.18	0.082141	10	0.036	-4.78	0.084911
P22147	XRN1	24	8.919	3.16	0.08399	23	0.059	-4.08	0.12295
P39998	EDC3	4	8.661	3.11	0.087901	4	0.041	-4.60	0.093604
P36102	PAN3	7	8.600	3.10	0.08887	5	0.054	-4.22	0.11465
P38922	HRB1	4	8.577	3.10	0.089244	5	0.027	-5.22	0.065687
P53010	PAN2	8	8.546	3.10	0.089735	3	0.046	-4.45	0.10142
Q06698	YLR419W	17	8.525	3.09	0.090075	5	0.039	-4.70	0.088747
P38934	BFR1	26	8.306	3.05	0.093744	17	0.046	-4.43	0.1026
P26321	RPL5	2	7.665	2.94	0.1057	2	0.157	-2.67	0.23324
P0CX47; P0CX48	RPS11A; RPS11B	2	7.602	2.93	0.107	6	0.150	-2.74	0.22754
P14126	RPL3	3	7.532	2.91	0.10846	8	0.051	-4.28	0.1109
P39729	RBG1	8	7.306	2.87	0.11336	7	0.108	-3.21	0.18623
P38203	LSM2	2	7.303	2.87	0.11343	1	0.111	-3.17	0.18901
P38788	SSZ1	16	7.117	2.83	0.11771	11	0.074	-3.75	0.14509
P05317	RPP0	5	7.042	2.82	0.1195	6	0.701	-0.51	0.47409
P10664	RPL4A	6	7.010	2.81	0.12027	5	0.140	-2.84	0.21793
P07281	RPS19B	4	6.997	2.81	0.1206	3	0.230	-2.12	0.28865
P40047	ALD5	19	6.920	2.79	0.12249	21	0.056	-4.16	0.11841
P0CX37; P0CX38	RPS6A; RPS6B	5	6.843	2.77	0.12444	7	0.094	-3.42	0.16962
P32527	ZUO1	10	6.719	2.75	0.12767	10	0.079	-3.65	0.1519
P53297	PBP1	3	6.704	2.75	0.12806	8	0.058	-4.12	0.12098
P24000	RPL24B	2	6.645	2.73	0.12965	3	0.408	-1.29	0.38021
P04456	RPL25	4	6.576	2.72	0.13154	3	0.055	-4.19	0.11629
P25443	RPS2	6	6.141	2.62	0.14437	2	0.293	-1.77	0.32603
P39015	STM1	19	5.800	2.54	0.15573	18	0.048	-4.39	0.10514
P05755	RPS9B	5	5.603	2.49	0.16286	5	0.227	-2.14	0.28625
Q02753	RPL21A	2	5.600	2.49	0.16297	4	0.633	-0.66	0.45615
Q3E7Y3; P0C0W1	RPS22B; RPS22A	6	5.525	2.47	0.1658	4	0.207	-2.27	0.27294
P47017	LSM1	2	5.511	2.46	0.16634	1	0.130	-2.94	0.20872
P48567	PUS4	2	5.419	2.44	0.16997	6	0.038	-4.71	0.088203

***ENO2-6MS2L* (RNase eluate)**		**Forward experiment**				**Reverse experiment**			
Protein ID	Gene	P	H/L	log_2	Significance	P	H/L	log_2	Significance
P48589	RPS12	5	5.343	2.42	0.17301	3	0.126	-2.99	0.20474
P0C0V8; Q3E754	RPS21A; RPS21B	3	5.280	2.40	0.17561	1	0.100	-3.32	0.17754
P38011	ASC1	16	5.228	2.39	0.17782	9	0.078	-3.68	0.15031
Q03862	ARX1	10	5.033	2.33	0.18635	5	0.064	-3.97	0.13
P0C2H8	RPL31A	4	4.979	2.32	0.18883	2	0.086	-3.54	0.15992
Q01477	UBP3	13	4.808	2.27	0.197	9	0.064	-3.96	0.13065
P38174	MAP2	3	4.756	2.25	0.19955	4	0.126	-2.99	0.20513
P26786	RPS7A	16	4.638	2.21	0.20562	13	0.117	-3.09	0.19588
P50094; O42831	IMD4; YAR075W	8	4.462	2.16	0.21509	7	0.076	-3.72	0.1469
P05740; P46990	RPL17A; RPL17B	3	4.402	2.14	0.2185	3	0.075	-3.74	0.14582
P40150	SSB2	20	4.362	2.13	0.22078	17	0.077	-3.70	0.14893
P02407; P14127	RPS17A	5	4.333	2.12	0.22251	4	0.139	-2.84	0.21767
P04147	PAB1	25	4.332	2.12	0.22253	27	0.079	-3.66	0.15141
P39730	FUN12	7	4.249	2.09	0.22747	5	0.094	-3.41	0.1698
P12945	NAT1	8	4.233	2.08	0.22843	2	0.174	-2.52	0.24782
P39936	TIF4632	20	4.123	2.04	0.23531	14	0.077	-3.70	0.14875
P29453	RPL8B	10	4.091	2.03	0.23738	8	0.199	-2.33	0.26696
P40850	MKT1	4	3.997	2.00	0.24348	8	0.129	-2.95	0.20803
P53235	YGR054W	8	3.848	1.94	0.25376	5	0.142	-2.82	0.22024
P09440	MIS1	19	3.537	1.82	0.27732	12	0.065	-3.95	0.13178
P50095; REV_P38882	IMD3; REV_UTP9	12	3.499	1.81	0.28036	13	0.125	-3.00	0.20378
P41805	RPL10	1	3.459	1.79	0.28369	2	0.270	-1.89	0.3133
P05738	RPL9A	12	3.453	1.79	0.28419	8	0.148	-2.76	0.22555
P46784; Q08745	RPS10B; RPS10A	2	3.356	1.75	0.29251	2	0.098	-3.35	0.17471
P07347	ARD1	2	3.192	1.67	0.30732	2	0.188	-2.41	0.25896
P25567	SRO9	6	3.135	1.65	0.31273	5	0.077	-3.69	0.14902
Q12129	NMD4	5	3.135	1.65	0.31274	3	0.056	-4.17	0.11771
P05750	RPS3	7	2.990	1.58	0.32715	6	0.043	-4.54	0.09673
P38697; P39567	IMD2; IMD1	10	2.927	1.55	0.33374	11	0.097	-3.37	0.17348
Q12517	DCP1	3	2.877	1.52	0.33903	4	0.153	-2.71	0.22987
Q12211	PUS1	2	2.804	1.49	0.34717	2	0.189	-2.40	0.25951
Q06106	MRD1	8	2.539	1.34	0.37881	5	0.165	-2.60	0.24048
P38701	RPS20	7	2.412	1.27	0.39554	6	0.099	-3.34	0.17557
P33322	CBF5	4	2.381	1.25	0.39985	2	0.244	-2.03	0.29762
P20459	SUI2	4	2.376	1.25	0.40047	5	0.422	-1.25	0.38591
Q02792	RAT1	5	2.310	1.21	0.4098	3	0.350	-1.52	0.35459
P0CX43; P0CX44	RPL1A; RPL1B	13	2.206	1.14	0.4251	10	0.136	-2.88	0.21485
Q12186	MSL5	5	2.171	1.12	0.43048	5	0.670	-0.58	0.46607
Q08208	NOP12	5	2.105	1.07	0.44086	5	0.310	-1.69	0.33514
Q12000	TMA46	7	2.104	1.07	0.44092	7	0.110	-3.19	0.18774
P48164	RPS7B	15	2.081	1.06	0.44465	14	0.375	-1.42	0.36605
P39517	DHH1	5	2.069	1.05	0.44656	6	0.197	-2.34	0.26541
P32481	GCD11	5	2.022	1.02	0.45431	3	0.396	-1.34	0.37523
Q02326	RPL6A	7	1.999	1.00	0.45827	4	0.156	-2.68	0.23298

