Identification of Aurora Kinase Substrates in *Schizosaccharomyces pombe* by Chemical Genetics and Quantitative Phosphoproteomics

Dissertation

der Mathematisch-Naturwissenschaftlichen Fakultät

der EBERHARD KARLS UNIVERSITÄT TÜBINGEN

zur Erlangung des Grades eines

Doktors der Naturwissenschaften

(Dr. rer. nat.)

vorgelegt von

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Tübingen
2012

Tag der mündlichen Qualifikation:	18.07.2012
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ACKNOWLEDGEMENTS

First and foremost, I would like to express my sincere gratitude to Dr. Silke Hauf for giving me the opportunity to do my PhD thesis in her lab. I am grateful for her guidance, her generous support and the time she spent discussing ideas and experiments with me. Her patience, encouragement and support had a great impact on the success of my work and I am glad being part of her lab.

I would like to thank Prof. Dr. Boris Maček for giving me the opportunity to perform this great mass spectrometry study in his lab. I am grateful for his patience and his support in teaching me all the things I needed to know about mass spectrometry. Without his expertise and guidance, this project would not have been possible. I am also thankful to all his lab members, especially Karsten Krug and Stuart Pengelley.

I would like to thank Prof. Dr. Daniel Rauh for all his support and his ideas during our collaboration. He is an inspiring person and I have learnt a lot from him. I am also thankful to his lab members, especially Haridas B. Rode and André Richters.

I would like to thank the members of my PhD advisory committee, Prof. Dr. Doron Rapaport, Prof. Dr. Alfred Nordheim, Dr. Dmitri Ivanov and Dr. Oliver Weichenrieder, for participating in my annual progress report and discussing my results as well as for their helpful advice and suggestions.

I am very thankful to all Hauf lab members for the nice working atmosphere and the enjoyable activities outside the lab. I want to thank Asha, Yu-Hua, Sabine, Hanna, Stephanie, Maria, Julia and Eva S. for great support and fruitful discussions, but especially for all the fun I had during my time in and outside the lab. In particular I want to thank Eva Illgen for her excellent technical help and lab organization.

I am also thankful to other people around the campus who made these years an enjoyable and wonderful time.

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ABBREVIATIONS

1NM-PP1	4-Amino-1-tert-butyl-3-(1'-naphthylmethyl)pyrazolo[3,4-d]pyrimidine
Ala	Alanine (A)
APC/C	Anaphase promoting complex/Cyclosome
Arg	Arginine (R)
Ark1	Aurora related kinase 1
Asp	Aspartate (D)
ATP	Adenosinetriphosphate
AQUA	Absolute quantification
BIR	Baculovirus inhibitor of apoptosis repeat
C	Carbon
C. albicans	*Candida albicans*
C. elegans	*Caenorhabditis elegans*
Cdc	Cell division cycle
Cdk	Cyclin-dependent kinase
CPC	Chromosomal passenger complex
CPT	Camptothecin
cut	Cell untimely torn
Da	Dalton
DHB	2,5-dihydroxybenzoic acid
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
ESI	Electrospray ionization
Gln	Glutamine (Q)
Glu	Glutamate (E)
Gly	Glycine (G)
H	Hydrogen
His	Histidine (H)
HP1	Heterochromatin protein 1
HU	Hydroxyurea
Ile	Isoleucine (I)
ICAT	Isotope-coded affinity tags
IEF	Isoelectric focusing

IMAC	Immobilized metal affinity chromatography
Ipl1	Increase in ploidy 1
iTRAQ	Isobaric tags for relative and absolute quantification
KMN	KNL1/Mis12 complex/Ndc80 complex
Leu	Leucine (L)
Lys	Lysine (K)
MBC	Carbendazim, methyl-2-benizimidazole carbamate
MOAC	Metal oxide affinity chromatography
MS	Mass spectrometry
N	Nitrogen
O	Oxygen
P	Phosphorus
PP1	Protein phosphatase 1 (Protein)
PP1	Pyrazolo[3,4-d]pyrimidine (Inhibitor)
Pro	Proline (P)
PTM	Posttranslational modification
RNAi	RNA interference
Ser	Serine (S)
SAC	Spindle assembly checkpoint
S. cerevisiae	*Saccharomyces cerevisiae* (budding yeast)
Sgo	Shugoshin
SILAC	Stable isotope labelling with amino acids in cell culture
SPB	Spindle pole body
S. pombe	*Schizosaccharomyces pombe* (fission yeast)
Thr	Threonine (T)
TiO_2	Titaniumdioxide
Tyr	Tyrosine (Y)
Val	Valine (V)
wt	Wild type
X. laevis	*Xenopus laevis*

SUMMARY

Aurora kinases are conserved throughout eukaryotes and are crucial for the proper execution of mitosis. The identification of Aurora substrates is important to elucidate the molecular function of the kinase. Based on the most obvious phenotypes of Aurora depletion or inhibition, several substrates had been identified through candidate-based approaches. However, Aurora kinases are known to contribute to processes for which the relevant substrates are unknown. In order to identify Aurora substrates on a proteome-wide scale, we applied site-specific quantitative phosphoproteomics in conjunction with chemical inhibition of an analogue-sensitive version of the fission yeast Aurora kinase Ark1 (Ark1-as3). Out of more than 8000 phosphorylation events that we detected, 70 phosphorylation sites (on 42 proteins) were probable targets of Aurora. Apart from a few known substrates, the majority of detected Aurora targets were previously unknown to be phosphorylated by Aurora. Given that several of these newly identified targets are implicated in diverse aspects of chromatin dynamics we propose that Aurora, apart from its role in controlling chromosome compaction and chromosome attachment to the mitotic spindle, has a more wide-spread function in modulating chromatin architecture during mitosis.

To identify Aurora targets, we combined chemical inhibition and quantitative phosphoproteomics. Chemical inhibition often leads to unwanted side effects due to limited specificity of the small molecule inhibitor. To exclude such side effects from the analysis, we took a combined approach of genetic mutation and chemical inhibition that increases specificity and we controlled for side effects in the phosphoproteomics experiment. To further increase inhibitor specificity and erase possible side effects, we additionally developed covalent inhibitors for analogue-sensitive Aurora, which require two selectivity filters for binding and inhibition. We further engineered analogue-sensitive Aurora kinase to contain a binding site for such inhibitors and screened for the optimal electrophile in the chemical inhibitor. This enabled us to covalently inhibit Aurora and characterize the potential of the designed molecules as specific kinase inhibitors.

ZUSAMMENFASSUNG

Aurora-Kinasen sind konserviert in Eukaryoten und sind entscheidend für die korrekte Abwicklung der Zellteilung. Die Identifikation von Aurora-Substraten ist wichtig, um die molekulare Funktion der Kinase zu verstehen. Basierend auf den offensichtlichsten Phänotypen einer Aurora-Depletion oder -hemmung wurden viele der Substrate in Kandidatenstudien identifiziert. Jedoch sind Aurora-Kinasen an Prozessen beteiligt für welche die relevanten Substrate nicht bekannt sind. Wir haben quantitative Phosphoproteomik in Verbindung mit der chemischen Hemmung einer analogsensitiven Variante der Aurora Kinase Ark1 (Ark1-as3) aus der Spalthefe angewendet, um eine proteomweite Identifizierung von Aurora-Substraten zu erreichen. Von mehr als 8000 Phosphorylierungsereignissen waren 70 Phosporylierungsstellen (auf 42 Proteinen) mögliche Ziele der Aurora-Kinase. Abgesehen von ein paar wenigen bekannten Substraten war die Mehrzahl der entdeckten Aurora-Ziele nicht als solche bekannt. Aufgrund der Tatsache, dass viele der neu identifizierten Ziele in diversen Bereichen der Chromatindynamik beteiligt sind, schlagen wir vor, dass Aurora, abgesehen von ihrer Rolle in der Chromosomenkondensation und -anheftung an die mitotische Spindel, eine weitreichende Funktion bei der Bestimmung des Chromatinaufbaus während der Zellteilung hat.

Wir haben chemische Hemmung und quantitative Phosphoproteomik kombiniert, um Aurora-Substrate zu identifizieren. Aufgrund der unzureichenden Spezifität von kleinmolekularen Hemmstoffen hat die chemische Hemmung oft unerwünschte Nebeneffekte zur Folge. Mit der Kombination einer genetischen Mutation und der chemischen Hemmung haben wir eine Erhöhung der Spezifität erzielt, und in unserem Phosphoproteomikexperiment eine Kontrolle hinzugefügt, um solche Nebeneffekte von der Analyse auszuschließen. Zusätzlich haben wir kovalente Hemmstoffe, welche zwei Selektivitätsfaktoren für die Bindung und Hemmung erfordern, für die analogsensitive Aurora-Kinase entwickelt, um die Hemmstoffspezifität weiter zu erhöhen und die unerwünschten Nebeneffekte zu beseitigen. Wir haben sowohl die analogsensitive Aurora-Kinase verändert, so dass sie eine Bindungsstelle für solch einen Hemmstoff besitzt, als auch eine Suche für ein optimales Elektrophil des chemischen Hemmstoffes durchgeführt. Dies

ermöglichte es uns Aurora kovalent zu hemmen und das Potenzial der erzeugten Moleküle als spezifische Kinase-Hemmstoffe zu charakterisieren.

1 Introduction

1.1 Discovery of cells

All organisms, whether it is a unicellular bacterium, a plant, an animal or a human have something in common: they are composed of single or even billions of cells. The first discovery of cells reaches back to the year 1665 when Robert Hooke described that cork is composed of little chambers, which he called cells, based on the Latin word *cellula* ('small room'). More than 150 years later, in the year 1838, the German botanist Matthias Jakob Schleiden and in 1839 the zoologist Theodor Schwann reported that plants and animals are composed of or formed by cells. These findings are considered the official formulation of the 'cell theory'. Schleiden hypothesized that cells are formed by a process he called 'free cell formation', the spontaneous crystallisation inside another cell. In 1852 Robert Remak and in 1858 Rudolf Virchow, among other scientists, refuted this theory and showed that new cells are formed through scission of pre-existing cells, which Virchow summarized in the term '*omnis cellula e cellula*' (every cell from pre-existing cell), thus completing the classical cell theory.

1.2 The cell cycle

The discovery that cells are formed through scission of pre-existing cells and the postulation of the 'cell theory' was the basis of research that led to the discovery that the eukaryotic cell reproduction follows a highly regulated series of events, called the cell cycle. This process can be divided into phases. In 1882, the German biologist Walther Flemming published the first report on a cell cycle phase when naming the process of nuclear division 'mitosis' (from the Greek *mito*, or 'thread') after the appearance of condensed chromosomes (Flemming, 1965). It initially appeared that cells were only active during mitosis and so the time the cell did not spend in mitosis was called interphase (or resting stage). Studies from Oscar Hertwig and other scientists in the early 20th century proposed the concept of the karyoplasmic ratio, arguing that the ratio between nuclear and cytoplasmic volume is constant, and that cell cleavage takes place when this ratio becomes unbalanced as a consequence of cell growth (reviewed in (Nurse, 2000)). This is

not entirely the case but reports correctly that progression through the cell cycle is coupled to cellular growth. Another hallmark of the discovery of cell cycle phases took place in the 1940s when Avery, MacLeod and McCarty showed that DNA was the carrier of the genetic information (Avery et al., 1944). This finding suggested that DNA must be duplicated some time during interphase in order to ensure that both daughter cells receive a full complement of genetic material. The relationship between the timing of DNA synthesis and the mitotic cycle was elucidated in 1953, placing the synthesis of DNA before nuclear division (Howard and Pelc, 1953). In the same year, Watson and Crick published their model on the structure of DNA as a double-helix. This model elucidated the mechanism of base pairing and provided an explanation how genetic information is stored and replicated in living organisms (Watson and Crick, 1953).

All together these studies, from the first discovery of cells to the elucidation of the processes and phases of the cell cycle, shaped the understanding of the cell cycle that we have today. Since the 1950s, the cell cycle is generally described as a succession of four distinct phases. Two daughter cells derive from the segregation of genetic material (mitosis) and the division of the cytoplasm (cytokinesis), together called M phase. This is followed by a phase called G1 (‘gap’) before the duplication of the genetic material in S phase (synthesis phase), when DNA replication takes place. After the completion of S phase, the cell prepares entry into the next M phase and traverses a phase called G2. The cycle is completed after the cell enters M phase again and produces two new daughter cells.

A regulatory network, called the cell cycle control mechanism, helps to ensure the correct and timely order of these events. Key players in regulation of proper cell cycle progression are the cyclin-dependent kinases (Cdks). Cdk activities rise and fall as the cell progresses through the cell cycle and leads to cyclical changes in the phosphorylation of Cdk targets. The binding to activator proteins called cyclins is responsible for the oscillation of Cdk activity, while Cdk protein abundance is constant throughout the cell cycle (Morgan, 1995). Cyclin abundances oscillate due to synthesis and degradation in distinct cell cycle phases (Morgan, 1997). A complex of Cdk and cyclin B for example ensures the progression from G2 to M phase. The exit of M phase requires a decrease of Cdk activity, which is accomplished by the degradation of cyclin B. This in turn is

mediated by the anaphase promoting complex/cyclosome (APC/C), an E3 ubiquitin ligase, that ubiquitinates cyclin B and thus targets it to degradation by the proteasome (Morgan, 2007).

1.3 Mitosis

Mitosis is the stage of the cell cycle where the genetic material is distributed equally to the two daughter cells. Mitosis was first described by Walther Flemming in 1882 and is divided into several sub-phases that are based on distinct morphological changes of the nucleus or the cell (Flemming, 1965). The first step of mitosis is called prophase and starts with the visible condensation of chromosomes, which later appear as a pair of sister chromatids. In vertebrate cells, the centrosomes, also called the 'microtubule organizing centres', separate in prophase and generate an elaborate microtubule array, the mitotic spindle (Wittmann et al., 2001). In the following prometaphase, the nuclear envelope breaks down and spindle microtubules emanating from the centrosomes make contact to the chromosomes and attach to them at specialized proteinaceous structures, called kinetochores, that are located at the centromere of each sister chromatid (Takeuchi and Fukagawa, 2012). In yeast, the nuclear envelope does not break down during mitosis, and the spindle forms inside the nucleus. This is called a 'closed' mitosis, as opposed to the 'open' mitosis of metazoan cells. Recent studies in human cells showed that chromosomes form a ring at the equatorial plane of the mitotic spindle during prometaphase and kinetochores seem to initially predominantly make contact to the side of microtubules. Stable 'end-on' attachments between microtubules and kinetochores form during late prometaphase, and in metaphase all chromosomes are stably attached to the two centrosomes and aligned at the centre of the spindle (also called metaphase plate) (Magidson et al., 2011). During the formation of microtubule-kinetochore attachments, errors can occur and are resolved by an error correction mechanism, which involves the kinase Aurora B (see later). This mechanism resolves erroneous attachments, thus creating free kinetochores that have a new chance to interact with microtubules to form a proper bi-oriented attachment. In addition, cells contain a signalling pathway called the spindle assembly checkpoint (SAC), which avoids progression beyond metaphase until all chromosomes have

achieved proper bi-orientation (Musacchio and Salmon, 2007). The SAC senses unattached kinetochores and blocks progression into anaphase by binding of SAC proteins to Cdc20, an activator and substrate recognition factor of the APC/C (Fang et al., 1998). The APC/C is a ubiquitin ligase that marks target proteins for destruction by the proteasomes by 'decorating' them with ubiquitin. Once all kinetochores are properly attached to the mitotic spindle, the SAC is satisfied and stops targeting Cdc20, which in turn can now activate the APC/C leading to the destruction of securin and cyclin B. The protein securin builds a complex with a protease called separase and keeps it in its inactive form. The degradation of cyclin B and securin leads to a reduction in Cdk1-activity and the release and activation of separase. Active separase cleaves cohesin (Oliveira and Nasmyth, 2010), a protein complex that holds sister chromatids together from their generation in S phase. The microtubules emanating from opposite spindle poles now start to separate the sister chromatids and segregate them to the opposing poles of the cell. This stage of mitosis is called anaphase. Mitosis is completed in telophase, when the spindle is disassembled and the decondensing chromosomes and other nuclear components are repacked into the daughter nuclei by the reforming nuclear envelope (Sullivan and Morgan, 2007). Cell division is completed by the division of the cytoplasm (cytokinesis) (Guertin et al., 2002).

1.4 The role of Aurora kinases in mitotic progression

Aurora kinases are members of a family of serine/threonine kinases that are found in eukaryotic organisms from yeast to mammals. The founding member of this family was isolated in 1987 (Tearle and Nüsslein-Volhard, 1987) in a screen for *Drosophila melanogaster* (*D. melanogaster*) mutants and the gene was found to be required for centrosome separation. Because the mutation of the respective gene lead to monopolar spindles, the gene was named after a phenomenon of the night sky in the polar regions, called Aurora (Glover et al., 1995). In mammals three members of the Aurora family have been identified (Aurora A, Aurora B and Aurora C). *D. melanogaster*, *Xenopus laevis* (*X. laevis*) and *Caenorhabditis elegans* (*C. elegans*) each have two Aurora kinases (Aurora A and B), whereas in yeast only one Aurora kinase was identified, which is functionally related to the metazoan Aurora B kinase. In *Saccharomyces cerevisiae* (*S. cerevisiae*, 'budding

yeast') the Aurora kinase is called Ipl1 ('increase in ploidy 1') and in *Schizosaccharomyces pombe* (*S. pombe*, 'fission yeast') Ark1 ('Aurora-related kinase 1'). Sequence comparison of several Aurora target sites suggested a preferred phosphorylation consensus sequence for Aurora, which is [RK]-X-[ST]-[ILV] (Cheeseman et al., 2002).