***ENO2-6MS2L* (RNase eluate)**		**Forward experiment**				**Reverse experiment**			
Protein ID	Gene	P	H/L	log_2	Significance	P	H/L	log_2	Significance
P39935	TIF4631	16	1.898	0.92	0.47584	14	0.190	-2.39	0.26033
Q08647	PUS7	5	1.886	0.91	0.47805	6	0.259	-1.95	0.30637
P26785	RPL16B	4	1.852	0.89	0.48408	5	0.497	-1.01	0.414
Q06704	IMH1	9	1.835	0.88	0.48726	6	0.295	-1.76	0.32723
P11412	ZWF1	4	1.823	0.87	0.48955	3	0.595	-0.75	0.44521
P26783	RPS5	6	1.818	0.86	0.4904	9	0.121	-3.05	0.1993
P00925	ENO2	22	1.737	0.80	0.49217	19	0.152	-2.72	0.22881
P35997; P38711	RPS27A; RPS27B	3	1.682	0.75	0.47765	2	0.097	-3.37	0.17344
P53883	NOP13	10	1.617	0.69	0.4598	4	0.380	-1.40	0.36837
P35732	DEF1	2	1.579	0.66	0.44918	14	0.184	-2.45	0.25534
P50109	PSP2	8	1.575	0.66	0.44804	9	0.656	-0.61	0.46239
P08566	ARO1	4	1.550	0.63	0.44095	6	0.655	-0.61	0.46197
P0C0W9; Q3E757	RPL11A; RPL11B	8	1.550	0.63	0.44082	6	0.237	-2.08	0.29261
P40070	LSM4	3	1.500	0.58	0.4262	5	0.567	-0.82	0.43678
P38779	CIC1	6	1.444	0.53	0.40934	2	0.436	-1.20	0.3916

6MS2L-RNA (RNase eluate)		**Forward experiment**				**Reverse experiment**			
Protein ID	Gene	P	H/L	log_2	Significance	P	H/L	log_2	Significance
P36102	PAN3	9	58.517	5.87	0.00016	11	0.037	-4.74	0.000934
P04147	PAB1	29	40.286	5.33	0.00057	28	0.021	-5.58	0.000119
P39936	TIF4632	11	39.738	5.31	0.00059	21	0.018	-5.76	7.16E-05
P34160	STO1	9	29.565	4.89	0.00149	13	0.024	-5.38	0.000196
P38205	NCL1	9	27.439	4.78	0.00186	11	0.053	-4.24	0.002804
Q08920	CBC2	3	25.617	4.68	0.00227	3	0.038	-4.74	0.000948
P33753	TRM2	8	24.868	4.64	0.00248	13	0.013	-6.24	1.87E-05
P53010	PAN2	8	24.695	4.63	0.00253	13	0.031	-4.99	0.000521
Q12129	NMD4	3	23.554	4.56	0.00289	3	0.120	-3.06	0.024294
P39935	TIF4631	14	22.795	4.51	0.00317	20	0.036	-4.78	0.000857
P30771	NAM7	6	21.883	4.45	0.00355	14	0.059	-4.08	0.003887
P53316	YGR250C	4	16.236	4.02	0.00782	5	0.086	-3.53	0.010985
P40561	SGN1	3	15.179	3.92	0.00926	5	0.104	-3.27	0.017253
P32380	SPC110	10	14.339	3.84	0.01066	2	0.339	-1.56	0.17003
P32505	NAB2	4	13.960	3.80	0.01137	6	0.074	-3.75	0.007407
P07260	CDC33	9	13.203	3.72	0.013	9	0.122	-3.03	0.025588
P10080	SBP1	4	11.406	3.51	0.01824	5	0.245	-2.03	0.10136
Q07362	PBP4	2	11.274	3.49	0.01873	5	0.157	-2.67	0.044024
P40850	MKT1	7	10.773	3.43	0.02073	8	0.136	-2.88	0.032266
P40070	LSM4	4	10.730	3.42	0.02092	4	0.214	-2.23	0.079971
P22147	XRN1	11	10.461	3.39	0.02213	23	0.165	-2.60	0.04875
P48567	PUS4	7	10.085	3.33	0.02397	6	0.106	-3.24	0.018093
P53297	PBP1	7	9.366	3.23	0.02808	8	0.126	-2.99	0.027155

6MS2L-RNA (RNase eluate)		Forward experiment				Reverse experiment			
Protein ID	Gene	P	H/L	log_2	Significance	P	H/L	log_2	Significance
P25644	PAT1	6	9.124	3.19	0.02967	7	0.114	-3.13	0.021672
P38199	HEK2	4	8.279	3.05	0.03625	6	0.192	-2.38	0.065225
P50094; O42831	IMD4; YAR075W	7	7.720	2.95	0.04167	12	0.188	-2.41	0.062731
P32588	PUB1	4	7.420	2.89	0.04503	4	0.214	-2.22	0.080211
P50095; REV_P38882	IMD3; REV_UTP9	12	6.892	2.78	0.05188	15	0.259	-1.95	0.11132
P40047	ALD5	21	6.149	2.62	0.06403	20	0.205	-2.29	0.074
P26783	RPS5	4	6.018	2.59	0.06656	5	0.393	-1.35	0.20909
P38011	ASC1	10	5.741	2.52	0.07235	11	0.272	-1.88	0.12083
P38934	BFR1	16	5.707	2.51	0.07312	15	0.315	-1.67	0.15247
Q08647	PUS7	6	5.633	2.49	0.0748	6	0.281	-1.83	0.12736
P25443	RPS2	2	4.682	2.23	0.10196	3	0.548	-0.87	0.31352
P0CX29; P0CX30	RPS23A, RPS23B	2	4.610	2.20	0.10453	2	0.167	-2.58	0.049618
P26786	RPS7A	9	4.569	2.19	0.10603	10	0.446	-1.16	0.24621
P38701	RPS20	2	4.564	2.19	0.10622	5	0.409	-1.29	0.22022
P33442	RPS1A	7	4.563	2.19	0.10625	8	0.358	-1.48	0.18395
P0CX35; P0CX36	RPS4A, RPS4B	5	4.481	2.16	0.10933	3	0.324	-1.63	0.15863
P38697; P39567	IMD2	9	4.475	2.16	0.10955	12	0.279	-1.84	0.12632
P0CX47; P0CX48	RPS11A; RPS11B	3	4.445	2.15	0.11073	3	0.229	-2.13	0.090389
P05750	RPS3	4	4.346	2.12	0.11469	4	0.383	-1.39	0.20154
P48164	RPS7B	7	4.263	2.09	0.11819	9	0.345	-1.54	0.17396
P48589	RPS12	2	4.253	2.09	0.11863	4	0.340	-1.56	0.17075
P0CX37; P0CX38	RPS6A; RPS2B	3	4.200	2.07	0.12095	3	0.356	-1.49	0.18228
P0CX43; P0CX44	RPL1A; RPL1B	9	4.012	2.00	0.12963	12	0.520	-0.94	0.29549
P05738	RPL9A	6	3.974	1.99	0.1315	8	0.426	-1.23	0.23238
P09440	MIS1	3	3.914	1.97	0.13454	6	0.364	-1.46	0.18822
Q05022	RRP5	8	3.900	1.96	0.13523	25	0.303	-1.72	0.14387
P05755	RPS9B	3	3.877	1.95	0.13644	4	0.234	-2.10	0.093922
P38889	SKN7	2	3.765	1.91	0.14244	3	0.389	-1.36	0.2059
Q02326	RPL6A	4	3.740	1.90	0.14381	5	0.363	-1.46	0.18736
P05739	RPL6B	5	3.647	1.87	0.14912	7	0.398	-1.33	0.21267
P26785	RPL16B	3	3.637	1.86	0.1497	2	0.282	-1.83	0.12792
P10664	RPL4A	3	3.606	1.85	0.15156	3	0.321	-1.64	0.15686
P39015	STM1	8	3.535	1.82	0.1559	14	0.373	-1.42	0.19473
P0CX82; P0CX83	RPL19A; RPL19B	4	3.508	1.81	0.1576	4	0.167	-2.58	0.049946
P0C0W9; Q3E757	RPL11A; RPL11B	5	3.496	1.81	0.15838	7	0.385	-1.38	0.20328
P05737	RPL7A	8	3.492	1.80	0.15866	10	0.348	-1.52	0.17633
Q02753	RPL21A	4	3.337	1.74	0.169	5	0.267	-1.91	0.11728
P29453	RPL8B	7	3.284	1.72	0.17269	5	0.330	-1.60	0.16357
P40150	SSB2	15	3.201	1.68	0.17881	19	0.355	-1.49	0.18141
Q03690	CLU1	4	3.172	1.67	0.181	9	0.436	-1.20	0.23893
P40212; Q12690	RPL13B; RPL13A	3	3.166	1.66	0.18144	3	0.329	-1.60	0.16287
P05740; P46990	RPL17A; RPL17B	3	3.137	1.65	0.18371	3	0.299	-1.74	0.14043
P0CX49; P0CX50 [a]	RPL18A; RPL18B	2	3.077	1.62	0.18846	2	0.248	-2.01	0.10362
P0CX63 [b]	YGR161W-B	5	3.066	1.62	0.18938	5	0.417	-1.26	0.22616

6MS2L-RNA (RNase eluate)		Forward experiment				Reverse experiment			
Protein ID	Gene	P	H/L	log_2	Significance	P	H/L	log_2	Significance
P05759 [c]	RPS31	2	3.041	1.60	0.19145	4	0.342	-1.55	0.17168
P39730	FUN12	3	3.036	1.60	0.19186	6	0.387	-1.37	0.20483
P05317	RPP0	5	3.022	1.60	0.19299	3	0.483	-1.05	0.27155
P06105	SCP160	29	2.807	1.49	0.21221	41	0.419	-1.25	0.22742
P41805	RPL10	2	2.795	1.48	0.21338	2	0.595	-0.75	0.34297
Q06106	MRD1	3	2.416	1.27	0.2545	8	0.589	-0.76	0.33898
P35732	DEF1	8	2.387	1.25	0.25811	14	0.652	-0.62	0.37596
P38249	RPG1	6	2.335	1.22	0.26466	4	0.505	-0.99	0.28573
P37292	SHM1	8	2.314	1.21	0.26745	8	0.484	-1.05	0.27227
Q04215 [d]	YMR046C	5	2.234	1.16	0.2782	4	0.610	-0.71	0.3516
P06634	DED1	4	2.121	1.09	0.29439	7	0.641	-0.64	0.36965
P38788	SSZ1	12	2.113	1.08	0.29564	14	0.646	-0.63	0.37271
Q06704	IMH1	2	2.086	1.06	0.29971	11	0.688	-0.54	0.39626
P32527	ZUO1	9	2.021	1.01	0.31006	12	0.650	-0.62	0.37505
P47047	MTR4	4	1.986	0.99	0.3157	11	0.662	-0.60	0.38175
P07281	RPS19B	1	1.742	0.80	0.35985	2	0.607	-0.72	0.34995
P38879	EGD2	3	1.724	0.79	0.36344	6	0.697	-0.52	0.40135
P46655; REV_P50275	GUS1; REV_ASE1	5	1.683	0.75	0.37186	7	0.574	-0.80	0.32974
P03962	URA3	2	1.512	0.60	0.40961	4	0.476	-1.07	0.26668
P04801	THS1	5	1.508	0.59	0.4106	9	0.541	-0.89	0.30925
P34730	BMH2	6	1.473	0.56	0.41905	7	0.627	-0.67	0.36144
P46672	ARC1	3	1.457	0.54	0.42295	3	0.513	-0.96	0.29132
P00815	HIS4	2	1.417	0.50	0.43304	1	0.630	-0.67	0.36371

Additional protein IDs forming one proteinGroup:
[a] P27809
[b] P0CX64; P25384; Q12472; P0C2J3; Q03494; Q12113; Q12337; Q12501; Q12491; P0C2J5; P0C2J2; P0C2J4; P0C2J6; P0CX61; P0CX62; P25383; Q03483; Q12293; Q12392; Q12439; Q99303; Q12260
[c] P0CG63; P0CH08; P0CH09
[d] Q12266

Table 2. RNase elution efficiency as assessed by the comparison of enriched proteins in RNase eluate and Boiled Beads sample. After *PGK1-6MS2L* affinity purification the beads were first treated with RNase and subsequently boiled in SDS sample buffer. The resulting protein samples – RNase eluate and Boiled Beads (BB) sample – were analysed by mass spectrometry. The enriched proteins were grouped into three categories (Unique, Possible Common, Common) depending if the protein classified as enriched in one MS data set, possibly in both or in both MS data sets. Table depicts the log_2 (H/L) ratios of the enriched proteins.