Aurora kinases have been implicated in the regulation of different processes during cell division. Aurora A subfamily members associate with centrosomes and with regions of microtubules that are proximal to the centrosome. They are known to regulate mitotic entry, centrosome maturation and bipolar spindle formation (Marumoto et al., 2005). Aurora B is the enzymatically active member of a complex called the 'chromosomal passenger complex' (CPC). This complex has several functions during mitosis, including chromosome condensation, proper bipolar spindle formation, spindle stability and cytokinesis (Ruchaud et al., 2007; Carmena, 2008; Kelly and Funabiki, 2009; Liu and Lampson, 2009). Apart from Aurora B, the CPC includes the scaffolding member INCENP, and the targeting subunits Survivin and Borealin/DasraB (Ruchaud et al., 2007). INCENP interacts with all three other members of the complex and its highly conserved C-terminus (also called IN-box) is important for the binding and activation of Aurora B. The activation is a two-step mechanism: binding of INCENP leads to a partial activation of Aurora B and full activation requires phosphorylation of INCENP by Aurora B at two serines in a conserved threonine-serine-serine (TSS) motif close to the IN-box (Bishop and Schumacher, 2002; Honda et al., 2003; Sessa et al., 2005).

The localization of the CPC is dynamic with distinct changes throughout mitosis. In early mitosis, it is localized along chromosome arms and progressively enriches at the inner centromere during prometaphase. As cells progress into anaphase the CPC translocates to the spindle midzone and finally concentrates at the midbody between dividing cells (Ruchaud et al., 2007). These localization changes are the result of protein interactions within the complex and with outside factors. Whereas the C-terminus of INCENP is important for the interaction with Aurora B, the N-terminus of INCENP forms a three-helix bundle involving the N-terminus of Borealin and the C-terminus of Survivin (Jeyaprakash et al., 2007). This interaction is important for the spindle and midbody localization of the complex. The inner centromere localization of the complex has been analysed recently. Several studies have shown that the CPC is anchored to the inner centromere by the

interaction of the BIR domain of Survivin and the C-terminal domain of Borealin with phosphorylation marks present on histones in centromeric chromatin. Phosphorylation of histone H2A by the mitotic kinase Bub1 creates a binding site for an adaptor molecule, Shugoshin, which in turn binds to the CPC (Yamagishi et al., 2010; Tsukahara et al., 2010). The BIR domain of Survivin recognises a phosphorylation on Thr3 of histone H3 which is generated by the mitotic kinase Haspin (Yamagishi et al., 2010; Kelly et al., 2010; Wang et al., 2010; Jeyaprakash et al., 2011).

Whereas Aurora A and Aurora B are predominantly found in somatic cells, the expression of Aurora C is restricted to mammalian germ cells and Aurora C has been implicated in spermatogenesis and male fertility (Dieterich et al., 2007; Kimmins et al., 2007).

1.4.1 The mitotic functions of Aurora B kinase

The function of the Aurora B kinase family has been extensively studied over the past decade and the identification of small molecule inhibitors of Aurora kinases made an important contribution to this research (Taylor and Peters, 2008; Katayama and Sen, 2010). Several direct substrates of the kinase have been identified and their phosphorylation plays a role for proper progression through mitosis (Fig. 1.1):

The phosphorylation of histone H3 at Ser10 is a hallmark of mitosis, and is conducted by Aurora B (Hsu et al., 2000; Giet and Glover, 2001; Murnion et al., 2001; Kaitna et al., 2002; Petersen and Hagan, 2003). When cells enter mitosis, histone H3 is phosphorylated all along the chromosome and the phosphorylation persists until early anaphase. Although the presence of this phosphorylation is well known, its function is still under debate (Prigent and Dimitrov, 2003). Expression of non-phosphorylatable histone H3 (mutation of Ser10 to Ala) in budding yeast exhibited no obvious phenotype (Hsu et al., 2000), but caused defects in chromosome segregation in fission yeast (Mellone et al., 2003). In the ciliated protozoan *Tetrahymena thermophila*, chromosome segregation as well as chromosome condensation were defective when H3-S10 was mutated (Wei et al., 1999). In human cells, histone H3 Ser10 phosphorylation has been implicated in dissociation of the heterochromatin protein 1 (HP1) from mitotic chromosomes (Fischle et al., 2005; Hirota et al., 2005), which may also be the case in fission

yeast (Kloc et al., 2008). The dissociation of HP1 is believed to be important for heterochromatin maintenance (Kloc et al., 2008; Chen et al., 2008).

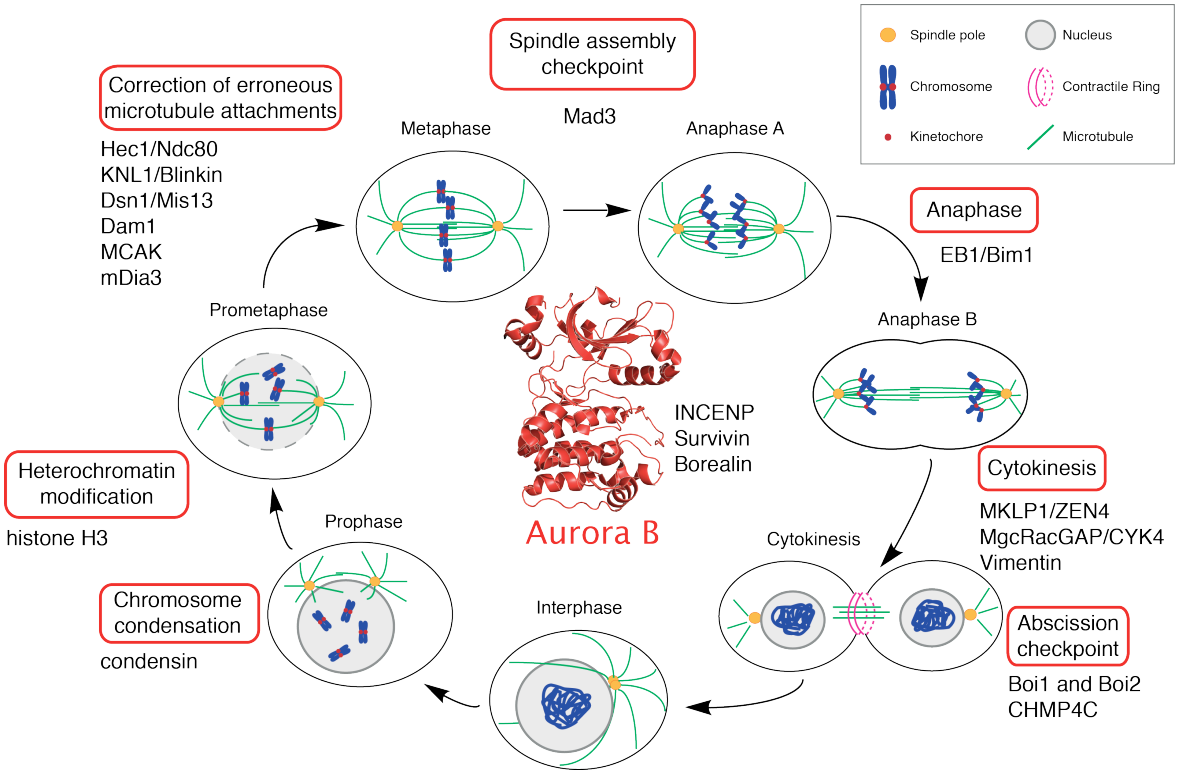

Figure 1.1 Schematic representation of the multiple functions and substrates of Aurora B or yeast Aurora during mitosis

Aurora B is the enzymatically active member of the chromosomal passenger complex (CPC). The CPC regulates multiple mitotic processes by phosphorylation of various substrates (Carmena et al., 2009). Aurora B also phosphorylates the other CPC members INCENP (Bishop and Schumacher, 2002; Honda et al., 2003), Survivin (Delacour-Larose et al., 2007; Wheatley et al., 2004) and Borealin (Gassmann et al., 2004), which *inter alia* is important for the activation of Aurora B. At the beginning of mitosis Aurora phosphorylates histone H3 all along the chromosome arms (Hsu et al., 2000; Giet and Glover, 2001; Murnion et al., 2001; Kaitna et al., 2002; Petersen and Hagan, 2003), which has been implicated in the dissociation of the heterochromatin protein1 (HP1) in human cells and fission yeast (Fischle et al., 2005; Hirota et al., 2005; Kloc et al., 2008). Aurora B was identified as a crucial factor for chromosome condensation by regulating the loading of the condensin complex to mitotic chromosomes in several organisms (Lipp et al., 2007; Takemoto et al., 2007; Nakazawa et al., 2008). In prometaphase, Aurora B regulates kinetochore-microtubule attachments through phosphorylating kinetochore proteins that interact directly with spindle microtubules, including Hec1/Ndc80 (Cheeseman et al., 2002; DeLuca et al., 2006; Ciferri et al., 2008), KNL1/Blinkin (Liu et al., 2010; Welburn et al., 2010), Dsn1/Mis13 (Welburn et al., 2010), the Dam1/Dash complex (Cheeseman et al., 2002; Tien et al., 2010), kinesin-13 family member MCAK (Andrews et al., 2004; Kline-Smith et al., 2004; Lan et al., 2004; Zhang et al., 2007) and the formin mDia3 (Cheng et al., 2011). Recent data indicates that Aurora B has a conserved role in the SAC (Kallio et al., 2002; Petersen and Hagan, 2003; Santaguida et al., 2011; Saurin et al., 2011; Maldonado and Kapoor, 2011), but apart from budding yeast, where Mad3 was identified as a substrate of Aurora B (King et al., 2007), the target(s) involved are still unknown. In budding yeast, Bim1, the homologue of human EB1, is phosphorylated in anaphase and this is required for normal spindle elongation kinetics and an efficient disassembly of the spindle midzone in cytokinesis (Zimniak et al., 2009). Later in mitosis, Aurora B phosphorylates the central spindle proteins MKLP1/ZEN4 and MgcRacGAP/Cyk4 (Guse et al., 2005; Minoshima et al.) as well as the intermediate filament protein Vimentin (Goto et al., 2003). In budding yeast and human cells Aurora

B is implicated in a checkpoint, which delays abscission in the presence of chromatin bridges. In budding yeast Aurora accomplishes this by phosphorylating Boi1 and Boi2 (Norden et al., 2006; Mendoza et al., 2009). In human cells a similar mechanism has been identified and recent data shows that CHMP4C is phosphorylated by Aurora B and delays the abscission in case of chromosome bridges (Steigemann et al., 2009; Carlton et al., 2012).

Apart from phosphorylating histone H3, the kinase activity of Aurora B has been implicated in the condensation of mitotic chromosomes presumably through localizing the condensin complex to chromatin (Bazile et al., 2010). In Drosophila and *C. elegans,* the depletion of Aurora B abolished localization of the condensin subunits Barren (Giet and Glover, 2001), SMC-2 and SMC-4 (Hagstrom et al., 2002) to mitotic chromosomes. In *X. laevis* egg extracts and in human cells, Aurora B activity is important for the chromosome localization of condensin I subunits in mitosis (Lipp et al., 2007; Takemoto et al., 2007). Peters and co-workers showed in addition that the three non-SMC subunits of condensin I (Cap-D2, CAP-G and CAP-H) are Aurora B substrates *in vitro* and their mitosis-specific phosphorylation depends on Aurora B *in vivo* (Lipp et al., 2007). Also in fission yeast, the condensin complex failed to localize to mitotic chromosomes in the absence of Ark1 activity (Nakazawa et al., 2008).

The CPC has also been shown to be important for the stability of the mitotic spindle (Adams et al., 2001; Gassmann et al., 2004). In *X. laevis* egg extracts, Aurora B phosphorylates and inhibits antagonizing factors of a ‘chromatin-driven spindle assembly pathway’, the microtubule-destabilizing protein Stathmin/Op18 (Gadea and Ruderman, 2006) and the kinesin-13 microtubule depolymerase MCAK (Ohi et al., 2004). Although this suggests that Aurora is important for spindle formation, it has been shown that spindles can nevertheless form when CPC components are depleted (Adams et al., 2001; Gassmann et al., 2004; Yue et al., 2008).

An important function of the Aurora B kinase is the regulation of attachment of microtubules to the kinetochores ensuring proper sister chromatid segregation. It was first described in budding yeast and later also in metazoan cells that Aurora B regulates attachment by destabilizing erroneous kinetochore-microtubule attachments such as syntelic (both kinetochores attached to the same spindle pole) and merotelic (one kinetochore attached to both spindle poles) attachments (Tanaka et al., 2002; Hauf et al., 2003; Dewar et al., 2004; Lampson et al., 2004).

It has been suggested that Aurora B creates a kinetochore-microtubule disruption signal until the kinetochores are attached in an amphitelic fashion (a pair of kinetochores attached to microtubules from opposite sites) and the centromere/kinetochore region comes under tension. According to this model, amphitelic attachment stretches the kinetochores away from the inner centromere and thus separates Aurora B from its substrates (Kelly and Funabiki, 2009). In 2009, Lens and colleagues provided strong support for this model by tethering Aurora B to different locations within the centromere and kinetochore (Liu et al., 2009). They used a phosphorylation biosensor (Fuller et al., 2008) to directly read out Aurora B activity *in vivo*. When the sensor was tethered to the centromere, it was always phosphorylated regardless of tension. Tethering the sensor to the kinetochore showed phosphorylation when tension on the chromosomes was low, and dephosphorylation when tension was high. However, when Aurora B was tethered artificially to the kinetochore, the sensor at the kinetochore remained phosphorylated, which coincided with microtubule detachment (Liu et al., 2009). This strongly suggested that Aurora B regulates kinetochore-microtubule attachments dependent on the physical vicinity to its substrates.

The molecular basis of error-correction is partially understood and seems conserved throughout eukaryotes. The conserved outer kinetochore protein network composed of KNL1, Mis12 complex and Ndc80 complex (KMN) is crucial for the interaction with microtubules (Cheeseman et al., 2006). It has been shown that Aurora B phosphorylates the N-terminal tail of Ndc80/Hec1 at several sites in mammalian cells, *C. elegans* and budding yeast (Cheeseman et al., 2002; Ciferri et al., 2008; Akiyoshi et al., 2009). These phosphorylations have been shown to decrease the affinity of microtubule binding to Ndc80 (Cheeseman et al., 2006; Ciferri et al., 2008). Recent studies showed that also KNL1 and Dsn1 (a member of the Mis12 complex) are substrates of Aurora B. The phosphorylation of two serines in conserved PP1-phosphatase binding motifs of KNL1 influences PP1-binding and is important for the regulation of proper kinetochore-microtubule attachment (Liu et al., 2010; Welburn et al., 2010). The phosphorylation of Dsn1 was shown to sensitize the microtubule binding activity of the KMN network (Welburn et al., 2010). In addition to phosphorylation of the KMN network, the phosphoregulation of MCAK activity by Aurora also seems to promote the

correction of microtubule mis-attachments (Andrews et al., 2004; Kline-Smith et al., 2004; Lan et al., 2004; Zhang et al., 2007).

Apart from its role in the error-correction mechanism, the Aurora B kinase has also been implicated in the SAC, although this topic has been discussed controversially (Musacchio, 2011). Recent data indicates that the role of Aurora B in the SAC is conserved throughout eukaryotic organisms (Kallio et al., 2002; Petersen and Hagan, 2003; Santaguida et al., 2011; Saurin et al., 2011; Maldonado and Kapoor, 2011), but the target(s) involved are still unknown.

At the onset of anaphase the CPC dramatically changes its localization away from the centromere to the central spindle and midbody, where it is required to ensure proper cytokinesis and regulating the abscission checkpoint (Carmena, 2008). Studies in *C. elegans* and human cells have shown that Aurora B phosphorylates MKLP1/ZEN4 and that this is essential for completion of cytokinesis (Guse et al., 2005). In addition it has been shown that Aurora B phosphorylates MgcRacGAP *in vitro*, and the overexpression of a nonphosphorylatable mutant of MgcRacGAP impaired cytokinesis (Minoshima et al., 2003).

In budding yeast and human cells, Aurora B delays the abscission of daughter cells when chromosome bridges are present between these two. This prevents chromosome breakage or protects against tetraploidization (Norden et al., 2006; Steigemann et al., 2009). Apart from chromatin bridges, defects in nuclear pore assembly also lead to activation of an Aurora B-mediated abscission checkpoint (Mackay et al., 2010). In budding yeast, Aurora B regulates the association of the two anillin-like proteins Boi1 and Boi2 to the contractile ring to delay abscission in response to chromatin bridges (Norden et al., 2006; Mendoza et al., 2009). Recent work in mammalian cells identified ESCRT-III subunit charged multivescular body protein 4C (CHMP4C) as a substrate of Aurora B and an important regulator of the Aurora B-mediated abscission checkpoint. Upon Aurora B phosphorylation CHMP4C localizes to the midbody and delays abscission in response to chromatin bridges and thus prevents DNA damage (Carlton et al., 2012).

All these studies shed light on how Aurora phosphoregulates and coordinates chromosome segregation with cytokinesis. Nevertheless, future work will be necessary to fully understand Aurora's role in mitosis and the identification of all its cellular substrates is a crucial requisite for this.

1.5 Phosphorylation as an important posttranslational modification in regulation of cellular processes

The Aurora kinase Ark1 is one out of 109 kinases in the fission yeast genome, which encodes about 5000 genes in total (www.pombase.org; (Wood et al., 2012)). Hence, kinases constitute about 2% of the genome, which is similar in human cells where out of 20,000 genes, around 500 encode kinases (Manning et al., 2002b; Miranda-Saavedra and Barton, 2007). Kinases catalyse protein phosphorylation, which is a reversible posttranslational modification (PTM) seen in prokaryotes and eukaryotes. In eukaryotes, phosphorylation usually occurs on serine, threonine, tyrosine and histidine residues (Ciesla et al., 2011). Phosphorylation in prokaryotes occurs in addition on lysine and arginine residues (Ciesla et al., 2011). The origin of the added phosphate is the γ-phosphate group from ATP. Attaching this phosphate group to proteins introduces a hydrophilic, twofold negatively charged group, which can lead to conformational changes of the modified protein or influence binding of the protein to other molecules (Cohen, 2000). Phosphorylation is one of the most frequent PTMs and was estimated to be present in up to 30% of proteins in a cell (Cohen, 2000). Phosphorylation affects almost every basic cellular process including metabolism, membrane transport, growth, division, differentiation, and motility (Manning et al., 2002a). Phosphorylation of proteins can be reversed by the activity of counteracting enzymes, called phosphatases. Since the discovery of the first kinases and phosphatases, the search for targets of these enzymes has been intense (Burnett and Kennedy, 1954; Anderson, 1992; Cohen, 2002).