BB sample									
Gene	Unique				Gene	Possible Common			
	BB		RNase			BB		RNase	
	for	rev	for	rev		for	rev	for	rev
YLR419W	2.41	-2.67	-3.30		MIS1	2.52	-2.96	2.10	
RPL19A; RPL19B	2.20	-2.21	-0.15	0.22	RPS11A; RPS11B	2.31	-3.06	0.63	-0.30
RPL15A; RPL15B	2.13	-1.91			BRE5	2.21	-2.55	2.25	
YNL050C	2.07	-0.91			RPL30	2.00	-1.50		-1.31

BB sample									
Gene	Unique				Gene	Possible Common			
	BB		RNase			BB		RNase	
	for	rev	for	rev		for	rev	for	rev
RPL13B; RPL13A	1.82	-1.97	-1.26	1.47	RPL5	1.72	-0.89		-0.84
TRA1	1.41	-1.07			RPL9A	1.61	-2.06	1.77	-0.43
VPH1	1.32	-1.55			PBP1	1.55	-2.00	1.30	
RPL10	1.26	-1.86	-0.83	0.83	RPL1A; RPL1B	1.54	-2.10	1.12	-0.05
RRP7	1.26	-0.52			RPL4B	1.32	-1.96		-1.61
UTP22	1.07	-1.01			CBF5	1.24	-1.12	0.89	
KRI1	0.96	-1.10			RPS25B	0.78	-0.53	1.67	
ACT1	0.86	-2.45	-0.59	-0.59	RPS17A; RPS17B	0.71	-2.41	0.45	-0.51
KRR1	0.82	-0.88			RPS23A; RPS23B	0.69	-2.95	0.45	-0.42
SEC16	0.76	-1.13			RPS4A; RPS4B	0.66	-3.20	0.61	-0.39
MSS116	0.74	-0.97			RPS13	0.55	-0.78	0.48	-0.34
NOP4	0.73	-0.63			DIM1	0.51	-1.18	1.39	
RPP1	0.70	-2.40							
NUG1	0.56	-0.75							

RNase eluate									
Gene	Unique				Gene	Possible Common			
	BB		RNase			BB		RNase	
	for	rev	for	rev		for	rev	for	rev
RPP2B	0.02	0.77	3.82	-1.55	PAT1		-3.82	3.76	-4.70
TRM2			3.46	-3.49	TRM44	3.26		3.71	-4.93
PAN3			3.13	-4.30	EDC3		-4.04	3.54	-4.00
PAN2			3.09	-3.88	RPS3	0.32	-3.06	2.53	-2.19
RPS5	-0.25	-2.65	2.03	-1.17	RPS7A	0.40	-2.68	2.00	-1.30
WHI3			2.01	-2.37	ARB1	0.43	-0.40	1.57	-1.40
DCP1			1.91	-2.29	TIF1	1.29		1.40	-0.54
HRB1			1.77	-1.87	RPS6A;RPS6B	0.49	-2.76	1.29	-0.63
LSM4			1.59	-1.55	YGR250C	0.67		1.23	-1.37
ALD5			1.38	-1.26	RPS27A; RPS27B		-1.14	1.11	-1.13
SRO9			1.30	-1.69	RPS7B	0.49	-2.48	0.96	-1.33
RPP0	-0.13	-0.80	1.28	-1.50	SGN1		-1.07	0.95	-1.28
TMA64			1.21	-1.67	LEU2	2.09	-0.30	0.83	-0.54
MET6	-0.02		1.17	-0.65	RPS21A; RPS21B		-0.31	0.81	-1.25
NAB2			1.16	-1.25	RPS22B; RPS22A	0.47	-0.24	0.81	-1.06
TDH3	-2.44	1.47	1.13	-0.70	NOP58	0.60	-0.26	0.74	-0.66
ADH3			1.08	-0.71	RPS16A; RPS16B	0.00	-1.90	0.74	-1.07
PGI1	0.06		1.05	-1.84	SBP1	1.97	-0.39	0.66	-0.91
RPL35A; RPL35B	-0.03	-1.44	0.99	-0.97	RPS20	0.53	-0.15	0.57	-0.59
HXK2			0.94	-1.51	HSP60	0.29	-0.28	0.54	-0.59
LHP1			0.91	-1.05	RPS16A	0.63	-0.23	2.37	-0.97
IMD4; YAR075W			0.81	-1.36					
ADH1	-1.85	1.33	0.77	-0.54					
RPS31; UBI4	-0.12	-0.45	0.74	-1.19					

RNase eluate									
Gene	Unique				Gene	Possible Common			
	BB		RNase			BB		RNase	
	for	rev	for	rev		for	rev	for	rev
PUB1			0.65	-0.73					
IMD3			0.62	-1.10					
HEK2			0.54	-0.76					

BB sample and RNase eluate Common									
Gene	BB		RNase		Gene	BB		RNase	
	for	rev	for	rev		for	rev	for	rev
NAM7	6.78	-7.41	5.92	-6.08	RPL4A	1.25	-1.81	1.51	-1.21
EBS1	4.99	-5.20	4.47	-5.49	RPS2	1.48	-2.60	1.40	-0.86
DCP2	4.38	-5.01	4.28	-4.10	MKT1	1.49	-1.67	1.34	-1.60
XRN1	2.99	-3.42	2.89	-2.98	ARX1	1.28	-1.44	1.27	-1.50
TMA46	4.05	-2.96	2.70	-3.25	TIF4631	1.42	-2.08	1.23	-1.76
ASC1	0.90	-2.61	2.68	-3.02	ZUO1	0.75	-1.45	1.10	-1.37
PAB1	2.46	-3.26	2.58	-3.36	RPS9B	2.02	-3.14	1.08	-1.16
STM1	2.50	-2.76	2.51	-2.97	SSZ1	0.90	-0.89	1.08	-1.42
RBG1	3.95	-2.76	2.49	-3.07	RPS8A; RPS8B	2.93	-3.13	0.89	-1.50
UBP3	2.30	-2.57	1.97	-2.22	DEF1	1.14	-1.02	0.89	-0.83
SSB2	1.08	-1.55	1.89	-1.74	SCP160	0.79	-1.18	0.87	-0.76
BFR1	2.01	-2.61	1.79	-2.60	RPS1A	0.62	-3.03	0.77	-0.97
RPL3	3.17	-1.96	1.77	-1.75	PGK1	1.68	-5.09	0.75	-3.58
TIF4632	1.78	-2.34	1.76	-2.37	RPS19B	0.73	-0.58	0.75	-0.60
YEF3; HEF3	0.73	-0.82	1.52	-1.38	RPS1B	0.56	-2.64	0.60	-1.26

Table 3. Literature-based analysis of MS2L-tagged RNA co-purifying proteins (>1.41-fold enriched) to identify their previously known target RNAs and function. Comparison of our data set of enriched proteins to RBPs identified in yeast (S. F. Mitchell et al. 2013) or in mammalian cells (Castello et al. 2012; Baltz et al. 2012).

PGK1-6MS2L						
Gene	mRNA Target	Function[a]	Mitchell et al.	Homologue/Related Human Protein[b]	Castello et al.	Baltz et al.
ACT1	none	Other	no	ACTG1	no	no
ADH1	none	Metabolism	no	ADH1A	no	no
ADH3	none	Metabolism	no	Opisthokonta		
ALD5	none	Metabolism	no	ALDH2	no	no
ARB1	rRNA	Rs Biogenesis	no	ABCF2	cand	no
ARX1	rRNA	Rs Biogenesis	no	EBP1	no	no
ASC1	rRNA	Translation	no	GNB2L1	**yes**	**yes**
BFR1	mRNA (Hogan et al. 2008a)	Translation	**yes**	MDR1	no	no
BRE5	mRNA	Other	**yes**	Saccharomycetaceae		
CBF5	snoRNA, rRNA	Rs Biogenesis	**yes**	DKC1	**yes**	no
DCP1	mRNA	Decay	no	DCP1A	no	no
DCP2	mRNA	Decay	no	DCP2	no	no

PGK1-6MS2L

Gene	mRNA Target	Function[a]	Mitchell et al.	Homologue/Related Human Protein[b]	Castello et al.	Baltz et al.
DEF1	none	Other	no	N/A		
DIM1	rRNA	Rs Biogenesis	no	DIMT1L	**yes**	**yes**
EBS1	mRNA (Luke et al. 2007)	Decay	no	SMG7	no	no
EDC3	mRNA	Decay	no	EDC3	no	no
HRB1	mRNA	Export	**yes**	MYEF2	no	**yes**
HSP60	none	Other	no	HSPD1	**yes**	no
HXK2	none	Metabolism	no	HK1	no	no
IMD3; REV_UTP9	none	Metabolism	**yes**	IMPDH3	no	no
IMD4; YAR075W	none	Metabolism	**yes**	IMPDH4	no	no
KHD1	mRNA	Localization	**yes**	PCBP3	**yes**	no
KRI1	rRNA	Rs Biogenesis	no	KRI1	**yes**	no
KRR1	rRNA	Rs Biogenesis	no	KRR1	**yes**	**yes**
LEU2	none	Metabolism	no	Eukaryota		
LHP1	tRNA, snRNA	Other	no	Saccharomycetaceae		
LSM4	mRNA	Decay	no	LSM4	**yes**	**yes**
MET6	none	Metabolism	no	Eukaryota		
MIS1	none	Metabolism	no	Ascomycota		
MKT1	none	Unknown	no	Ascomycota		
MSS116	mRNA	Splicing	**yes**	Saccharomycetaceae		
NAB2	mRNA	Export	**yes**	ZC3H14	**yes**	**yes**
NOP4	rRNA	Rs Biogenesis	**yes**	RBM28	**yes**	**yes**
NOP58	snoRNA	Rs Biogenesis	**yes**	NOP58	**yes**	**yes**
NUG1	rRNA	Rs Biogenesis	**yes**	GNL3L	**yes**	no
PAB1	mRNA	Translation	**yes**	PABPC1	**yes**	**yes**
PAN2	mRNA	Decay	no	PAN2	no	no
PAN3	mRNA	Decay	no	PAN3	no	no
PAT1	mRNA	Decay	**yes**	PATL1	**yes**	**yes**
PBP1	mRNA	Decay	**yes**	ATXN2	**yes**	**yes**
PGI1	none	Metabolism	no	GPI	no	no
PGK1	none/mRNA (Castello et al. 2012)	Metabolism	no	PGK1	cand	no
PUB1	mRNA	Decay	**yes**	TIA1	**yes**	**yes**
RBG1	none	Translation	no	DRG1	cand	no
RPL10	rRNA	Translation	no	RPL10	**yes**	**yes**
RPL13B; RPL13A	rRNA	Translation	no	RPL13	**yes**	no
RPL15A; RPL15B	rRNA	Translation	no	RPL15	**yes**	**yes**
RPL16B	rRNA	Translation	no	RPL13A	**yes**	no
RPL19A; RPL19B	rRNA	Translation	no	RPL19	**yes**	no
RPL1A; RPL1B	rRNA	Translation	no	RPL10A	**yes**	**yes**
RPL3	rRNA	Translation	no	RPL3	**yes**	**yes**
RPL30	rRNA	Translation	no	RPL30	**yes**	**yes**
RPL35A; RPL35B	rRNA	Translation	no	RPL35	**yes**	no
RPL4A	rRNA	Translation	no	RPL4	**yes**	**yes**
RPL4B	rRNA	Translation	no	RPL4	**yes**	**yes**
RPL5	rRNA	Translation	no	RPL5	**yes**	**yes**

PGK1-6MS2L

Gene	mRNA Target	Function[a]	Mitchell et al.	Homologue/Related Human Protein[b]	Castello et al.	Baltz et al.
RPL9A	rRNA	Translation	no	RPL9	cand	no
RPP0	rRNA	Translation	no	RPLP0	**yes**	**yes**
RPP1	rRNA, tRNA	Rs Biogenesis	no	RPP30	**yes**	no
RPP2B	rRNA	Translation	no	Saccharomycetaceae		
RPS11A; RPS11B	rRNA	Translation	no	RPS11	**yes**	no
RPS13	rRNA	Translation	no	RPS13	cand	**yes**
RPS16A; RPS16B	rRNA	Translation	no	RPS16	no ev	**yes**
RPS17A; RPS17B	rRNA	Translation	no	RPS17	no	**yes**
RPS19B	rRNA	Translation	no	RPS19	no ev	**yes**
RPS1A	rRNA	Translation	no	RPS3A	**yes**	**yes**
RPS1B	rRNA	Translation	no	RPS3A	**yes**	**yes**
RPS2	rRNA	Translation	no	RPS2	**yes**	**yes**
RPS20	rRNA	Translation	**yes**	RPS20	**yes**	**yes**
RPS21A; RPS21B	rRNA	Translation	no	RPS21	**yes**	no
RPS22B; RPS22A	rRNA	Translation	no	RPS15A	**yes**	**yes**
RPS23A; RPS23B	rRNA	Translation	no	RPS23	cand	**yes**
RPS25B	rRNA	Translation	no	Saccharomycetaceae		
RPS27A; RPS27B	rRNA	Translation	no	RPS27A	**yes**	**yes**
RPS3	rRNA	Translation	no	RPS3	**yes**	**yes**
RPS31	rRNA	Translation	no	RPS27A	**yes**	**yes**
RPS4A; RPS4B	rRNA	Translation	no	RPS4X	**yes**	**yes**
RPS5	rRNA	Translation	no	RPS5	**yes**	**yes**
RPS6A; RPS6B	rRNA	Translation	no	RPS6	**yes**	no
RPS7A	rRNA	Translation	no	RPS7	**yes**	**yes**
RPS7B	rRNA	Translation	no	RPS7	**yes**	**yes**
RPS8A; RPS8B	rRNA	Translation	no	RPS8	**yes**	**yes**
RPS9B	rRNA	Translation	no	RPS9	**yes**	no
RRP7	rRNA	Rs Biogenesis	no	RRP7A	**yes**	no
SBP1	mRNA	TL Repression	**yes**	Saccharomycetaceae		
SCP160	mRNA	Translation	**yes**	HDLBP	**yes**	**yes**
SEC16	none	Other	no	SEC16A; SEC16B	no	no
SGN1	mRNA (Winstall et al. 2000)	Unknown	no	ASCL3	no	no
SRO9	mRNA	Translation	**yes**	Saccharomycetaceae		
SSB2	unknown	Co-TL NP Mat	no	N/A		
SSZ1	none	Co-TL NP Mat	no	HSP70L1	no	no
STM1	mRNA (Hogan et al. 2008a)	TL Repression	no	Saccharomycetaceae		
ZUO1	rRNA	Co-TL NP Mat	no	MPP11	no	no
TDH3	none	Metabolism	no	GAPDH	cand	no
TIF1	mRNA	Translation	no	EIF4A1	**yes**	**yes**
TIF4631	mRNA	Translation	**yes**	EIF4G1	**yes**	**yes**
TIF4632	mRNA	Translation	**yes**	EIF4G2	**yes**	**yes**
TMA46	unknown	Translation	**yes**	ZC3H15	**yes**	**yes**
TMA64	none	Unknown	no	Saccharomycetaceae		
TRA1	none	Transcription	no	TRRAP	no	no