That an estimated 30% of the proteome is subject to phosphorylation means that 6,000 out of 20,000 human proteins may become phosphorylated at some point. Given that ‘only’ 500 genes encode for kinases, each kinase must have more than one target and indeed the number of substrates for a given kinase can differ widely. The Ser/Thr kinase ‘Phosphorylase b kinase’ presumably only has a single target. It phosphorylates and thus activates glycogen phosphorylase to release glucose-1-phosphate from glycogen (Krebs et al., 1958; Fischer, 2010). In contrast, the Cyclin-dependent kinases that are important in cell cycle progression phosphorylate hundreds of proteins (Ubersax et al., 2003; Holt et al., 2009).

The identification of phosphorylation sites and the identification of the kinase that a given phosphorylation depends on has been and still is a challenging task. It

can be addressed by numerous methods ranging from genetics to biochemistry (reviewed in (Manning and Cantley, 2002)). Traditional approaches were often time-consuming biochemical methods, which typically focussed on single phosphorylation sites on the protein of interest. Phosphorylation was either detected and investigated with a phosphospecific antibody against the target site or by using radioactive ^{32}P labelled γ-ATP for *in vitro* kinase reactions followed by phosphopeptide mapping and sequencing by Edman degradation. Mutational analysis to localize and verify the modified residue then completed the analysis (Sefton and Shenolikar, 2001; Johnson and Hunter, 2005). Phosphorylation studies on a larger scale have been possible by combining 2D-electrophoresis with ^{32}P-labelling. However these methods were time-intensive, low-throughput and carried safety concerns due to the radioactive labelling. In addition, identification of a phosphorylation *in vitro* is no guarantee that the phosphorylation is also present *in vivo*. Phosphorylation site detection in cell lysates incubated with recombinant kinase for example abolishes the cellular context such as localization of substrate and kinase. Therefore secondary validation methods are needed to verify phosphorylation results obtained *in vitro* (Manning and Cantley, 2002).

With the advances in mass spectrometry (MS) in the late 1990s, proteomic and phosphoproteomic studies on a larger scale have become possible. Since then, new technologies are regularly reported. These include the development and improvement of mass spectrometers and advances in methods for peptide fractionation or phosphopeptide enrichment. A great advantage of the MS method is the direct and simultaneous detection of numerous *in vivo* phosphorylation sites. The following chapter discusses recent advances in the field of phosphoproteomics that helped tremendously in the identification of novel phosphorylation sites.

1.6 Analysis of the phosphoproteome through mass spectrometric techniques

In general, MS analysis of a proteome uses the following workflow: Cells are disrupted to obtain a cell extract, which is then fractionated followed by protein digestion to peptides (or the other way around). Enrichment for PTM-bearing peptides can be added. Peptides are then separated by capillary chromatography,

ionized using a process called electrospray ionization (ESI) and analysed with a tandem mass spectrometer. The mass spectra of peptides are detected and selected for further fragmentation. The obtained data reflects the amino acid sequence of the measured peptides. Matching the mass information of the peptide as well as the amino acid sequence with protein databases leads to identification of the corresponding protein (Steen and Mann, 2004).

Fractionation of proteins can be achieved by gel electrophoresis or by immunoprecipitation of proteins of interests and their associated protein complexes. Proteins are then converted into peptides by enzymatic digestion with proteases. Peptides can be fractionated by isoelectric focusing (IEF; separation of peptides according to their isoelectric point (pI)) or by strong cation exchange chromatography (SCX). Due to the often substoichiometric nature of PTMs, unmodified peptides are generally in excess over peptides carrying PTMs. Given that only the most abundant spectra are typically analysed in an MS analysis, PTM-bearing peptides may be found with low efficiency. In order to circumvent this problem, enrichment methods are used to increase the percentage of modified peptides. One possibility is the enrichment *via* antibodies against certain PTMs, which is usually done in acetylation studies (Kim et al., 2006; Choudhary et al., 2009). For phosphorylations this is a common strategy when analysing tyrosine-phosphorylated residues, which are little abundant (Hunter and Sefton, 1980; Lemeer and Heck, 2009). This approach was originally described by Vandekerckhove and co-workers in 1999 (De Corte et al., 1999) and has been used to map thousands of tyrosine phosphorylation sites in different cancer cell lines (Rush et al., 2005; Rikova et al., 2007). In order to generate an unbiased catalogue of phosphorylation sites, different phosphopeptide enrichment techniques have been developed in addition. One commonly used technique is immobilized metal affinity chromatography (IMAC (Andersson and Porath, 1986)), which is based on the high affinity of phosphate groups for certain chelated metal ions such as Fe^{3+}, Zn^{2+}, and Ga^{3+} (Posewitz and Tempst, 1999). One of the main challenges in IMAC has been the nonspecific retention of strongly acidic peptides, due to the weak affinity between negatively charged carboxylates and positively charged metal ions. In order to increase the selectivity of IMAC for phosphopeptides, White and co-workers avoided nonspecific binding of carboxylates by methyl-esterification of carboxyl groups prior to IMAC enrichment

(Ficarro et al., 2002). Since then, IMAC has been used in a variety of phosphoproteomic studies (Li et al., 2007; Wilson-Grady et al., 2008; Zhai et al., 2008; Huttlin et al., 2010). Another method for selectively enriching phosphopeptides is metal oxide affinity chromatography (MOAC), e.g. by titanium dioxide (TiO_2) spheres (Pinkse et al., 2004). This technique is based on the same principle as IMAC, but the metal does not need to be chelated as it is usually coated on beads. This method is similarly prone to nonspecific retention of acidic peptides. Jørgensen and co-workers used 2,5-dihydroxybenzoic acid (DHB) to outcompete carboxylic acids from adsorbing to TiO_2 while phosphopeptides are retained (Larsen et al., 2005). This led to a reduction of nonspecific binding of acidic peptides and thus improved the selectivity of this purification. In general TiO_2 phosphopeptide enrichment has become a popular method for large-scale phosphoproteomics and has shown success in many research areas (Olsen et al., 2006; Macek et al., 2008; Beltrao et al., 2009; Bodenmiller et al., 2010).

1.7 SILAC as a quantitative technique to analyse changes in protein and PTM abundance

In recent years the identification of proteins and their PTMs with the help of MS has generated a huge amount of data (Lemeer and Heck, 2009). Often these data are generated from a specific tissue or from cells at a specific time point of development or the cell cycle. In order to get a better understanding of highly dynamic cellular processes and signalling networks (e.g. the influence of a growth factor) a sensitive and largely qualitative approach such as MS needs to be modified to yield quantitative data. One strategy that allows direct quantitative comparison between two or more samples is stable isotope labelling. Stable isotopes such as ^{2}H (instead of ^{1}H), ^{13}C (instead of ^{12}C), ^{15}N (instead of ^{14}N) and ^{18}O (instead of ^{16}O) are chemically identical to their native counterpart and behave similarly in chromatographic or MS analyses. The sensitivity of the mass spectrometer allows detecting the mass change introduced by the heavy isotope. In one of the first studies, Chait and co-workers grew one yeast culture on medium containing the natural abundant isotope of nitrogen (^{14}N, 99.6%; ^{15}N, 0.4%), while another culture was grown on the same medium enriched in ^{15}N (**>** 96%). The authors analysed protein mixtures and could detect expression differences by

peptide mass mapping. In addition to protein expression changes they also investigated changes in the phosphorylation states of proteins (Oda et al., 1999).

In general, isotope labels can be introduced (i) as an external standard using spiked synthetic peptides, (ii) enzymatically, (iii) chemically, or (iiii) metabolically (Ong and Mann, 2005). The only requirement for any stable isotope labelling approach is that the heavy-labelled peptide can be clearly distinguished. For quantification in survey MS spectra, it is essential that the mass shift by the label is at least 4 Da in order to distinguish the isotopomer clusters of the labelled and unlabelled forms of the peptide (Bantscheff et al., 2007). The simplest approach for the introduction of stable isotope-labelled peptides is to 'spike' known quantities of chemically synthesized peptides into the sample as internal standards. This absolute quantification (AQUA) strategy has been used by Gygi and co-workers *inter alia* to quantify low abundant yeast proteins involved in gene silencing and to quantitatively determine the cell cycle-dependent phosphorylation of human separase (Gerber et al., 2003). This approach has the drawback that it is introduced late in the stage of sample processing. Any quantitative variations prior to this step are not corrected by the internal standard (Havlis and Shevchenko, 2004).

The enzymatic labelling approach makes use of the incorporation of oxygen (O) during protein digestion by certain proteases. Incorporation of heavy ^{18}O in peptides results in a mass shift of 2 Da per ^{18}O atom. Due to a high rate of different labelling efficiencies between peptides, this method has not been widely applied in quantitative proteomics (Miyagi and Rao, 2007). Incorporating heavy isotopes by chemical labelling can be achieved by using stable isotope-bearing chemical reagents, which are targeted toward reactive sites on a protein or a peptide. One such method (isotope-coded affinity tags; ICAT) (Gygi et al., 1999) has the disadvantage of only labelling, and hence being able to quantify, cysteine-containing peptides. Better coverage is obtained by iTRAQ (isobaric tags for relative and absolute quantification), which has been employed for studies of phosphorylation status (Ross et al., 2004). There are numerous methods available for chemical labelling of peptides and proteins for absolute quantification (Bantscheff et al., 2007). The general drawback is that they are prone to side reactions, which can lead to unexpected products that may influence quantification results (for a review see (Bantscheff et al., 2007)).

Metabolic labelling, in difference to the other methods, introduces the metabolic label early in the experimental process during cell growth and division. The already mentioned study from Chait and co-workers using ^{15}N-enriched cell culture medium was a metabolic labelling approach (Oda et al., 1999). In 2002, Mann and co-workers advertised a technique called ‘stable isotope labelling with amino acids in cell culture’ (SILAC) (Fig. 1.2) (Ong et al., 2002). Amino acids containing stable isotopes, such as arginine bearing six ^{13}C atoms or lysine bearing six ^{13}C atoms, are supplied in the cell culture medium. Cell populations are grown in media containing either the ‘heavy’ or the ‘light’ form of an essential amino acid, which is thus introduced in each newly synthesized protein. The two different cell populations can now be mixed prior to cell lysis when cells are still intact. Using the protease trypsin (cleaves after arginine and lysine) ensures that cleaved peptides carry at least one of the labelled amino acids. In the MS analysis each peptide pair is separated by the mass difference introduced by the labelled amino acid. Relative quantification is performed by comparing the intensities of isotope clusters of the intact peptide in the MS spectrum (Fig. 1.2) (Mann, 2006). The combination of samples early during processing makes metabolic labelling by SILAC a very accurate quantitative MS method, because any quantitative variation arising during the sample processing influences both biological samples equally. A limitation to the SILAC approach is the availability of the repertoire of heavy forms of amino acids, which restricts the number of cellular states that can be directly compared. Although experiments with up to five different states can be addressed (Harsha et al., 2008), these 3-, 4- or 5-state experiments have to be performed with deuterated amino acids (^{2}H instead of ^{1}H), which might affect separation by reversed phase chromatography (Zhang et al., 2002). Multistate experiments are therefore often performed by combining two 3-state experiments with one overlapping condition as an internal standard (Ong and Mann, 2006).

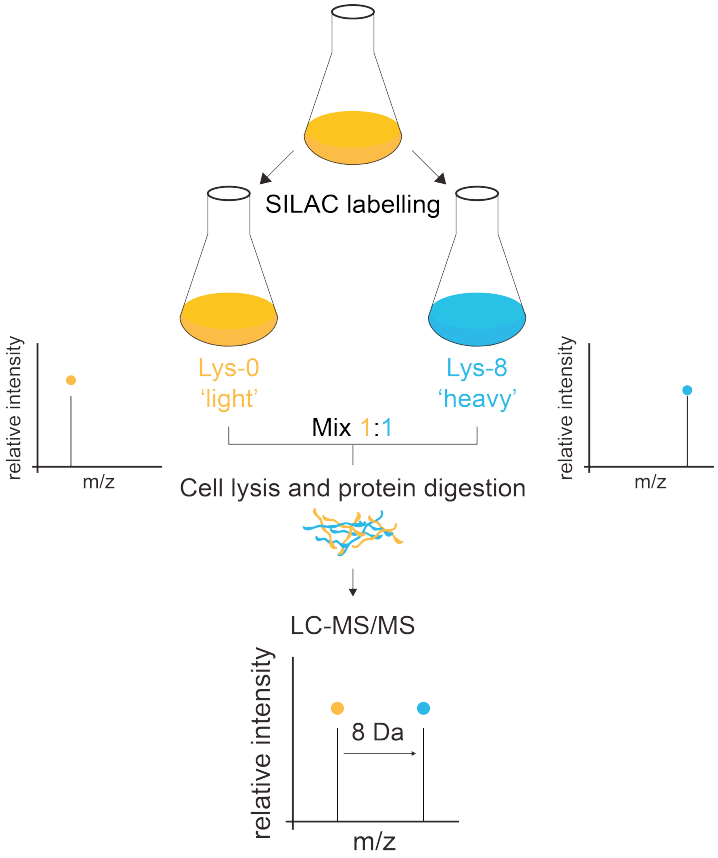

Figure 1.2 Schematic representation of a SILAC (stable isotope labelling with amino acids in cell culture) experiment

Cells are either cultured in normal, 'light' medium (Lys-0, highlighted in yellow) or transferred to medium containing 'heavy', stable isotope-labelled amino acid lysine with $^{13}C_6$ and $^{15}N_2$ (Lys-8, highlighted in blue). Cell growth and protein synthesis in 'heavy' medium leads to the metabolic incorporation of the isotope-labelled amino acid throughout the proteome. Cells are mixed equally and lysed prior to protein digestion. Digestion with Lys-C results in peptides containing Lys-0 or Lys-8. The residue-specific mass shift of 8 Da allows distinction and relative quantification of the 'light' and the 'heavy' peptide by mass spectrometry.

The restriction that the isotope-labelled amino acids need to be supplemented during cell growth makes it difficult to use this approach in adult animals. Early studies have been performed with immortalized cell lines or single-celled eukaryotes like yeast (Ong et al., 2002; Gruhler et al., 2005). Supplying nutrients with the isotope-labelled amino acids enables the use of SILAC also in mouse (Kruger et al., 2008; Zanivan et al., 2012), Drosophila (Sury et al., 2010), zebrafish (Westman-Brinkmalm et al., 2011), and plant cells (Gruhler et al., 2005).

1.8 Analogue-sensitive kinase alleles as chemical genetics tool

The relationship between genes and phenotypes has been mainly investigated by classical genetic methods. In general, forward and reverse genetics can be applied. In forward genetics the target genes or pathways are identified by studying phenotypes resulting from random mutagenesis, whereas in reverse genetics the focus is on a particular gene of interest. This gene is mutated (e.g. temperature sensitive mutation leading to conditional inactivation) or altered in gene expression to elucidate its cellular phenotype. The development of new techniques such as RNA interference (RNAi) and knock-out/knock-in animals have made reverse genetic the leading approach in genetics. Nevertheless these genetic methods have certain drawbacks that complicate the interpretation of phenotypes. Permanent presence of a mutant or permanent depletion of a protein can lead to adaptive processes that partially compensate the defect. In cell cycle studies, the requirement of a gene at a certain step will hide effects that the same gene may have on later stages. Conditional alleles such as temperature sensitive mutants are often used to circumvent such problems, but the temperature shift has been shown to alter cellular processes (Causton et al., 2001). Small chemical molecules have been a powerful tool to overcome this problem seen with classical genetic approaches. Chemical approaches are akin to the genetic approaches and therefore often termed chemical genetics. Through phenotype-based (forward) screening of small molecules, compounds that induce a phenotype of interest can be selected, followed by identifying the target of the compound (Kawasumi and Nghiem, 2007). An early example of the power of the forward chemical genetic approach was the identification of monastrol. The drug was identified in a high throughput screen testing chemicals that specifically arrest cells in mitosis. Through biochemical and microscopic studies monastrol was subsequently found to inhibit the mitotic motor protein kinesin Eg5 (Mayer et al., 1999). The advantage of chemicals is that they are easy to apply, work rapidly and are often reversible. Another advantage of chemicals over genetics is that they can be used to disrupt an enzymatic or other activity of a protein, while leaving the protein itself (and other activities) intact (Knight and Shokat, 2007). Schreiber and co-workers showed that Tubacin inhibits the tubulin deacetylase activity of histone deacetylase 6, while not disturbing its histone deacetylase activity (Haggarty et al.,

2003). Tubacin accomplishes this by binding and thus inhibiting only one of the two catalytic domains of the protein. Such a specific inhibition would not have been possible by RNAi depletion or gene knockout. Nevertheless the combination of RNAi and chemical methods can be helpful to understand the cellular function of proteins (Weiss et al., 2007).

Despite the success of small chemical molecules in biological research, target specificity of these molecules can vary greatly and off-targets are likely. Examples for this are small molecules targeting protein kinases. Kinases have a high sequence and structural similarity so that inhibitors targeting one kinase often hit more than this single target (Davies et al., 2000). The Aurora B inhibitor Hesperadin for example targets at least seven other kinases (Hauf et al., 2003). In the year 2000, Shokat and co-workers reported a method to overcome poor kinase specificity. The authors showed that mutating a single residue in the ATP binding pocket of protein kinases increased sensitivity to a range of small molecule inhibitors (Bishop et al., 2000). This residue, termed the gatekeeper, is conserved as a large hydrophobic amino acid (methionine, leucine, phenylalanine or threonine) in a conserved position among kinases (Hanks and Hunter, 1995; Zhang et al., 2005). Originally the authors tested this approach with members of the oncogenic Src-kinase family that is inhibited strongly by synthetic pyrazolo[3,4-d]pyrimidine (PP1) (Hanke et al., 1996). This inhibitor is not specific for single members of this family making it difficult to assess their different cellular functions. Shokat and colleagues mutated the gatekeeper of v-Src into a smaller amino acid such as glycine or alanine and searched for PP1-derivatives inhibiting the activity of the modified kinase (Bishop et al., 1998). They identified compounds (such as 4-Amino-1-tert-butyl-3-(1'-naphthylmethyl)pyrazolo[3,4-d]pyrimidine, short 1NM-PP1) that were able to specifically inhibit different sensitized kinases and not the unmodified wild type kinases (Fig. 1.3) (Bishop et al., 2000). This design strategy of kinase sensitization can be called 'bump-and-hole' approach. The gatekeeper mutation creates a 'hole' in the ATP-binding pocket of the target kinase that makes it accessible for a small-molecule inhibitor that has a 'bump' fitting into this newly created 'hole'. Such an engineered kinase is often referred to as 'analogue-sensitive' (as) kinase. The advantage of this method is that cross-reactivity of these chemicals with other kinases is low, due to the natural occurring 'bulky' gatekeeper residue. This method is a good example for the successful

combination of genetics (mutation of the gatekeeper) and chemistry. This approach has now been used in a wide variety of studies to specifically sensitize kinases and decipher their cellular functions (Kung et al., 2006; Larochelle et al., 2006; Pinsky et al., 2006; Burkard et al., 2007; Kang et al., 2008; Windecker et al., 2009; Maciejowski et al., 2010; Cipak et al., 2011).