PGK1-6MS2L

Gene	mRNA Target	Function[a]	Mitchell et al.	Homologue/Related Human Protein[b]	Castello et al.	Baltz et al.
TRM2	tRNA	tRNA Mod	no	TRMT2A; TRMTB	**yes**	no
TRM44	tRNA	tRNA Mod	no	TRMT44	no	no
UBP3	mRNA	Other	**yes**	USP10	**yes**	**yes**
UPF1	mRNA	Decay	**yes**	UPF1	**yes**	**yes**
UTP22	rRNA	Rs Biogenesis	no	NOL6	**yes**	no
WHI3	mRNA	Unknown	no	N/A		
VPH1	none	Other	no	ATP6V0A1	no	no
XRN1	mRNA	Decay	**yes**	XRN1	**yes**	**yes**
YEF3; HEF3	mRNA	Translation	no	Ascomycota		
YGR250C	unknown	Unknown	**yes**	CELF1	**yes**	**yes**
YLR419W	unknown	Unknown	**yes**	DHX36	**yes**	**yes**
YNL050C	unknown (Scherrer et al. 2010)	Unknown	no	Saccharomycetaceae		

ENO2-6MS2L

Gene	mRNA Target	Function[a]	Mitchell et al.	Homologue/Related Human Protein[b]	Castello et al.	Baltz et al.
ALD5	none	Metabolism	no	ALDH2	no	no
ARB1	rRNA	Rs Biogenesis	no	ABCF2	cand	no
ARD1	none	Co-TL NP Mat	no	NAA11	no	no
ARO1	none	Metabolism	no	Eukaryota		
ARX1	rRNA	Rs Biogenesis	no	EBP1	no	no
ASC1	rRNA	Translation	no	GNB2L1	**yes**	**yes**
BFR1	mRNA (Hogan et al. 2008)	Translation	**yes**	MDR1	no	no
CBF5	snoRNA, rRNA	Rs Biogenesis	**yes**	DKC1	**yes**	no
CIC1	none	Unknown	no	Saccharomycetaceae		
DCP1	mRNA	Decay	no	DCP1A	no	no
DCP2	mRNA	Decay	no	DCP2	no	no
DEF1	none	Other	no	N/A		
DHH1	mRNA	Decay	**yes**	DDX6	**yes**	**yes**
EBS1	mRNA (Luke et al. 2007)	Decay	no	SMG7	no	no
EDC3	mRNA	Decay	no	EDC3	no	no
ENO2	none	Metabolism	no	ENO2	no	no
FUN12	mRNA	Translation	no	Ascomycota		
GCD11	mRNA	Translation	no	Eukaryota		
HRB1	mRNA	Export	**yes**	MYEF2	no	**yes**
IMD2; IMD1	none	Metabolism	**yes**	IMPDH1	no	no
IMD3; REV_UTP9	none	Metabolism	**yes**	IMPDH3	no	no
IMD4; YAR075W	none	Metabolism	**yes**	IMPDH4	no	no
IMH1	none	Other	no	saccharomyceta		
LSM1	mRNA	Decay	no	LSM1	**yes**	**yes**
LSM2	mRNA	Decay	no	LSM2	**yes**	**yes**
LSM4	mRNA	Decay	no	LSM4	**yes**	**yes**
MAP2	none	Co-TL NP Mat	no	METAP2	**yes**	no

ENO2-6MS2L

Gene	mRNA Target	Function[a]	Mitchell et al.	Homologue/Related Human Protein[b]	Castello et al.	Baltz et al.
MGM101	mRNA	Splicing	no	saccharomyceta		
MIS1	none	Metabolism	no	Ascomycota		
MKT1	none	Unknown	no	Ascomycota		
MRD1	rRNA	Rs Biogenesis	no	RBM19	**yes**	**yes**
MSL5	mRNA	Splicing	no	Ascomycota		
NAT1	unknown	Co-TL NP Mat	no	NAA15	**yes**	no
NMD4	unknown	Unknown	no	Saccharomycetaceae		
NOP12	rRNA	Rs Biogenesis	no	Ascomycota		
NOP13	rRNA	Rs Biogenesis	no	Ascomycota		
PAB1	mRNA	Translation	**yes**	PABPC1	**yes**	**yes**
PAN2	mRNA	Decay	no	PAN2	no	no
PAN3	mRNA	Decay	no	PAN3	no	no
PAT1	mRNA	Decay	**yes**	PATL1	**yes**	**yes**
PBP1	mRNA	Decay	**yes**	ATXN2	**yes**	**yes**
PBP4	none	Unknown	no	N/A		
PSP2	unknown (Castello et al. 2012)	Unknown	**yes**	N/A		
PUS1	tRNA, snRNA	tRNA Mod	**yes**	PUS1	**yes**	**yes**
PUS4	tRNA	tRNA Mod	no	Ascomycota		
PUS7	tRNA, rRNA, snRNA	tRNA Mod	no	PUS7	**yes**	**yes**
RAT1	mRNA and others	Decay	**yes**	XRN2	**yes**	**yes**
RBG1	none	Translation	no	DRG1	cand	no
RNY1	tRNA, rRNA	Decay	no	Saccharomyceta		
RPL10	rRNA	Translation	no	RPL10	**yes**	**yes**
RPL11A; RPL11B	rRNA	Translation	no	RPL11	**yes**	**yes**
RPL16B	rRNA	Translation	no	RPL13A	**yes**	no
RPL17A; RPL17B	rRNA	Translation	no	RPL17	**yes**	**yes**
RPL19A; RPL19B	rRNA	Translation	no	RPL19	**yes**	no
RPL1A; RPL1B	rRNA	Translation	no	RPL10A	**yes**	**yes**
RPL21A	rRNA	Translation	no	RPL21	**yes**	**yes**
RPL24B	rRNA	Translation	no	RPL24	**yes**	**yes**
RPL25	rRNA	Translation	no	RPL23A	**yes**	**yes**
RPL3	rRNA	Translation	no	RPL3	**yes**	**yes**
RPL31A	rRNA	Translation	no	RPL31	**yes**	no
RPL4A	rRNA	Translation	no	RPL4	**yes**	**yes**
RPL5	rRNA	Translation	no	RPL5	**yes**	**yes**
RPL6A	rRNA	Translation	no	Opisthokonta		
RPL8B	rRNA	Translation	no	RPL7A	**yes**	**yes**
RPL9A	rRNA	Translation	no	RPL9	cand	no
RPO41	unknown	Transcription	no	Eukaryota		
RPP0	rRNA	Translation	no	RPLP0	**yes**	**yes**
RPS10B; RPS10A	rRNA	Translation	no	RPS10	**yes**	**yes**
RPS11A; RPS11B	rRNA	Translation	no	RPS11	**yes**	no
RPS12	rRNA	Translation	no	RPS12	**yes**	**yes**
RPS17A; RPS17B	rRNA	Translation	no	RPS17	no	**yes**

ENO2-6MS2L

Gene	mRNA Target	Function[a]	Mitchell et al.	Homologue/Related Human Protein[b]	Castello et al.	Baltz et al.
RPS18A; RPS18B	rRNA	Translation	no	RPS18	no	**yes**
RPS19B	rRNA	Translation	no	RPS19	no ev	**yes**
RPS2	rRNA	Translation	no	RPS2	**yes**	**yes**
RPS20	rRNA	Translation	**yes**	RPS20	**yes**	**yes**
RPS21A; RPS21B	rRNA	Translation	no	RPS21	**yes**	no
RPS22B; RPS22A	rRNA	Translation	no	RPS15A	**yes**	**yes**
RPS27A; RPS27B	rRNA	Translation	no	RPS27A	**yes**	**yes**
RPS3	rRNA	Translation	no	RPS3	**yes**	**yes**
RPS4A; RPS4B	rRNA	Translation	no	RPS4X	**yes**	**yes**
RPS5	rRNA	Translation	no	RPS5	**yes**	**yes**
RPS6A; RPS6B	rRNA	Translation	no	RPS6	**yes**	no
RPS7A	rRNA	Translation	no	RPS7	**yes**	**yes**
RPS7B	rRNA	Translation	no	RPS7	**yes**	**yes**
RPS9B	rRNA	Translation	no	RPS9	**yes**	no
SHM1	none	Metabolism	no	SHMT2	cand	no
SRO9	mRNA	Translation	**yes**	Saccharomycetaceae		
SSB2	unknown	Co-TL NP Mat	no	N/A		
SSZ1	none	Co-TL NP Mat	no	HSP70L1	no	no
STM1	mRNA (Hogan et al. 2008)	TL Repression	no	Saccharomycetaceae		
SUI2	mRNA	Translation	no	EIF2S1	**yes**	**yes**
ZUO1	rRNA	Co-TL NP Mat	no	MPP11	no	no
ZWF1	none	Metabolism	no	G6PD	no	no
TIF4631	mRNA	Translation	**yes**	EIF4G1	**yes**	**yes**
TIF4632	mRNA	Translation	**yes**	EIF4G2	**yes**	**yes**
TMA46	unknown	Translation	**yes**	ZC3H15	**yes**	**yes**
TRM2	tRNA	tRNA Mod	no	TRMT2A; TRMTB	**yes**	no
TRM44	tRNA	tRNA Mod	no	TRMT44	no	no
UBP3	mRNA	Other	**yes**	USP10	**yes**	**yes**
UPF1	mRNA	Decay	**yes**	UPF1	**yes**	**yes**
XRN1	mRNA	Decay	**yes**	XRN1	**yes**	**yes**
YGR054W	rRNA	Translation	**yes**	EIF2A	cand	no
YLR419W	unknown	Unknown	**yes**	DHX36	**yes**	**yes**

6MS2L-RNA

Gene	mRNA Target	Function[a]	Mitchell et al.	Homologue/Related Human Protein[b]	Castello et al.	Baltz et al.
ALD5	none	Metabolism	no	ALDH2	no	no
ARC1	none	tRNA Aminoacy	no	Ascomycota		
ASC1	rRNA	Translation	no	GNB2L1	**yes**	**yes**
BFR1	mRNA (Hogan et al. 2008)	Translation	**yes**	MDR1	no	no
BMH2	none	Other	no	YWHAE	**yes**	no
CBC2	mRNA	Nuc Processing	no	NCBP2	**yes**	**yes**
CDC33	mRNA	Translation	no	EIF4E	no ev	**yes**

6MS2L-RNA						
Gene	mRNA Target	Function[a]	Mitchell et al.	Homologue/Related Human Protein[b]	Castello et al.	Baltz et al.
CLU1	unknown	Unknown	**yes**	Saccharomycetaceae		
DED1	mRNA	Translation	**yes**	DDX3Y	no	no
DEF1	none	Other	no	N/A		
EGD2	none	Co-TL NP Mat	no	NACA	no ev	no
FUN12	mRNA	Translation	no	Ascomycota		
GUS1; REV_ASE1	tRNA	tRNA Aminoacy	**yes**	EPRS	no ev	no
HIS4	none	Metabolism	no	Eukaryota		
IMD2; IMD1	none	Metabolism	**yes**	IMPDH1	no	no
IMD3; REV_UTP9	none	Metabolism	**yes**	IMPDH3	no	no
IMD4; YAR075W	none	Metabolism	**yes**	IMPDH4	no	no
IMH1	none	Other	no	saccharomyceta		
KHD1	mRNA	Localization	**yes**	PCBP3	**yes**	no
LSM4	mRNA	Decay	no	LSM4	**yes**	**yes**
MIS1	none	Metabolism	no	Ascomycota		
MKT1	none	Unknown	no	Ascomycota		
MRD1	rRNA	Rs Biogenesis	no	RBM19	**yes**	**yes**
MTR4	multiple RNA types	Nuc Processing	**yes**	SKIV2L2	**yes**	no
NAB2	mRNA	Export	**yes**	ZC3H14	**yes**	**yes**
NCL1	tRNA	tRNA Mod	no	NSUN2	**yes**	**yes**
NMD4	unknown	Unknown	no	Saccharomycetaceae		
PAB1	mRNA	Translation	**yes**	PABPC1	**yes**	**yes**
PAN2	mRNA	Decay	no	PAN2	no	no
PAN3	mRNA	Decay	no	PAN3	no	no
PAT1	mRNA	Decay	**yes**	PATL1	**yes**	**yes**
PBP1	mRNA	Decay	**yes**	ATXN2	**yes**	**yes**
PBP4	none	Unknown	no	N/A		
PUB1	mRNA	Decay	**yes**	TIA1	**yes**	**yes**
PUS4	tRNA	tRNA Mod	no	Ascomycota		
PUS7	tRNA, rRNA, snRNA	tRNA Mod	no	PUS7	**yes**	**yes**
RPG1	mRNA	Translation	**yes**	EIF3A	**yes**	**yes**
RPL10	rRNA	Translation	no	RPL10	**yes**	**yes**
RPL11A; RPL11B	rRNA	Translation	no	RPL11	**yes**	**yes**
RPL13B; RPL13A	rRNA	Translation	no	RPL13	**yes**	no
RPL16B	rRNA	Translation	no	RPL13A	**yes**	no
RPL17A; RPL17B	rRNA	Translation	no	RPL17	**yes**	**yes**
RPL18A; RPL18B	rRNA	Translation	no	RPL18	no ev	no
RPL19A; RPL19B	rRNA	Translation	no	RPL19	**yes**	no
RPL1A; RPL1B	rRNA	Translation	no	RPL10A	**yes**	**yes**
RPL21A	rRNA	Translation	no	RPL21	**yes**	**yes**
RPL4A	rRNA	Translation	no	RPL4	**yes**	**yes**
RPL6A	rRNA	Translation	no	Opisthokonta		
RPL6B	rRNA	Translation	no	Opisthokonta		
RPL7A	rRNA	Translation	no	RPL7	**yes**	**yes**
RPL8B	rRNA	Translation	no	RPL7A	**yes**	**yes**