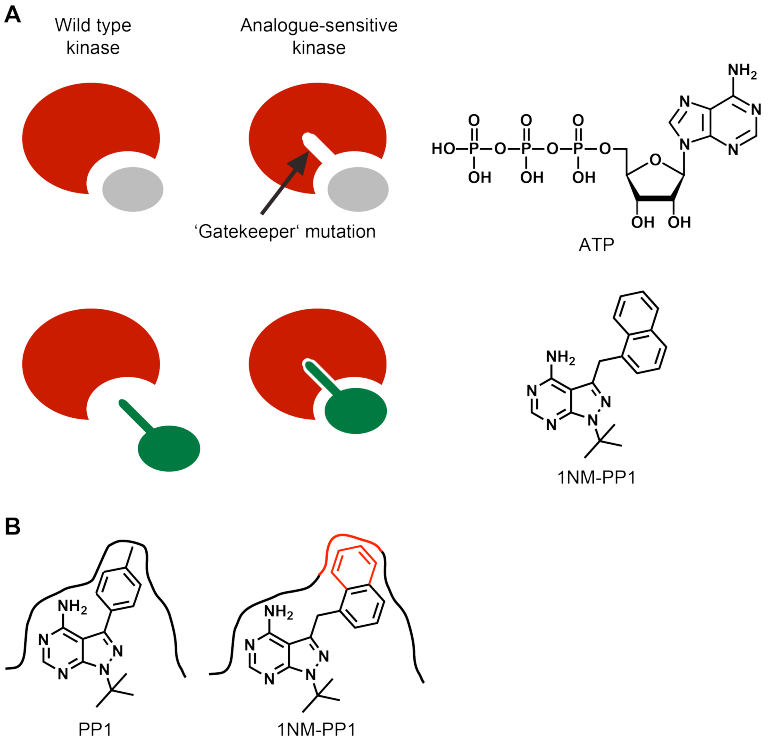

Figure 1.3 Schematic representation of mechanism of action of small molecule inhibitors in analogue-sensitive kinases

(**A**) Mutation of a specific hydrophobic residue ('gatekeeper') in the ATP-binding pocket of a kinase generates an analogue-sensitive kinase that can be specifically targeted by 1NM-PP1 (derivative of the kinase inhibitor PP1 (Hanke et al., 1996)). The binding of ATP is usually not influenced by this mutation. (**B**) Mutation of the gatekeeper residue in a protein kinase creates a new pocket (red) that can be uniquely accessed by the small molecule inhibitor analogue 1NM-PP1 (adapted from (Knight and Shokat, 2007).

1.9 Aim of the study

Aurora kinases are well conserved throughout eukaryotes and are crucial for the proper execution of mitosis. The identification of Aurora substrates is important for the understanding of its function during mitosis. Based on the most obvious phenotypes of Aurora depletion or inhibition, several substrates had been identified through candidate approaches. However, Aurora kinases are known to contribute to processes, for which the relevant substrates are entirely unknown. The aim of this study was to use an unbiased proteome approach in order to identify Aurora targets. Such an approach was expected to yield an unbiased picture, uninfluenced by the visually most obvious phenotypes, was expected to find the relevant substrates for known functions and was expected to complement our understanding of Aurora function. As unbiased strategy, I used a combination of chemical genetics and quantitative phosphoproteomics. First I altered the ATP-binding pocket of Aurora to make it susceptible to the small molecule inhibitor 1NM-PP1. This enabled me to specifically inhibit Aurora in mitosis and to compare the phosphoproteome of inhibitor-treated and untreated cells. Stable isotope labelling with amino acids in cell culture (SILAC) in combination with mass spectrometry made it possible to identify quantitative differences in phosphorylation between these two situations.

In addition to the search for new substrates, I extended our approach of Aurora kinase engineering. In addition to the ATP-binding pocket (gatekeeper) mutation I planned to introduce a cysteine residue at the lip of the ATP-binding pocket. This cysteine can serve as attachment point for covalent inhibitors with matching reactive group. The so modified kinase has two selectivity filters (the gatekeeper and the reactive cysteine), and matching inhibitors for this kinase therefore may be even more specific than those developed for analogue-sensitive kinases carrying only the gatekeeper mutation.

2 Results

2.1 Mitotic Substrates of the Kinase Aurora with Roles in Chromatin Regulation Identified through Quantitative Phosphoproteomics of Fission Yeast

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Published in: **Science Signaling**, Volume 4, Issue 179, rs6 (2011)

Author contributions:

I established the SILAC labelling protocol for fission yeast cells and conducted cell culture experiments for the three independent experiments (Fig. 1A), cell lysis and protein extraction, protein digestion, SCX and phosphopeptide enrichment with TiO_2, mutation of *spc7* and *orc1* with subsequent growth and tetrad analysis (Fig. 4), recombinant protein purification and *in vitro* kinase assays (Fig. 3 and Table S5 and S6), and participated in the analysis of the obtained MS data. In addition I contributed to the writing of the manuscript together with Silke Hauf and Boris Macek.

Karsten Krug performed bioinformatic analysis of the obtained MS results. He conducted the phosphorylation motif analysis in Fig. 2A, the GO term analysis (Table S4 and Fig. S7) that Fig. 3 is partially based on, the normalization of phosphopeptide ratios based on protein abundance in Fig. S2, and the analysis of phosphorylation site positioning in proteins in Table S2.

Stuart Pengelley taught me MS techniques and operated the mass spectrometer during several measurements.

Boris Macek supervised the study and operated the mass spectrometer during several measurements. He was responsible for the analysis of the MS raw data with the software MaxQuant.

Silke Hauf supervised the study and performed tetrad analysis seen in Fig. 5A and Fig. S8.

Mitotic Substrates of the Kinase Aurora with Roles in Chromatin Regulation Identified Through Quantitative Phosphoproteomics of Fission Yeast

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Abstract

Kinases of the Aurora family are essential for the proper execution of mitosis in eukaryotes and Aurora inhibitors are in clinical trials as anticancer drugs. We applied site-specific quantitative phosphoproteomics in conjunction with chemical inhibition of Aurora to identify mitotic Aurora substrates in fission yeast on a proteome-wide scale. We detected 8000 phosphorylation events, of which we assigned almost 6000 to a specific residue; 220 were reduced in cells exposed to the Aurora inhibitor. After controlling for unspecific effects of the inhibitor, we classified 70 sites (on 42 proteins) as probable targets of Aurora, which enabled refinement of the consensus sequence for phosphorylation by Aurora. Several of the substrate candidates were known targets of Aurora, validating the approach, but most represented newly detected Aurora substrates. The involvement of these Aurora substrates in diverse aspects of chromatin dynamics suggests that in addition to its established role in controlling chromosome compaction and attachment to the mitotic spindle, Aurora influences other aspects of chromatin architecture and function during mitosis.

Introduction

The serine-threonine protein kinases of the Aurora family are essential for the proper execution of mitosis in eukaryotes (1). Metazoan Aurora A is important for efficient entry into mitosis and bipolar spindle formation (2). Metazoan Aurora B and the single Aurora in yeast (known as Ipl1 in budding yeast and Ark1 in fission yeast) ensure that chromosomes become properly segregated by controlling chromosome compaction and chromosome attachment to the mitotic spindle, as well as cytokinesis and abscission (3). Aurora family kinases have been implicated in tumorigenesis, and both Aurora A and Aurora B are currently explored as targets for anticancer therapy (4, 5).

Crucial for understanding the molecular functions of Aurora is the identification of the cellular substrates of the kinase, but few in vivo substrates are known. In particular, kinetochore-associated proteins are phosphorylated by Aurora B or yeast Aurora, and this regulation is important for proper chromosome attachment (6-16). Members of the centralspindlin complex are phosphorylated by Aurora B to promote cytokinesis and control abscission timing (17-21). Furthermore, Aurora B and yeast Aurora phosphorylate histone H3 on serine-10 (Ser^{10}) during mitosis (22), leading to dissociation of heterochromatin proteins (23, 24), which may support propagation of heterochromatin through cell division (25, 26). To identify additional substrates of Aurora, we combined quantitative phosphoproteomics with chemical inhibition of Aurora in fission yeast and screened for proteome-wide changes in mitotic phosphorylation that depend on Aurora.

Results

Phosphoproteome comparison in the presence and absence of kinase activity identifies Aurora substrates

We sought to identify substrates of yeast Aurora by comparing the phosphoproteome in mitotic cells in the presence and absence of the kinase activity of Aurora. We created a fission yeast (*Schizosaccharomyces pombe*) strain expressing an analog-sensitive (AS) version of Aurora from the endogenous locus (*ark1-as3*) (27) and carrying a conditional *cdc25* mutation (28). When shifted to the nonpermissive temperature, these cells arrest just before mitosis. We then released the cells into mitosis in the presence or absence of 1NM-PP1, an inhibitor specific for AS kinases (29). We harvested the cultures when the cells had reached a mitotic index of 80 to 90%, meaning that most of the cells were in mitosis (Fig. 1A). We compared the protein phosphorylation events occurring in the absence or presence of Aurora activity by site-specific quantitative phosphoproteomics (30) using stable isotope labeling by amino acids in cell culture (SILAC) (31) (Fig. 1A). Cells in the inhibitor-treated culture were labeled with heavy lysine (H); cells in the control culture, to which the solvent dimethyl sulfoxide (DMSO) was added, were labeled with light lysine (L). Thus, phosphorylation events that were reduced by the inhibitor treatment are characterized by a low H/L ratio. Because Aurora is expected to phosphorylate some of its substrates only when chromosomes are not properly attached to the mitotic spindle (32), we repeated the experiment with the microtubule-depolymerizing drug methyl 2-benzimidazole carbamate (MBC) to perturb chromosome attachment under otherwise identical conditions (Fig. 1A, experiment 2). MBC efficiently depolymerizes fission yeast microtubules (33), and most of the cells in the treated cultures failed to separate their spindle pole bodies in mitosis (Fig. 1A,

experiment 2), indicating a failure to assemble a mitotic spindle. To ensure that changes in phosphorylation were specific to inhibition of Aurora and not resulting from inhibition of other kinases that could be targeted by 1NM-PP1, we subjected a strain expressing wild-type *ark1+* to the same inhibitor and control treatment (Fig. 1A, experiment 3). Phosphorylation events that depend on the kinase activity of Aurora were expected to have a reduced H/L ratio in experiment 1 or 2 or both, but not in experiment 3. We performed two technical replicates for each experiment and after phosphopeptide enrichment observed 8254 phosphorylation events on 1544 proteins (about one-third of the fission yeast proteome); 530 of the observed phosphoproteins had not been identified in either of two previous fission yeast phosphoproteome analyses (fig. S1) (34, 35). More than 6000 of the phosphorylation events were detected in all three experiments (Fig. 1B) and we could assign more than 5800 to a specific residue with a median localization probability >98% (table S1). Most (>94%) of the phosphorylation events quantified in each experiment did not significantly change upon inhibitor treatment (Fig. 1A and table S1). Parallel quantitative analysis of the proteome indicated that changes in protein abundance upon inhibitor treatment were negligible (fig. S2). Strong reduction of phosphorylation events upon inhibitor treatment was more pronounced in cells expressing the inhibitor-sensitive *ark1-as3* than in cells expressing wild-type *ark1+* (Fig. 1A), suggesting that we had identified specific Aurora targets. We grouped the phosphorylation events that were reduced in experiment 1 or 2 into five classes of confidence on the basis of their repeated detection and mode of regulation in experiment 1 to 3 (Fig. 1C and table S1). We considered the 70 phosphorylation sites on 42 proteins in the highest confidence classes 1 and 2 as Aurora-dependent. Most of these phosphorylation sites mapped into protein regions outside well-defined domains

(table S2), in agreement with the global analysis of substrates of cyclin-dependent kinase 1 (Cdk1), another major cell cycle kinase (36). Among the 150 phosphorylation events in classes 3 to 5 (Fig. 1C and table S1), there are likely additional Aurora-dependent sites, but the current data are too sparse to distinguish them from nonspecific inhibitor-dependent or stochastically occurring changes.

The presence of proteins in classes 1 and 2 that have previously been identified as substrates of yeast Aurora, or whose orthologs in metazoan organisms have been identified as substrates of Aurora B, validated the results. Class 1 contains Spc7 (homologous to *Caenorhabditis elegans* KNL-1 and human KNL1, which is also known as Blinkin) (table S1). In vertebrate cells, phosphorylation of this kinetochore protein by Aurora B is required for proper chromosome attachment to the mitotic spindle (12, 37). Furthermore, Bir1 (also known as Survivin), Pic1 (also known as INCENP), and Sgo2, all of which are interaction partners and reported substrates of yeast Aurora or whose orthologs are substrates of metazoan Aurora B (3, 38-40), were present in class 1 or 2, as was the condensin subunit Cnd2 (homologous to *Drosophila* Barren and human CAP-H). Condensin localization to mitotic chromosomes requires Aurora in fission yeast and Aurora B in metazoans (41-44). Human Aurora B phosphorylates CAP-H (45), and fission yeast Aurora-dependent phosphorylation of Cnd2 is required for condensin function (46, 47). Fission yeast Aurora is also required for the localization of the protein phosphatase Clp1 to the contractile ring during anaphase (48), and a direct interaction partner of Clp1, called Nsk1, was among the proteins in class 2. Across all eukaryotes, the best-established Aurora B or yeast Aurora substrate is histone H3, which is phosphorylated on Ser^{10} (22). We observed the doubly modified peptide with Ser^{10} phosphorylation and lysine 14 (Lys^{14}) acetylation, and the abundance of this

peptide was substantially reduced upon Aurora inhibition in both experiments 1 and 2, but did not change in experiment 3 (fig. S3), corroborating that our strategy identified direct Aurora substrates.

Sequence analysis of the Aurora-dependent phosphorylation sites reveals a common motif

Analysis of the large set of potential yeast Aurora phosphorylation sites in the highest confidence classes 1 and 2 should reveal a common amino acid motif surrounding the phosphorylated site. Comparison of these sites to all phosphorylated sites in the data set revealed a strong preference for arginine (R, Arg) at position -2 (Fig. 2A). This partly matches the previously established motif for yeast Aurora-dependent phosphorylation, [RK]-X-[ST]-[ILV] (6). However, hydrophobic amino acids at position +1 were not prominent in class 1 and 2 sites (Fig. 2A and fig. S4). We examined the previously reported yeast Aurora and metazoan Aurora B phosphorylation sites and found that more than 65% of them did not have a hydrophobic amino acid at position +1 (fig. S5 and table S3). One example is the sequence surrounding Ser^{10} of histone H3, which has threonine (T) at position +1. Furthermore, among the published yeast Aurora and metazoan Aurora B sites, Arg at position –2 was more prevalent than Lys (K) (45 versus 4 occurrences) (table S3). On the basis of these published phosphorylation sites, as well as those that we identified here, we propose that R-X-[ST] is the common consensus motif for phosphorylation by Aurora B–type kinases. Aurora A and Aurora B have different functions, different localization and different binding partners during mitosis (1, 49). However, the amino acid sequence similarity between Aurora A and Aurora B is high (75% identity within the kinase domain of the human proteins), and a single amino acid change converts human Aurora A into a protein that binds the Aurora

B–interacting proteins INCENP and Survivin and complements for Aurora B (50, 51). Furthermore, some studies found Aurora A and Aurora B phosphorylate the same site on target proteins (15, 52-55), and [RKN]-R-X-[ST]-[ILVM] was identified as consensus for Aurora A on the basis of phosphorylation of synthetic peptides in vitro (56). We therefore hypothesize that R-X-[ST] is recognized by Aurora A as well as Aurora B. The R-X-[ST] consensus is furthermore shared with protein kinase A (57), and other determinants, like localization, must provide specificity.

Class 1 and 2 sites that are directly targeted by Aurora should conform to the R-X-[ST] consensus. About 30% of all class 1 and 2 sites matched this consensus and the percentage was higher (~60%) when we considered only those sites that exhibited the greatest reduction in phosphorylation in Aurora-inhibited cells (H/L < 0.25) (Fig. 2B). Sites that exhibited reduced phosphorylation due to nonspecific effects of the inhibitor (table S1) were not enriched for the R-X-[ST] motif (Fig. 2B).

There are several possible reasons for the occurrence of class 1 and 2 sites that did not match the consensus. Four sites matched the amino acid sequence K-X-[ST] (Id 556, 3455, 4377, and 5021; table S1). Because both Lys and Arg are positively charged and some of the yeast Aurora or metazoan Aurora B sites reported in the literature have Lys at position –2 (table S3), it is likely that these sites are targeted by Aurora, just like the R-X-[ST] sites. Other Aurora-dependent sites that did not match the R-X-[ST] consensus may have been falsely reported for technical reasons: Some of the sites were in the vicinity of another site matching the consensus. When these sites appeared together on one doubly phosphorylated peptide and when there were no other quantified phosphopeptides containing only one of the sites, we could not distinguish whether either or both sites were exhibiting the reduction in phosphorylation. In such cases, we

reported both as reduced in order not to miss any potential sites. For several such doubly phosphorylated peptides (Id 2826, 3455, 5021, 6068, and 9099; table S1), there was independent evidence from another peptide (Id 2825, 3456, 5020, and 9346; table S1) that phosphorylation of the R-X-[ST] site was reduced upon Aurora inhibition, but it was impossible to determine whether phosphorylation of the neighboring non-R-X-[ST] site was also reduced. In two cases of adjacent sites that we examined closely (Elg1-Thr^{34} Ser^{36}; Spc7-Ser^{4} Ser^{9}), the R-X-[ST] site was phosphorylated by fission yeast Aurora in vitro, but the adjacent non–R-X-[ST] site was not (table S5). Thus, our motif analysis may contain sites that are falsely reported as reduced and Aurora-dependent. This technical issue does not jeopardize the substrate analysis: Even though we cannot say with certainty whether phosphorylation of both sites was reduced upon Aurora inhibition, phosphorylation of the peptide did depend on Aurora activity.

We repeated the analysis of the frequency of motif occurrence by including the K-X-[ST] sites and excluding non-[RK]-X-[ST] sites from doubly phosphorylated peptides (fig. S6). In this analysis, only two of the sites exhibiting strong reduction (H/L <0.25) did not fit the [RK]-X-[ST] consensus. We confirmed that one of these sites (Orc1-Ser^{9}, Id 5035) was phosphorylated by fission yeast Aurora in vitro (table S5) despite the absence of the consensus motif.

The remaining class 1 and 2 sites that did not match the consensus may represent secondary sites that depend on kinases downstream of Aurora or sites that have passed the significance threshold by chance. We cannot distinguish these latter sites from secondary sites by formal parameters. Consistent with the possible identification of secondary sites, enrichment for the R-X-[ST] motif was greater among the sites that exhibited a greater reduction in phosphorylation after Aurora inhibition and, thus, are

more likely to represent direct Aurora substrates (Fig. 2B and fig. S6). Phosphorylation of secondary sites still depends on the kinase activity of Aurora and, hence, gives an indication of pathways that are regulated by Aurora.

The spectrum of newly detected Aurora substrates suggests a role for the kinase in chromatin restructuring during mitosis

The presence of known Aurora substrates in class 1 and 2 and the motif analysis indicated that our strategy successfully identified direct Aurora substrates. Most of the proteins in class 1 or 2 had previously not been reported as Aurora targets. Most of these proteins are nuclear and many play a role in chromatin dynamics (Fig. 3, fig. S7, and table S4). Among these new candidate substrates are heterochromatin proteins and nucleolar proteins, as well as proteins implicated in the posttranslational modification of histones, in DNA replication, in DNA damage signaling, in RNA processing, and in nucleocytoplasmic transport (Fig. 3).

To confirm some of the newly identified putative targets as direct Aurora substrates, we expressed candidate targets from different functional classes (Fig. 3) as recombinant proteins and tested their phosphorylation by fission yeast Aurora in vitro. Ten out of 11 proteins tested were phosphorylated by Aurora, and 8 of those 10 were phosphorylated on at least one of the sites found by our in vivo analysis (Fig. 3 and table S5), confirming that these proteins are direct Aurora targets. Sixteen out of the 19 phosphorylation sites found on these proteins after incubation with Aurora matched the [RK]-X-[ST] consensus; of the remaining 3, 2 had several Lys and Arg residues in position –3 to –6, and only for one site (Mdb1-Ser^{156}), we could not detect a resemblance to the consensus. This confirms that Aurora has a strong preference for [RK]-X-[ST] sites. Of the nine phosphorylation sites that had been found to be Aurora-

dependent in vivo, but that were not phosphorylated in vitro, five did not fit the [RK]-X-[ST] motif, and four of these (Id 2826, 6068, 6256, and 9099; table S1) may have been falsely reported because of their presence on a doubly phosphorylated peptide together with an [RK]-X-[ST] site. Another four sites were not phosphorylated in vitro despite matching the consensus. Because two of these sites belonged to class 1 and therefore had been found to be Aurora-dependent in vivo in two independent experiments, we suspect that the failure to phosphorylate these sites may have resulted from the conditions of the in vitro kinase assay, due to missing binding partners or improperly folded proteins. Hence, technical reasons may contribute to the failure to confirm all substrates in this assay.

We next addressed whether phosphorylation at identified sites is of functional consequence. Others have shown that phosphorylation of the condensin subunit Cnd2 at the site identified by us and at adjacent Aurora consensus sites is required for condensin function in mitosis (46, 47). Furthermore, we found that mutation of Aurora-dependent phosphorylation sites within the kinetochore protein Spc7 caused sensitivity to the microtubule-depolymerizing drug benomyl (Fig. 4), which is an indication of a mitotic defect. Combination of the Spc7 phosphorylation site mutants with mutations in Nuf2 (58), another outer kinetochore protein, caused a synthetic growth defect (Fig. 4C), suggesting that phosphorylation of Spc7 by Aurora is important for proper kinetochore-microtubule attachment. Mutation of Aurora-dependent phosphorylation sites in the origin recognition complex subunit Orc1 caused a synthetic growth defect and phenotypic enhancement when combined with deletion of the DNA damage checkpoint kinase gene *chk1*+ (Fig. 4, E and F). Similar observations have been made for *orc1* temperature-sensitive mutants (59). Hence, the functionality of Orc1 is impaired by

mutation of the phosphorylation sites. Together, these data suggest that both expected and unexpected Aurora-dependent phosphorylations identified in this study may be of functional relevance.

Yeast Aurora and metazoan Aurora B have been best characterized for their role in regulating mitotic chromosome segregation through phosphorylation of kinetochore proteins. In contrast, few substrates related to chromatin have previously been reported. In addition to histone H3, tousled-like kinase (TLK-1), which plays a role in chromatin assembly and the regulation of transcription; MYB-binding protein 1A (MYBBP1A), a nucleolar protein, which has been reported to bind transcription factors; and NSUN2, which is named for NOP2/sun domain and is a nucleolar RNA methyltransferase, have been identified as targets of Aurora B (60-62). Our data indicate that Aurora has a more widespread role in regulating chromatin.

To further corroborate a functional link between fission yeast Aurora and the newly identified substrates, we analyzed whether candidate substrate and Aurora mutants would show similar genetic interactions. When we searched for direct genetic interactors of the identified substrates (63-65), we found that several candidate substrate genes had common genetic interactors (Fig. 5A and fig. S8). As an example, six of the candidate substrates showed a negative genetic interaction with *cbp1* (CENP-B) (Fig. 5A and fig. S8). CENP-B is a conserved DNA-binding protein required for genome integrity in fission yeast (66). An *ark1* temperature-sensitive mutant was synthetically lethal with deletion of *cbp1* (fig. S8), indicating that Aurora and the candidate substrates could be functionally related. Similar observations were made for genetic interactions with *arp4* (also called *arp42*, encoding a subunit of the SWI-SNF and the RSC chromatin-remodeling complexes), *chk1* (encoding a DNA damage checkpoint kinase),

and *swi3* (encoding a protein homologous to vertebrate tipin, a component of the replication fork protection complex) (Fig. 5A). In all these cases, the *ark1* temperature-sensitive mutant exhibited genetic interactions similar to those reported for many of the substrates (Fig. 5A and fig. S8). Several candidate substrate genes or genes with which candidate substrates exhibited genetic interactions are required for resistance toward DNA-damaging agents (Fig. 5 and fig. S8). The temperature-sensitive *ark1* mutants also exhibited an increased sensitivity to DNA-damaging agents (Fig. 5B). These data confirm a functional relationship between Aurora and some of the newly identified substrates and suggest that Aurora is a major regulator of mitotic chromatin and has a role in protecting from DNA damage (Fig. 5C).

Discussion

The identification of all cellular substrates of a kinase is crucial for understanding its molecular mechanism of action, but comprehensive kinase substrate identification is still a challenging task (67). The approach we have taken here is powerful because substrates of the kinase are identified in vivo in the native cellular context. Similar strategies have been used to identify substrates of the kinase Cdk1 (36) and to assess global perturbations of kinases and phosphatases in yeast (68).

At present, one limitation of this approach is the inability to capture the entire phosphoproteome in one experiment, and therefore, despite excellent coverage, some substrates or phosphorylation sites escape detection. We alleviated this effect by including a technical replicate for each experiment. It is difficult to say whether we missed any substrates, because other than histone H3, Cnd2, and Bir1 (Survivin) (40,

47, 69), each of which we identified, no fission yeast Aurora targets have been described. However, on the basis of work in other organisms several conserved outer kinetochore proteins are likely phosphorylated by Aurora (6, 10, 12), and of these we identified only Spc7 (human KNL1, also known as Blinkin) (Fig. 3), suggesting that there are additional Aurora substrates not covered by our analysis.

The identification of a high number of unexpected targets raises the question of whether our analysis indeed specifically identified Aurora substrates. In particular for sites in class 1, we are, however, confident that their phosphorylation depends on Aurora, because (i) we have repeatedly detected these phosphorylation sites in cells with active Aurora, (ii) we have repeatedly found less of the phosphorylated peptide in Aurora-inhibited cells, and (iii) we did not find significant changes in the abundance of the phosphorylation of these sites when testing for nonspecific effects of the inhibitor (Fig. 1). It is still possible that some of these proteins are not direct Aurora substrates, but secondary targets. However, we can easily identify a common motif for most of these sites (Fig. 2), and most of the substrates that we tested (8 of 11) were phosphorylated by Aurora in vitro on the same sites identified in vivo (Fig. 3 and table S5). These data indicate that at least a subset of the identified sites that exhibited reduced phosphorylation upon fission yeast Aurora inhibition are directly targeted by Aurora. If we assume that a protein from class 1 or 2 is a direct substrate if it contains an [RK]-X-[ST] site that is less phosphorylated upon Aurora inhibition, then 12 of 13 proteins (92%) with at least one phosphorylation site strongly reduced (H/L <0.25) by Aurora inhibition and 21 of all 42 proteins in class 1 and 2 (50%) would be direct substrates (table S1).

The spectrum of candidate substrates that we identified suggests that Aurora influences chromatin during mitosis more broadly than previously thought (Fig. 5C). Given the generally high conservation in function between yeast Aurora and metazoan Aurora B, we speculate that such influence on chromatin may also occur in metazoans. Mitosis-specific modification of chromatin could serve a number of tasks. Changes to chromatin may be required to segregate chromosomes. Given the complex topology of chromatin and the many associated proteins and RNA molecules, it is generally surprising how the two DNA strands can be separated without causing damage. It has been proposed that proper chromosome segregation requires removal of many proteins and RNA from chromatin, because these would interfere with segregation (70). Aurora B-type kinases and condensin have been suggested to have a role in this "clearing" process (70). The Smc5-Smc6 complex is required for segregating chromosomes specifically after DNA damage, presumably by aiding in the removal of the cohesin complex from chromatin (71). The spectrum of Aurora substrates that we identified, which includes condensin and Smc6 (Fig. 3), is consistent with a potential role for Aurora in preparing chromosomes for segregation. Mitotic modification of chromatin may also help to prevent chromatin damage as a result of the condensation that occurs during mitosis (72) or it may modulate DNA damage signaling such that unperturbed mitotic progression is ensured (73). A role of Aurora in these processes is consistent with the increased DNA damage sensitivity of *ark1* mutants (Fig. 5, B and C). Furthermore, mitotic modification of chromatin may affect subsequent phases of the cell cycle. The mitotic phosphorylation of histone H3 by Aurora has been proposed to facilitate transcription from heterochromatin in the subsequent S phase, which reinforces heterochromatin silencing by processing of these transcripts into small interfering RNA

(26). Consistent with such a role, circumstantial evidence implicates mammalian Aurora B in maintaining epigenetic chromatin states in differentiated cells (74, 75). Thus, Aurora may have a role in heterochromatin inheritance (Fig. 5C). Because we found several proteins implicated in DNA replication as Aurora targets, Aurora may also contribute to the organization of the program of replication origin firing in S phase, which may be set up during mitosis (76, 77). Regardless of the precise consequences of the Aurora-dependent phosphorylation events, our data indicate that Aurora has previously unappreciated functions in regulating chromatin during mitosis and is involved in the resistance toward DNA damage. These additional functions should be considered in basic research, as well as in the clinical application of Aurora inhibitors.

Materials and Methods

S. pombe growth

All *S. pombe* yeast strains are listed in the Supplementary Materials. For SILAC experiments, a yeast strain carrying a temperature-sensitive *cdc25-22* mutation, the *ark1-as3* allele, and *plo1+-GFP* was grown for 10 cell cycles at 25°C in Edinburgh minimal medium (EMM) supplemented with $^{12}C_6{}^{14}N_2$ L-lysine (Lys0, "light", L) or $^{13}C_6{}^{15}N_2$ L-lysine (Lys8, "heavy", H) until it reached a concentration of 8 x 10^6 cells/ml. Cells were arrested before mitosis by shifting them to 36°C for 4.5 hours. Heavy-labeled cells were treated for 20 min with 5 µM 1NM-PP1 (4-amino-1-tert-butyl-3-(1'-naphthylmethyl)pyrazolo[3,4-d]pyrimidine; Toronto Research Chemicals) and light-labeled cells were treated with the same amount of the solvent DMSO. In the second experiment, MBC (25 µg/ml; Sigma-Aldrich) was additionally added 10 min before the addition of 1NM-PP1 or DMSO. The third experiment was identical to the first except that a strain expressing *ark1+* instead of *ark1-as3* was used. Cells were released from the *cdc25-22* arrest by reducing the temperature back to 25°C and were grown for 20 min at 25°C before harvesting. Cells were harvested by centrifugation (3000 g, 5 min, 4°C). The resulting cell pellet was washed three times in ice-cold double-distilled H_2O and finally resuspended in the residual amount of liquid left after decanting the supernatant. The suspension was dropped in liquid N_2 to freeze cells as droplets. The droplets were stored at –80°C until further processing. Typically 1.5 x 10^9 cells were used for each treatment (1NM-PP1 or DMSO) in one experiment.

Serial dilution growth tests were performed by growing cells to logarithmic growth phase in YEA (yeast extract containing adenine sulfate) medium and spotting a 1:5

serial dilution onto YEA plates, when indicated containing 10 μM camptothecin (CPT; Sigma-Aldrich), 4 mM hydroxyurea (HU; Sigma-Aldrich), or benomyl (8 μg/ml; Sigma-Aldrich).

Microscopy of S. pombe cells

Cells were fixed in –20°C methanol at the moment when freezing the rest of the culture in liquid N_2. After washing once with PEM [100 mM Pipes (pH 6.9), 1 mM EGTA, and 1 mM $MgSO_4$]/methanol and once with PEM, DNA was stained with DAPI (4',6-diamidino-2-phenylindole, 1 μg/ml; Sigma-Aldrich). Images were acquired on a Zeiss Axio Imager microscope coupled to a charged-coupled device camera and were processed with MetaMorph software (Molecular Devices Corporation). Typically, a Z–stack of about 3–μm thickness, with single planes spaced by 0.3 μm, was acquired and subsequently projected to a single image. Shown are color-combined pictures of maximum intensity projections of the GFP Z stack (green channel) with single plains of the DAPI Z stack (blue and red channel).

In vitro kinase assays

Full-length proteins or protein fragments (see table S6) were expressed in *Escherichia coli* strain BL21 and purified on Ni-NTA agarose beads (Qiagen). Ark1 and Pic1 were coexpressed in *E. coli* strain BL21 and similarly purified. Under these conditions, Pic1 copurified with His-Ark1. Proteins were stored in 50 mM tris-HCl (pH 7.5), 150 mM NaCl, and 20% glycerol at –80°C. For in vitro phosphorylation, 100 μg of substrate protein was incubated with 2 μg Ark1 and Pic1 in a buffer containing 50 mM tris-HCl (pH 7.5), 100 mM NaCl, 20 mM $MgCl_2$, 1 mM dithiothreitol, and 1 mM adenosine 5'-triphosphate in a total volume of 100 μl for 30 min at 30°C. The reaction was stopped

by adding 400 µl methanol, followed by chloroform-methanol precipitation of proteins. Precipitated proteins were resuspended in denaturation buffer (6 M urea, 2 M thiourea) and digested in solution by endoproteinase Lys-C (Waco) or trypsin (Promega) as described previously (78). About 20% of each digest was desalted on C_{18} Stage Tips (79) and analyzed directly by liquid chromatography–mass spectrometry (LC-MS), whereas the remainder of the digest was subjected to phosphopeptide enrichment by TiO_2 chromatography (78) before LC-MS analysis.

Protein extraction

The frozen cell droplets of the heavy and light cultures were mixed according to their cell count in a 1:1 ratio and protein extracts were prepared with a ball mill cell grinder (Mixer Mill 400; Retsch) under cryogenic conditions. The cell powder was resuspended in denaturation buffer [6 M urea, 2 M thiourea, and 1% n-octyl glucoside (NOG)], and insoluble material was removed by centrifugation (13,000g, 10 min, 4°C). Both supernatant (#I) and pellet were further processed. The pellet was resuspended in denaturation buffer, 500–µm glass beads were added and the mixture was subjected to 20-s shaking in a FastPrep machine (FP120, Qbiogene). The resulting suspension was centrifuged (13,000g, 10 min, 4°C), the pellet was discarded and the protein concentration in the supernatant of this step (#II) and the supernatant #I was determined with a BCA (bicinchoninic acid) assay (Novagen). Both supernatants were further processed in parallel for the in-solution proteolysis and phosphopeptide enrichment. The procedure was identical for each of the three SILAC experiments and was always repeated once (technical replicate). In the replicate procedure, only supernatant #I was processed, because supernatant #II typically had a low protein concentration.

In-gel proteolysis for proteome analysis

For proteome analysis, 150 µg of protein extract was separated by 4 to 12% gradient SDS–polyacrylamide gel electrophoresis (SDS-PAGE) (Invitrogen). The resulting lanes were cut into 15 slices, which were subjected to in-gel digestion as previously described (80), but with endoproteinase Lys-C instead of trypsin. The resulting peptide mixtures were desalted with C_{18} Stage Tips (79) before LC-MS measurements.