6MS2L-RNA

Gene	mRNA Target	Function[a]	Mitchell et al.	Homologue/Related Human Protein[b]	Castello et al.	Baltz et al.
RPL9A	rRNA	Translation	no	RPL9	cand	no
RPP0	rRNA	Translation	no	RPLP0	**yes**	**yes**
RPS11A; RPS11B	rRNA	Translation	no	RPS11	**yes**	no
RPS12	rRNA	Translation	no	RPS12	**yes**	**yes**
RPS19B	rRNA	Translation	no	RPS19	no ev	**yes**
RPS1A	rRNA	Translation	no	RPS3A	**yes**	**yes**
RPS2	rRNA	Translation	no	RPS2	**yes**	**yes**
RPS20	rRNA	Translation	**yes**	RPS20	**yes**	**yes**
RPS23A; RPS23B	rRNA	Translation	no	RPS23	cand	**yes**
RPS3	rRNA	Translation	no	RPS3	**yes**	**yes**
RPS31	rRNA	Translation	no	RPS27A	**yes**	**yes**
RPS4A; RPS4B	rRNA	Translation	no	RPS4X	**yes**	**yes**
RPS5	rRNA	Translation	no	RPS5	**yes**	**yes**
RPS6A; RPS6B	rRNA	Translation	no	RPS6	**yes**	no
RPS7A	rRNA	Translation	no	RPS7	**yes**	**yes**
RPS7B	rRNA	Translation	no	RPS7	**yes**	**yes**
RPS9B	rRNA	Translation	no	RPS9	**yes**	no
RRP5	rRNA	Rs Biogenesis	**yes**	PDCD11	**yes**	no
SBP1	mRNA	TL Repression	**yes**	Saccharomycetaceae		
SCP160	mRNA	Translation	**yes**	HDLBP	**yes**	**yes**
SGN1	mRNA (Winstall et al. 2000)	Unknown	no	ASCL3	no	no
SHM1	none	Metabolism	no	SHMT2	cand	no
SKN7	none	Transcription	no	Saccharomycetaceae		
SPC110	none	Other	no	Saccharomycetaceae		
SSB2	unknown	Co-TL NP Mat	no	N/A		
SSZ1	none	Co-TL NP Mat	no	HSP70L1	no	no
STM1	mRNA (Hogan et al. 2008)	TL Repression	no	Saccharomycetaceae		
STO1	mRNA	Nuc Processing	**yes**	NCBP1	cand	**yes**
ZUO1	rRNA	Co-TL NP Mat	no	MPP11	no	no
THS1	tRNA	tRNA Aminoacy	no	TARS	cand	no
TIF4631	mRNA	Translation	**yes**	EIF4G1	**yes**	**yes**
TIF4632	mRNA	Translation	**yes**	EIF4G2	**yes**	**yes**
TRM2	tRNA	tRNA Mod	no	TRMT2A; TRMTB	**yes**	no
UPF1	mRNA	Decay	**yes**	UPF1	**yes**	**yes**
URA3	none	Metabolism	no	Ascomycota		
XRN1	mRNA	Decay	**yes**	XRN1	**yes**	**yes**
YGR161W-B	none	Other	no	N/A		
YGR250C	unknown	Unknown	**yes**	CELF1	**yes**	**yes**
YMR046C	none	Other	no	N/A		

[a] Abbreviations: Co-TL NP Mat – Co-translational Nascent Peptide Maturation; Rs Biogenesis – Ribosome Biogenesis; TL Repression – Translation Repression; tRNA Mod – tRNA Modification; tRNA Aminoacy – tRNA Aminoacylation; Nuc Processing – Nuclear Processing; cand – candidate RBP; no ev – no evidence, N/A – no answer

[b] Homologues of the MS2L-tagged RNA co-purifying proteins were retreived from (S. F. Mitchell et al. 2013). To find human homologues to the remaing proteins, HomoloGene tool of the National Center for Biotechnology Information (NCBI) was used. If HomoloGene could not detect a human homolog of the yeast gene, the table shows the division of the eukaryotic kingdom where homologous genes were detected (read: Conserved in *Saccharomycetacea*). *ARX1*, *BFR1* and *EBS1* homologs are according to the literature *EBP1* (Hung and Johnson 2006), *MDR1* (Wilkinson and Millar 1998) and *SMG7* (Luke et al. 2007), respectively.

Table 4. Literature-based classification (Appendix, Table 3) of the enriched proteins according to the type of RNA bound by the protein. Table depicts percent of proteins known to bind a certain type of RNA. Note that some proteins classified under multiple categories. Proteins containing putative RNA-binding domains or known to be involved in mRNA biology but for whom the RNA target has not been identified, we classified under category "undefined RNA". snoRNA – small nucleolar RNA; snRNA – small nuclear RNA.

MS2L-tagged RNA	$\log_2$[a]	mRNA	rRNA	tRNA	snoRNA	snRNA	undefined RNA	no known RNA target
PGK1	0.5	27	43	3	2	1	4	19
	1	35	38	3	1	0	8	18
ENO2	0.5	26	43	6	1	2	7	17
	1	29	42	6	1	1	7	14
6MS2L	0.5	27	39	7	0	1	4	22
	1	32	41	6	0	1	6	15

[a] $\log_2$ 0.5 corresponds to threshold $\log_2$ (H/L) >0.5 or <-0.5; $\log_2$ 1 corresponds to threshold $\log_2$ (H/L) >1 or <-1.

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