In-solution proteolysis and phosphopeptide enrichment for phosphoproteome analysis

Six milligrams of total protein was further processed as described previously (78) with the following changes: In-solution digestion was performed with endoproteinase Lys-C; elution of bound peptides in the strong cation exchange (SCX) chromatography was done with a gradient of 0 to 50% SCX solvent B; the elution of bound phosphopeptides from the TiO_2 beads was performed with 3 x 100 µl of a modified elution buffer (40% NH_3, 60% ACN).

LC-MS/MS

All LC-MS analyses were performed on an EasyLC nano-HPLC (Proxeon Biosystems) coupled to an LTQ Orbitrap XL mass spectrometer (Thermo Scientific). Peptide separation was performed on a nano-HPLC column (75 µm by 15 cm) packed in-house with 3–µm C_{18} beads (Dr. Maisch). Peptides were loaded in a solvent containing 0.5% acetic acid and eluted with a segmented gradient of 5 to 60% of solvent containing 80% acetonitrile and 0.5% acetic acid.

The LTQ Orbitrap was operated in the positive ion mode. Precursor ions were recorded in the Orbitrap mass analyzer at a resolution of 60,000. Precursor ion fragmentation and acquisition were performed in the LTQ mass analyzer. For proteome analysis, the 10

most intense precursor ions were sequentially fragmented in each scan cycle, whereas for phosphoproteome analysis, the 5 most intense precursor ions were fragmented by activation of neutral loss ions at -98, -49 and -32.6 relative to the precursor ion (multistage fragmentation). In all measurements, sequenced precursor masses were excluded from further selection for 90 s. The target values for the LTQ were 5000 charges [tandem mass spectrometry (MS/MS)] and for the Orbitrap 10^6 charges (MS); the maximum allowed fill times were 150 and 500 ms, respectively.

MS data processing and analysis

The MS data were processed and quantified with MaxQuant (v1.0.14.3) (81). Generated peak lists were searched against a target-decoy database (82) consisting of the *S. pombe* proteome (http://www.sanger.ac.uk) and 262 frequently observed contaminants with the Mascot (Matrix Science) search engine (v.2.2.0). Carbamidomethylation of cysteine was set as fixed modification, whereas protein N-terminal acetylation, oxidation of methionine, and phosphorylation of serine, threonine, and tyrosine were set as variable modifications. Initial precursor mass tolerance was set to 7 parts per million (ppm) at the precursor ion and 0.5 dalton at the fragment ion level.

Identified MS/MS spectra were further processed by MaxQuant for statistical validation and quantitation of peptides, sites, and protein groups (83). False discovery rates (82) were set to 1% at phosphorylation site (P site), peptide, and protein group level. Downstream bioinformatics analysis was performed with R (v. 2.9.0; http://www.r-project.org). The interaction network in Fig. 5A and fig. S8C is based on the BioGRID database (84) and was visualized in Cytoscape 2.6.3 (85) with subsequent editing in Adobe Illustrator software. Information on DNA damage sensitivities and additional

protein-protein interactions were taken from the PombePD [http://www.proteome.com (86)].

Determination of phosphorylation sites

All phosphorylation events having a reported localization probability of at least 0.75 were considered to be assigned to a specific residue, and we refer to these as phosphorylation sites. This means that the sum of all remaining potential phosphorylation site probabilities was at most 0.25. We used the intensity weighted ratio significance values reported by MaxQuant to determine significantly changed phosphorylation sites. These values were further corrected for multiple hypothesis testing by the method proposed by Benjamini and Hochberg (87). For this data set, we report significant phosphorylation event ratios based on a Benjamini-Hochberg corrected *P* value threshold of 0.05.

Consensus motif analysis

We compared the occurrence of each amino acid at the –/+5 flanking positions of all class 1 and 2 sites exhibiting decreased phosphorylation to the corresponding frequencies among all identified and localized phosphorylation sites. To test whether the frequency of a particular amino acid at a specific flanking position was increased or decreased, we assumed the hypergeometric distribution as null distribution, and we derived *P* values by the application of Fisher's exact test.

Gene Ontology term enrichment analysis

Gene ontology (GO) term enrichment analysis was done with the BiNGO (88) plug-in for Cytoscape (85). GO annotation for *S. pombe* was downloaded from the GO consortium (http://www.geneontology.org/GO.downloads.annotations.shtml;

submission date 04/23/2010). The hypergeometric test was applied, and derived *P* values were adjusted for multiple hypothesis testing by the method of Benjamini and Hochberg (87). GO terms with adjusted *P* values <0.01 were considered to be significantly enriched. For grouping the candidate substrates into functional classes (Fig. 3), we preferentially used the fission yeast GO slim annotation (http://amigo.geneontology.org/cgi-bin/amigo/slimmer; submission date 03/15/2010).

Supplementary Materials

Fig. S1. Comparison of phosphorylated proteins identified in this and two other large-scale studies of fission yeast.

Fig. S2. Comparison of phosphopeptide ratios before and after normalization for protein abundance.

Fig. S3. Fragmentation mass spectra of the histone H3 peptide carrying the known Aurora target site (Ser^{10}).

Fig. S4. Consensus motif analysis of phosphorylation sites that depend on the kinase activity of fission yeast Aurora.

Fig. S5. Consensus motif analysis of previously identified yeast Aurora or metazoan Aurora B phosphorylation sites.

Fig. S6. Re–analysis of the frequency of motif occurrence by including the K-X-[ST] and excluding the doubly phosphorylated sites.

Fig. S7. GO term enrichment of proteins with class 1 and 2 phosphorylation sites.

Fig. S8. Genetic interaction tests of *ark1* temperature-sensitive mutants and previously identified genetic interactions of fission yeast Aurora substrates.

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90. **Acknowledgments**: We thank Y. Watanabe, O. Weichenrieder, D. Weigel and members of our laboratory for valuable comments on the manuscript; M. Mann, J.M. Peters and A. Köhler for advice; S.A. MacNeill, Y. Hiraoka, P. Nurse, and

the Yeast Genetic Resource Center for fission yeast strains; E. Illgen and S. Wahl for excellent technical support; D. Steiner for help with graphics.

Funding: Supported by the Max Planck Society, the Deutsche Forschungsgemeinschaft (SFB446 grant to A.K. and S.H.) and the Landesstiftung Baden-Württemberg (Juniorprofessorenprogramm grant to B.M.).

Author contributions: A.K., B.M., and S.H. designed the experiments; A.K. performed all experiments except those in Fig. 5A and fig. S8 (done by S.H.); to prepare samples for MS, A.K. had help from S.P. and B.M.; S.P. and B.M. processed and analyzed the MS results; K.K. performed additional computational analysis; S.H. and B.M. wrote the manuscript with input by A.K. and K.K. **Competing interests**: The authors declare that they have no competing interests. **Data availability**: The MS data associated with this manuscript can be downloaded from the ProteomeCommons.org Tranche network using the following hash: aDFbn22hraYj2G/L63ojoA0PN3p8JS/+7oHi17zlW0UUDFKerD9pXTauJ74R Wqfr/BK7V5e0qzBOTOpjWk1WhlVn35EAAAAAAAAAaHA==. The hash can be used to prove which files were published as part of this manuscript's data set, and the hash can be used to check that the data have not changed since publication.

Fig. 1. Comparison of the mitotic phosphoproteome in the presence and absence of kinase activity of Aurora.

(**A**) Schematic outline of the three experiments (Exp) (top), representative pictures of the cultures at the time of freezing in liquid N_2, the mitotic index (MI) of each culture, and diagrams showing for each quantified phosphorylation event the change in abundance between heavy (H; inhibitor-treated) and light (L; control-treated) culture (H/L; *x* axis) as a function of the signal intensity (*y* axis) recorded in the mass spectrometer. The dashed lines mark the border for a decrease or increase in phosphorylation by more than a factor of 4 [$\log_2$ (H/L) <–2 or >2]. Most phosphorylation events (small gray circles) did not change in abundance between the two cultures. Significantly regulated phosphorylation events ($P < 0.05$) are depicted as red circles, and sites additionally matching the R-X-[ST] motif as dark red triangles. In the pictures of the cultured cells, DNA is stained with DAPI and mitotic spindle pole bodies are labeled by the kinase Plo1, tagged with green fluorescent protein (Plo1-GFP).

(**B**) Overlap among the three experiments for all phosphorylation events (Total, n = 8254), all quantified phosphorylation events (Quantified, n = 7082), and phosphorylation events exhibiting a significantly decreased H/L ratio upon inhibitor treatment (P <0.05) (Decreased H/L ratio, n = 413).

(**C**) Classification of phosphorylation events (P events) significantly reduced in experiment 1 or experiment 2. A down arrow (↓) indicates significant reduction; an up arrow (↑) significant increase; a right arrow (→) no significant change upon inhibitor treatment. n.d., not detected. Phosphorylation events in class 5 could not be localized

(Local.) to a specific amino acid within the phosphopeptide, whereas all phosphorylation events in the other classes could be localized and are referred to as phosphorylation "sites" in the text. Phosphorylation sites in class 1 and 2 were considered dependent on Aurora activity. Five of the proteins with class 1 phosphorylation sites also have class 2 phosphorylation site, so that the total number of proteins with class 1 or class 2 sites is 42.

Fig. 2. Consensus motif analysis of phosphorylation sites that depend on the kinase activity of fission yeast Aurora.

(**A**) Class 1 and 2 sites were examined for the relative enrichment (red) or depletion (blue) of amino acids flanking the phosphorylation site (left side). The heat map depicts the ± $\log_{10}$ *P* values (derived by application of Fisher's exact test). The occurrence of arginine (R) at position –2 was significantly more frequent ($P = 2.03 \times 10^{-12}$) in the 70 class 1 and 2 sites compared to all 5812 phosphorylation sites in the data set. Consensus motif analysis according to biochemical properties of amino acids (right side) showed an enrichment of positively charged amino acids at position –2 ($P = 7.48 \times 10^{-7}$). No significant ($P < 1 \times 10^{-4}$) enrichment of hydrophobic amino acids was observed at position +1.

(**B**) Total number of considered sites (# sites) and percentage and number of sites matching the indicated R-X-[ST] consensus among all sites in a certain category. In contrast to sites from classes 1 and 2, phosphorylation sites that exhibited a decrease in phosphorylation due to nonspecific effects of the inhibitor (NS.↓) did not show enrichment for the R-X-[ST] motif. See table S1 for complete data.

Fig. 3. Aurora targets identified in this study.

Proteins with phosphorylation sites in classes 1 and 2 were grouped according to their GO Slim classification or GO classification for biological process with additional manual curation. The question mark group represents proteins without any well-defined function. Colored boxes indicate from left to right: (box 1) whether the protein is in class 1 (red) or class 2 (yellow); (box 2) whether at least one of the phosphorylation sites in the protein exhibited a strong reduction (H/L <0.25) by inhibitor treatment in experiment 1 or 2 (red, yes; yellow, no); (box 3) whether at least one of the phosphorylation sites in this protein that was reduced by Aurora inhibition matched the R-X-[ST] consensus (red, yes; yellow, no); (box 4) whether the protein localizes to the nucleus (red indicates nuclear localization of this protein or of a homologous protein from another organism; gray indicates cytoplasmic or otherwise non–nuclear localization; white indicates that no information was available); (box 5) whether any phosphorylation site in this protein exhibited reduced phosphorylation by inhibitor treatment in experiment 3 (gray, yes; red, no). The circles indicate whether the protein was phosphorylated by fission yeast Aurora in vitro (table S5) (red, yes at least one of the sites that depended on Aurora in vivo was phosphorylated in vitro; gray, phosphorylation by Aurora could not be detected; hatched red and gray, the protein was phosphorylated by Aurora but on sites different from the ones suggested by the in vivo analysis). The phosphorylation sites represented by box 5 were in all cases different from the sites that put the protein into class 1 or 2, which were sites that did not exhibit a reduction in phosphorylation in experiment 3. Protein names for *S. pombe* are given. Alternate names, homolog names, or names of the protein complex that the protein is part of are indicated in parentheses. *H.s., Homo sapiens*; *S.c. Saccharomyces cerevisiae*

Fig. 4. Mutation of Aurora-dependent phosphorylation sites in the kinetochore protein Spc7 or the origin recognition complex protein Orc1 impairs their functionality.

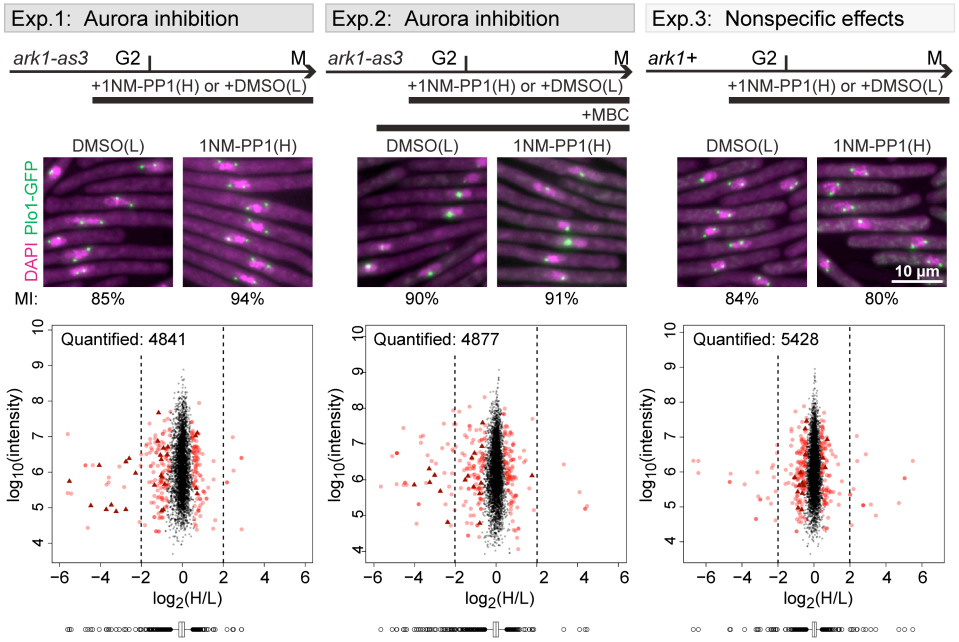
(**A**) Schematic diagram of the outer kinetochore protein Spc7 with phosphorylation sites identified in vivo (table S1) indicated above. The fragment tested for phosphorylation by fission yeast Aurora in vitro and the result from this assay (table S5) is indicated below. Aa, amino acids (**B**) Serial dilution growth test of various Spc7 phosphorylation site mutants compared to the wild-type (WT) strain. One of the plates contained the microtubule-depolymerizing drug benomyl, as indicated. Cells expressing *spc7*, in which three possible phosphorylation sites have been mutated (3A or 3D), showed an increased sensitivity to benomyl. (**C**) Serial dilution growth test of the indicated strains. Nuf2 is one subunit of the outer kinetochore Ndc80 complex. Cells expressing both the temperature-sensitive *nuf2-2* mutant (58) and *spc7-3A* or *-3D* exhibited a synthetic growth defect. (**D**) Schematic diagram of the origin recognition complex subunit Orc1 with phosphorylation sites identified in vivo (table S1) indicated above. Full-length Orc1 was tested for phosphorylation by Ark1 in vitro and the result from this assay (table S5) is indicated below. (**E**) Tetrads resulting from crossing a *chk1*Δ ("Δ") strain with an *orc1*+ ("1"), *orc1-S6A S9A* ("A") or *orc1-S6D S9D* ("D") strain. The *chk1*Δ *orc1-S6A S9A* double mutants showed reduced colony size compared to the single mutants, indicating a synthetic growth defect. Tetrads framed by a black box were chosen for the serial dilution growth test, shown in (F). (**F**) Serial dilution growth test for the tetrads indicated by a black box in (E). The hydroxyurea (HU) sensitivity of *chk1*Δ is increased by the presence of the *orc1* phosphorylation site mutants.

Fig. 5. Aurora shows similar genetic interactions as identified substrates and is required for resistance to DNA damage.

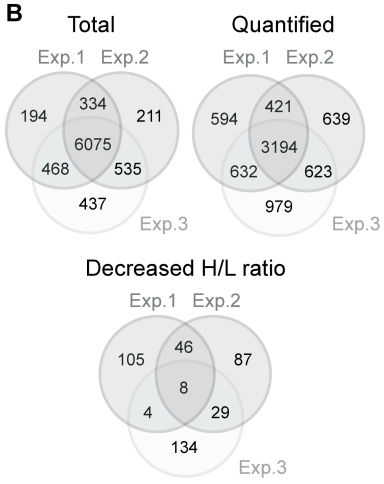
(**A**) Left side: Genetic interactions reported for some of the identified Aurora substrates (84). Genes required for resistance to DNA damage are shown in gray. Right side: Serial dilution growth test of the indicated yeast strains compared to the WT strain. One of the plates contained campthothecin (CPT), as indicated, to assay for sensitivity to DNA damage. (**B**) Serial dilution growth test of the indicated yeast strains. The degree of sensitivity of the *ark1* mutant strains to the DNA-damaging agents CPT and HU correlated with the severity of the temperature-sensitive allele (*ark1-T8* is the weakest allele). Ctf18, an alternative replication factor C subunit, is one of the newly identified Aurora substrates. Deletion of the *ctf18+* gene causes CPT and mild HU sensitivity (89). (**C**) Model showing well-characterized functions of Aurora on the left side and potential functions suggested by the range of identified substrates on the right side. The preferentially centromeric localization of Aurora during early mitosis is depicted by the red oval, and phosphorylation of kinetochore proteins, chromatin-associated proteins, and other nuclear proteins is symbolized by the small circles labeled with “P”.

Figure 1

A



B



C

	Exp.1	Exp.2	Exp.3	Local.	# P-events	# P-events (proteins)
Class 1	↓	↓	→	+	45	45 (24)
Class 2	↓	n.d.	→	+	15	25 (23)
	n.d.	↓	→	+	10	
Class 3	↓	→	→	+	42	89 (65)
	→	↓	→	+	45	
	↓	↑	→	+	1	
	↑	↓	→	+	1	
Class 4	↓	↓	n.d.	+	1	42 (31)
	↓	n.d.	n.d.	+	16	
	n.d.	↓	n.d.	+	9	
	↓	→	n.d.	+	11	
	→	↓	n.d.	+	3	
	↓	↑	n.d.	+	2	
Class 5	↓	n.d.	→	-	3	19 (17)
	n.d.	↓	→	-	1	
	↓	n.d.	n.d.	-	5	
	n.d.	↓	n.d.	-	10	

n.d. = not detected

Figure 2

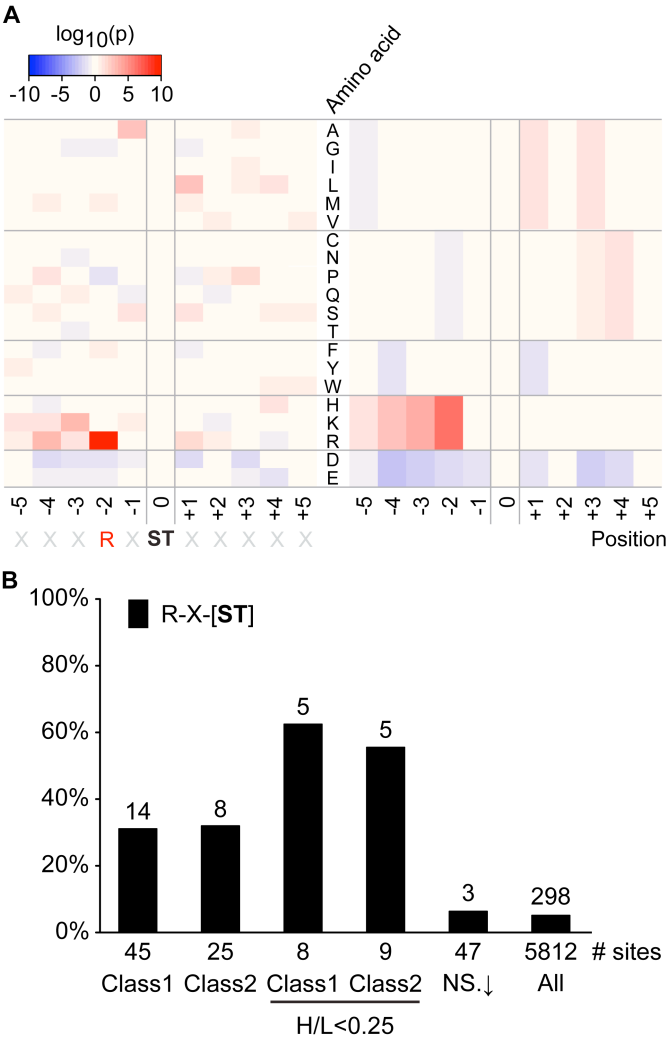

A
log10(p)
-10 -5 0 5 10
Amino acid
A G I L M V C N P Q S T F Y W H K R D E
-5 -4 -3 -2 -1 0 +1 +2 +3 +4 +5
X X X R X ST X X X X X
Position
B
100%
80%
60%
40%
20%
0%
R-X-[ST]
14
8
5
5
3
298
45 25 8 9 47 5812 # sites
Class1 Class2 Class1 Class2 NS.↓ All
H/L<0.25

Figure 3

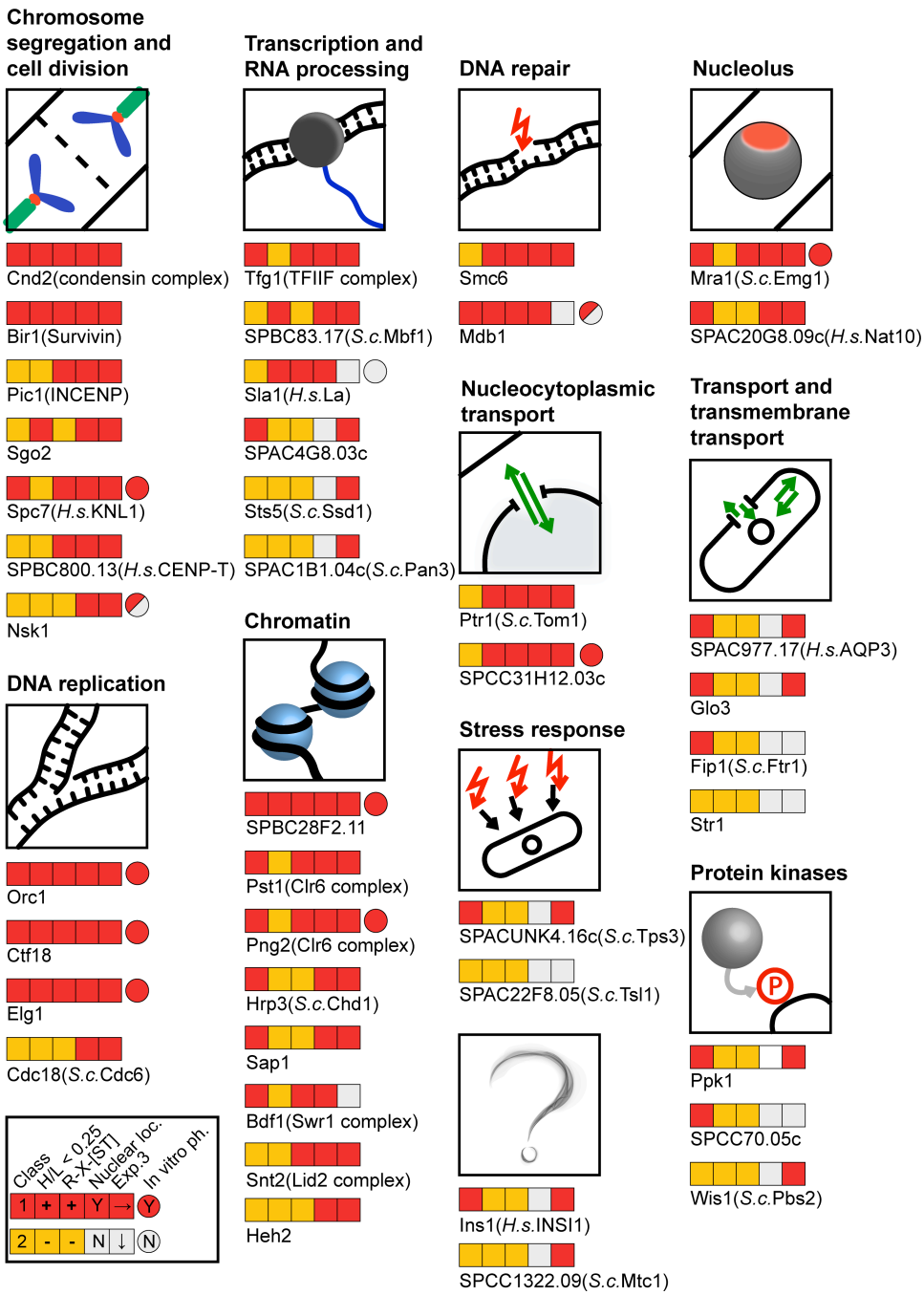

Chromosome segregation and cell division
Cnd2(condensin complex)
Bir1(Survivin)
Pic1(INCENP)
Sgo2
Spc7(H.s.KNL1)
SPBC800.13(H.s.CENP-T)
Nsk1
DNA replication
Orc1
Ctf18
Elg1
Cdc18(S.c.Cdc6)
Class
H/L < 0.25
R-X-[ST]
Nuclear loc.
Exp.3
In vitro ph.
1 + + Y → Y
2 - - N ↓ N
Transcription and RNA processing
Tfg1(TFIIF complex)
SPBC83.17(S.c.Mbf1)
Sla1(H.s.La)
SPAC4G8.03c
Sts5(S.c.Ssd1)
SPAC1B1.04c(S.c.Pan3)
Chromatin
SPBC28F2.11
Pst1(Clr6 complex)
Png2(Clr6 complex)
Hrp3(S.c.Chd1)
Sap1
Bdf1(Swr1 complex)
Snt2(Lid2 complex)
Heh2
DNA repair
Smc6
Mdb1
Nucleocytoplasmic transport
Ptr1(S.c.Tom1)
SPCC31H12.03c
Stress response
SPACUNK4.16c(S.c.Tps3)
SPAC22F8.05(S.c.Tsl1)
Ins1(H.s.INSI1)
SPCC1322.09(S.c.Mtc1)
Nucleolus
Mra1(S.c.Emg1)
SPAC20G8.09c(H.s.Nat10)
Transport and transmembrane transport
SPAC977.17(H.s.AQP3)
Glo3
Fip1(S.c.Ftr1)
Str1
Protein kinases
P
Ppk1
SPCC70.05c
Wis1(S.c.Pbs2)

Figure 4

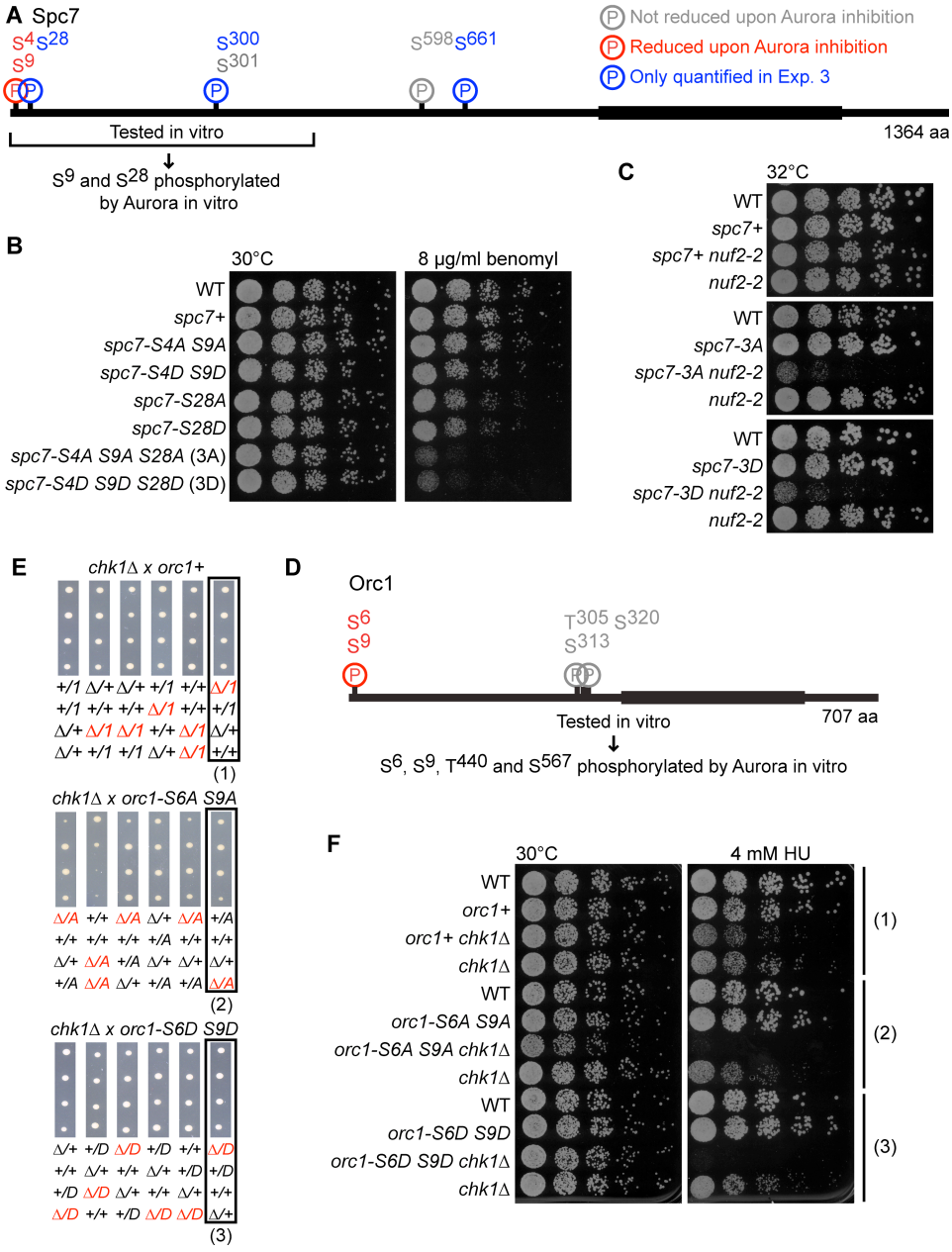
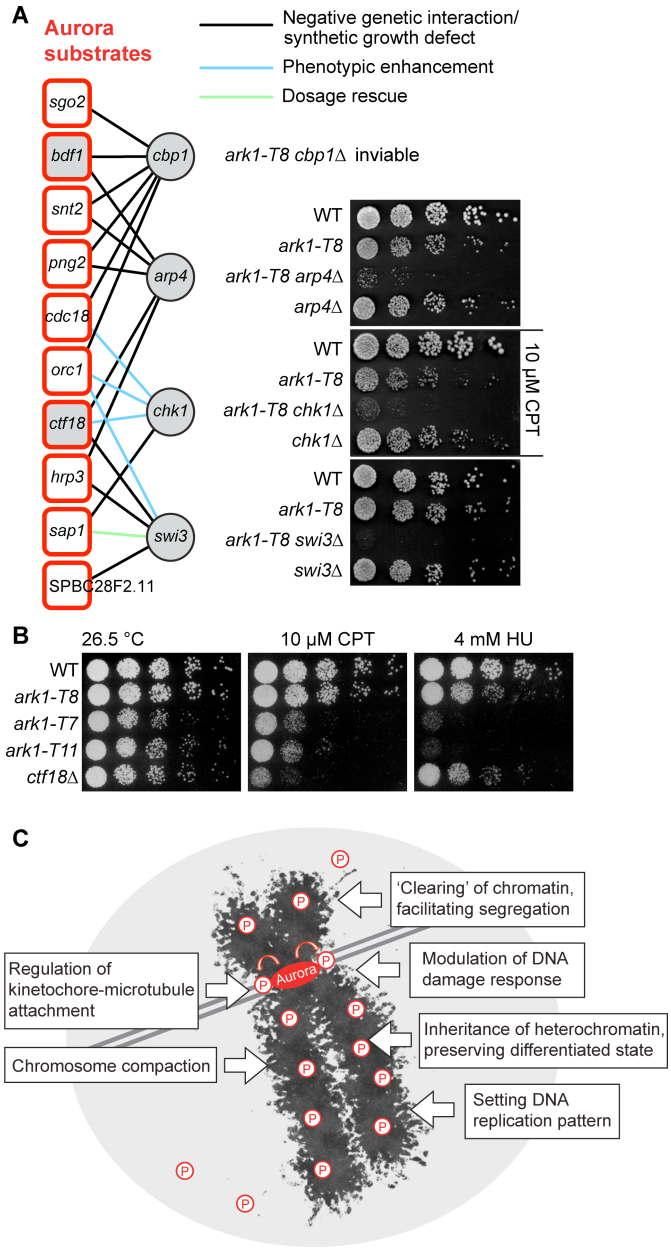

A Spc7
S4 S28
S9
S300
S301
S598 S661
Not reduced upon Aurora inhibition
Reduced upon Aurora inhibition
Only quantified in Exp. 3
Tested in vitro
1364 aa
S9 and S28 phosphorylated by Aurora in vitro
B
30°C
8 µg/ml benomyl
WT
spc7+
spc7-S4A S9A
spc7-S4D S9D
spc7-S28A
spc7-S28D
spc7-S4A S9A S28A (3A)
spc7-S4D S9D S28D (3D)
C
32°C
WT
spc7+
spc7+ nuf2-2
nuf2-2
WT
spc7-3A
spc7-3A nuf2-2
nuf2-2
WT
spc7-3D
spc7-3D nuf2-2
nuf2-2
D
Orc1
S6
S9
T305 S320
S313
Tested in vitro
707 aa
S6, S9, T440 and S567 phosphorylated by Aurora in vitro
E
chk1Δ x orc1+
(1)
chk1Δ x orc1-S6A S9A
(2)
chk1Δ x orc1-S6D S9D
(3)
F
30°C
4 mM HU
WT
orc1+
orc1+ chk1Δ
chk1Δ
WT
orc1-S6A S9A
orc1-S6A S9A chk1Δ
chk1Δ
WT
orc1-S6D S9D
orc1-S6D S9D chk1Δ
chk1Δ
(1)
(2)
(3)

Figure 5


A
Aurora substrates
Negative genetic interaction/ synthetic growth defect
Phenotypic enhancement
Dosage rescue
sgo2
bdf1
snt2
png2
cdc18
orc1
ctf18
hrp3
sap1
SPBC28F2.11
cbp1
arp4
chk1
swi3
ark1-T8 cbp1Δ inviable
WT
ark1-T8
ark1-T8 arp4Δ
arp4Δ
WT
ark1-T8
ark1-T8 chk1Δ
chk1Δ
10 μM CPT
WT
ark1-T8
ark1-T8 swi3Δ
swi3Δ
B
26.5 °C
10 μM CPT
4 mM HU
WT
ark1-T8
ark1-T7
ark1-T11
ctf18Δ
C
'Clearing' of chromatin, facilitating segregation
Modulation of DNA damage response
Inheritance of heterochromatin, preserving differentiated state
Setting DNA replication pattern
Regulation of kinetochore-microtubule attachment
Chromosome compaction
Aurora
P

Supplementary Materials for

Mitotic Substrates of the Kinase Aurora with Roles in Chromatin Regulation Identified Through Quantitative Phosphoproteomics of Fission Yeast

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Published 28 June 2011, Sci. Signal. 4 , rs6 (2011)
DOI: 10.1126/scisignal.2001588

This file includes:

Other Supplementary Material for this manuscript includes the following:
(available at www.sciencesignaling.org/cgi/content/full/4/179/rs6/DC1)

Table S1 (Microsoft Excel format). Summary of MS data on all identified phosphorylation events.

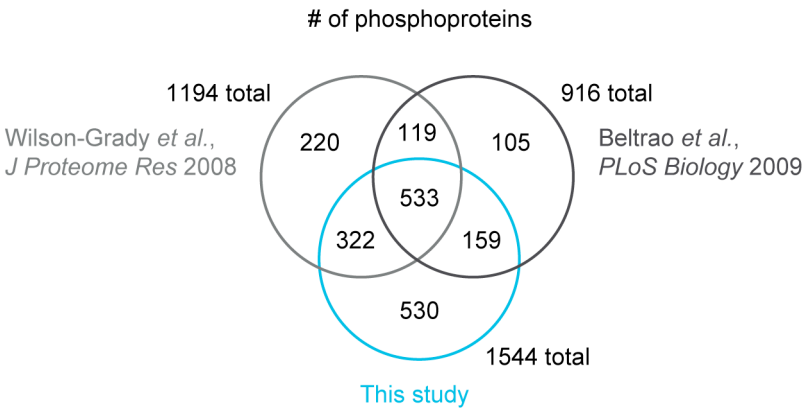


Fig. S1. Comparison of phosphorylated proteins identified in this and two other large-scale studies of fission yeast (1, 2). The Venn diagram shows the overlap among the three studies.

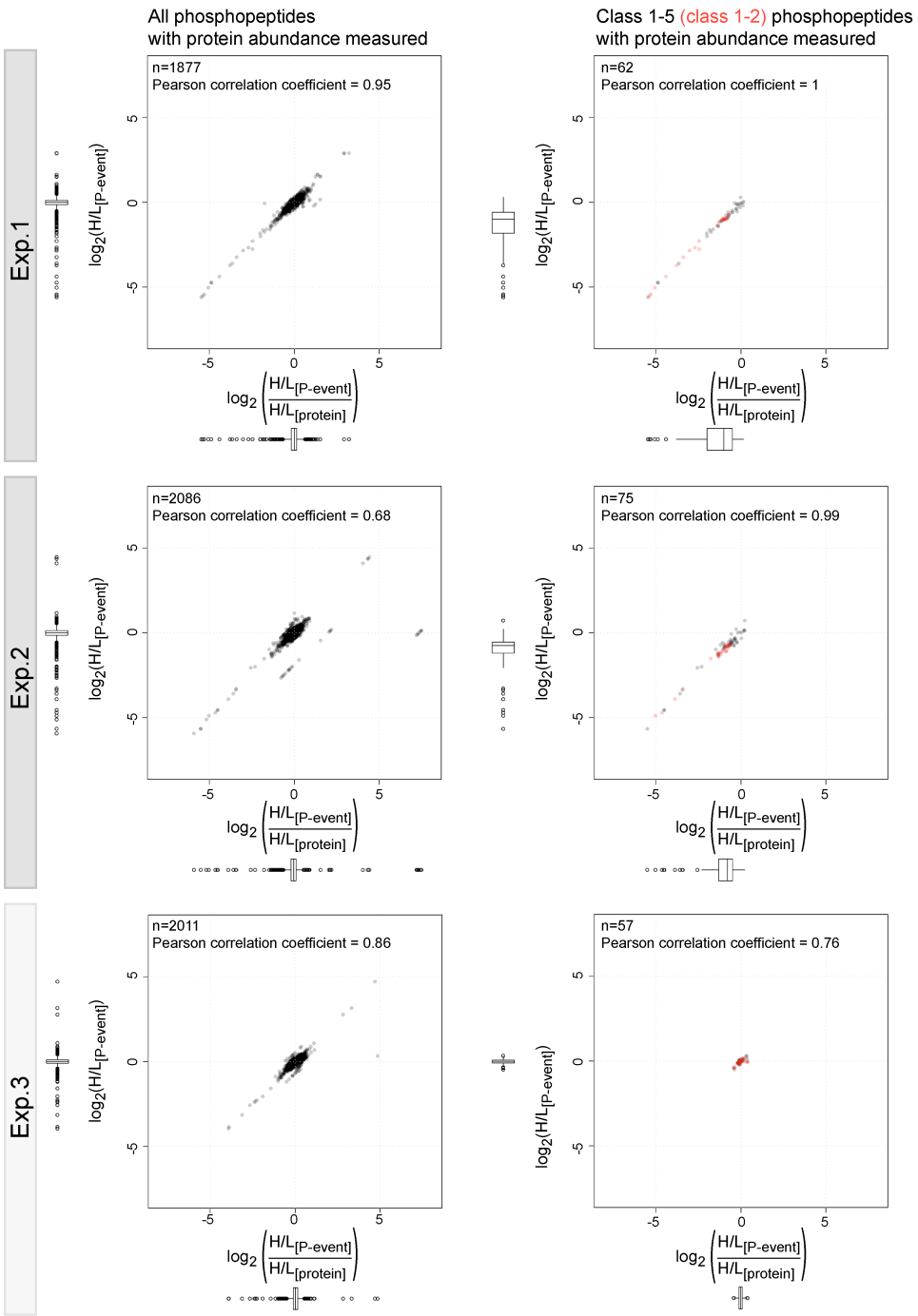
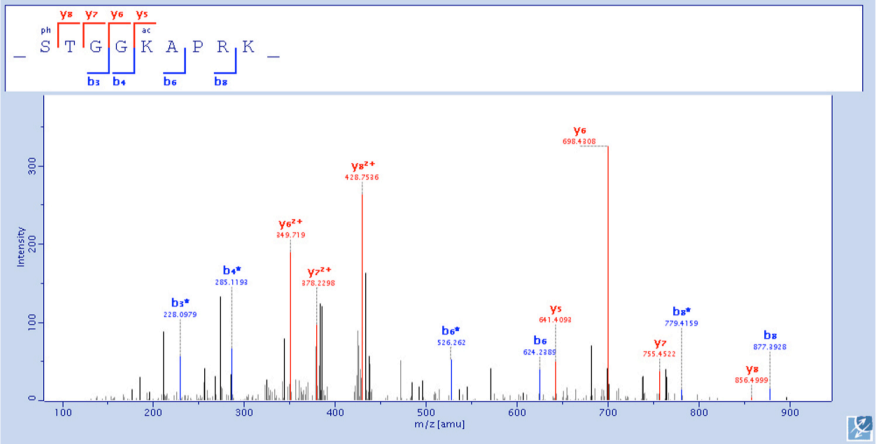


Fig. S2. Comparison of phosphopeptide ratios before and after normalization for protein abundance. The ratios of phosphopeptide abundance between 'heavy' (H) and 'light' (L) sample were compared before (*y* axis) and after normalization (*x* axis) for changes in protein abundance. Panels on the left side show all phosphorylation events for which quantitative protein information was available. Panels on the right side show such phosphorylation events from class 1 - 5 with class 1 and 2 phosphorylation sites marked in red. We excluded five phosphorylation events (all from one protein) that would have been assigned to class 3 - 5, because their apparent reduction in phosphorylation in Exp. 2 was due to a change in protein abundance. For the remaining class 1 - 5 phosphorylation events shown on the right side, the observed changes in phosphopeptide abundance between H and L sample were not due to changes in protein abundance.

Exp.1

Protein IDs: SPAC1834.04;SPBC1105.11c;SPBC8D2.04
Scannumber: 1477
SILAC State: Light
Source: 20090918_StPe_TL_AK_SN_SCX4
Method: CID; ITMS



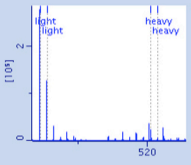
parent information

Mass:	1022.491
m/z:	512.25
Charge:	2+
Mass Error [ppm]:	-0.26338
PTM Score:	91.9
PEP:	0.0049608
Mascot Score:	22.33
Intensity:	1420808.1
Intensity L:	1320965.8
Intensity H:	99842.4

general information

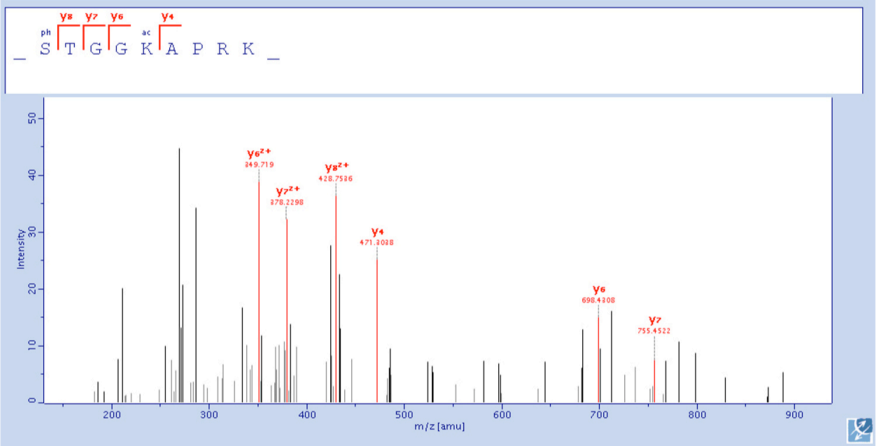
Annotation:	6 of 9 => 67 %
Intensity Coverage:	45 %
Protein Localisation:	11 ... 19

B-Ion					Y-Ion		Y-Ion²⁺	
Δ Da	mass		seq		mass	Δ Da	mass	Δ Da
NaN	168.0056	1	S	9	NaN	NaN	NaN	NaN
NaN	269.0533	2	T	8	856.5	0.16237	428.754	0.10681
0.02858	228.098	3	G	7	755.452	0.02262	378.23	0.13498
0.16465	285.119	4	G	6	698.431	0.17774	349.719	0.11569
NaN	553.2018	5	K	5	641.409	0.09196	641.4093	NaN
0.0264	526.262	6	A	4	471.3038	NaN	471.3038	NaN
NaN	721.2916	7	P	3	400.2667	NaN	400.2667	NaN
0.04538	779.416	8	R	2	303.2139	NaN	303.2139	NaN
NaN	NaN	9	K	1	147.1128	NaN	147.1128	NaN



Exp.2

Protein IDs: SPAC1834.04;SPBC1105.11c;SPBC8D2.04
Scannumber: 1948
SILAC State: Light
Source: 20100107_BoMa_SA_Spombe_MBC-Pellet-Ph_05
Method: CID; ITMS



parent information

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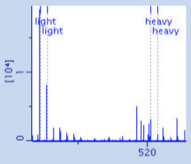
m/z:	512.25
Charge:	2+
Mass Error [ppm]:	-0.063049
PTM Score:	31.935
PEP:	0.71373
Mascot Score:	5.89
Intensity:	84877
Intensity L:	73549.6
Intensity H:	11327.3

general information

Annotation:	4 of 9 => 44 %
Intensity Coverage:	27 %
Protein Localisation:	11 ... 19

B-Ion					Y-Ion		Y-Ion²⁺	
Δ Da	mass		seq		mass	Δ Da	mass	Δ Da
NaN	168.0056	1	S	9	NaN	NaN	NaN	NaN
NaN	269.0533	2	T	8	856.4999	NaN	428.754	0.47412
NaN	326.0748	3	G	7	755.452	0.08858	378.23	0.16581
NaN	383.0962	4	G	6	698.431	0.0777	349.719	0.09164
NaN	553.2018	5	K	5	641.4093	NaN	641.4093	NaN
NaN	624.2389	6	A	4	471.304	0.14488	471.3038	NaN
NaN	721.2916	7	P	3	400.2667	NaN	400.2667	NaN
NaN	877.3928	8	R	2	303.2139	NaN	303.2139	NaN
NaN	NaN	9	K	1	147.1128	NaN	147.1128	NaN



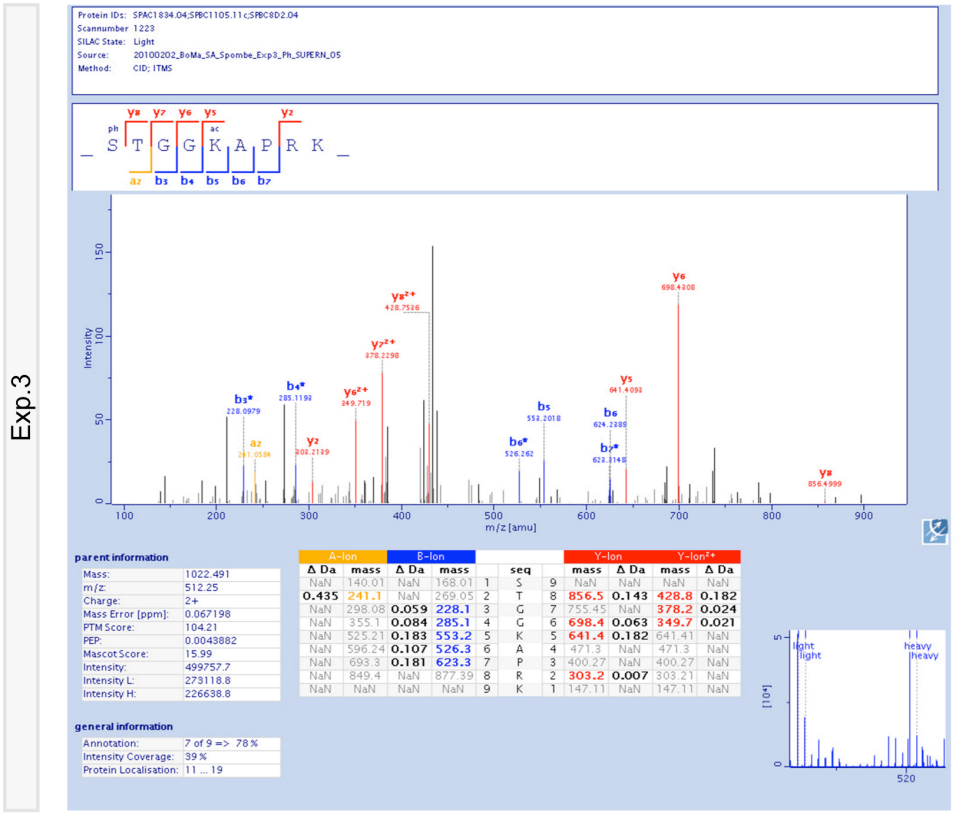


Fig. S3. Fragmentation mass spectra of the histone H3 peptide carrying the known Aurora target site (Ser^{10}). Shown are the fragmentation mass spectra of the histone H3-S^{10}ph K^{14}ac peptide in Exp. 1, Exp. 2, and Exp. 3. A survey scan insert showing the corresponding SILAC pair is shown in the lower right corner of each panel. Note that strong reduction of this modified peptide upon inhibitor treatment was found in Exps. 1 and 2, but not in Exp. 3.

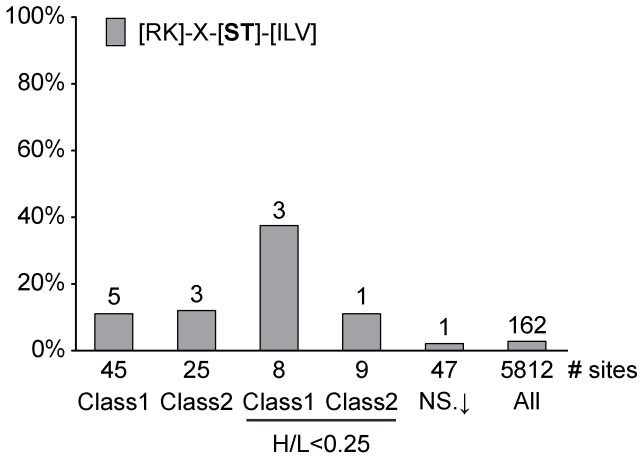


Fig. S4. Consensus motif analysis of phosphorylation sites that depend on the kinase activity of fission yeast Aurora. The percentage and number of sites matching the previously identified [RK]-X-[ST]-[ILV] consensus (3) among all sites in a certain category. NS.↓, phosphorylation sites reduced due to nonspecific effects of the inhibitor. See table S1 for complete data. See Fig. 2 for comparison to the number of sites that match the R-X-[ST] consensus.

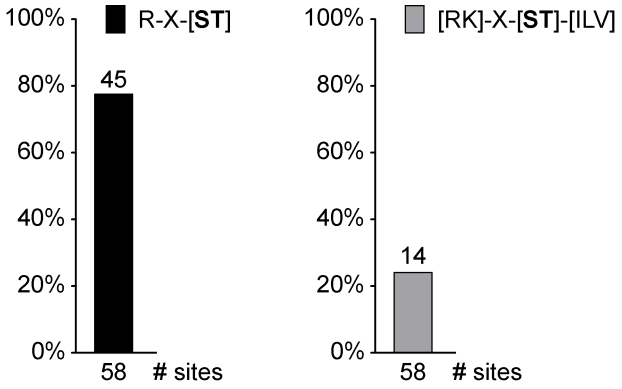


Fig. S5. Consensus motif analysis of previously identified yeast Aurora or metazoan Aurora B phosphorylation sites. Only sites that were phosphorylated by yeast Aurora or Aurora B in vitro and for which there was some evidence for their presence in vivo were considered (see table S3). Typically, immunoblotting or staining with a phosphorylation site-specific antibody or identification by mass spectrometry was such evidence. The total number of considered sites (# sites) and the percentage and number of sites matching the indicated consensus are given. A lack of preference for a hydrophobic amino acid at position +1 has previously also been noted after analyzing a much smaller number of Aurora B phosphorylation sites (4).

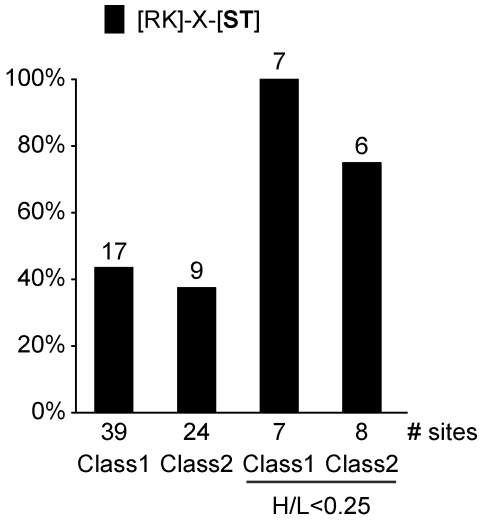


Fig. S6. Reanalysis of the frequency of motif occurrence by including the K-X-[ST] and excluding the doubly phosphorylated sites. Percentage and number of sites matching the indicated consensus among all sites in a certain category. The following phosphorylation sites were excluded: Id 2826, 3842, 3843, 4378, 6068, 6256, and 9099 (table S1).

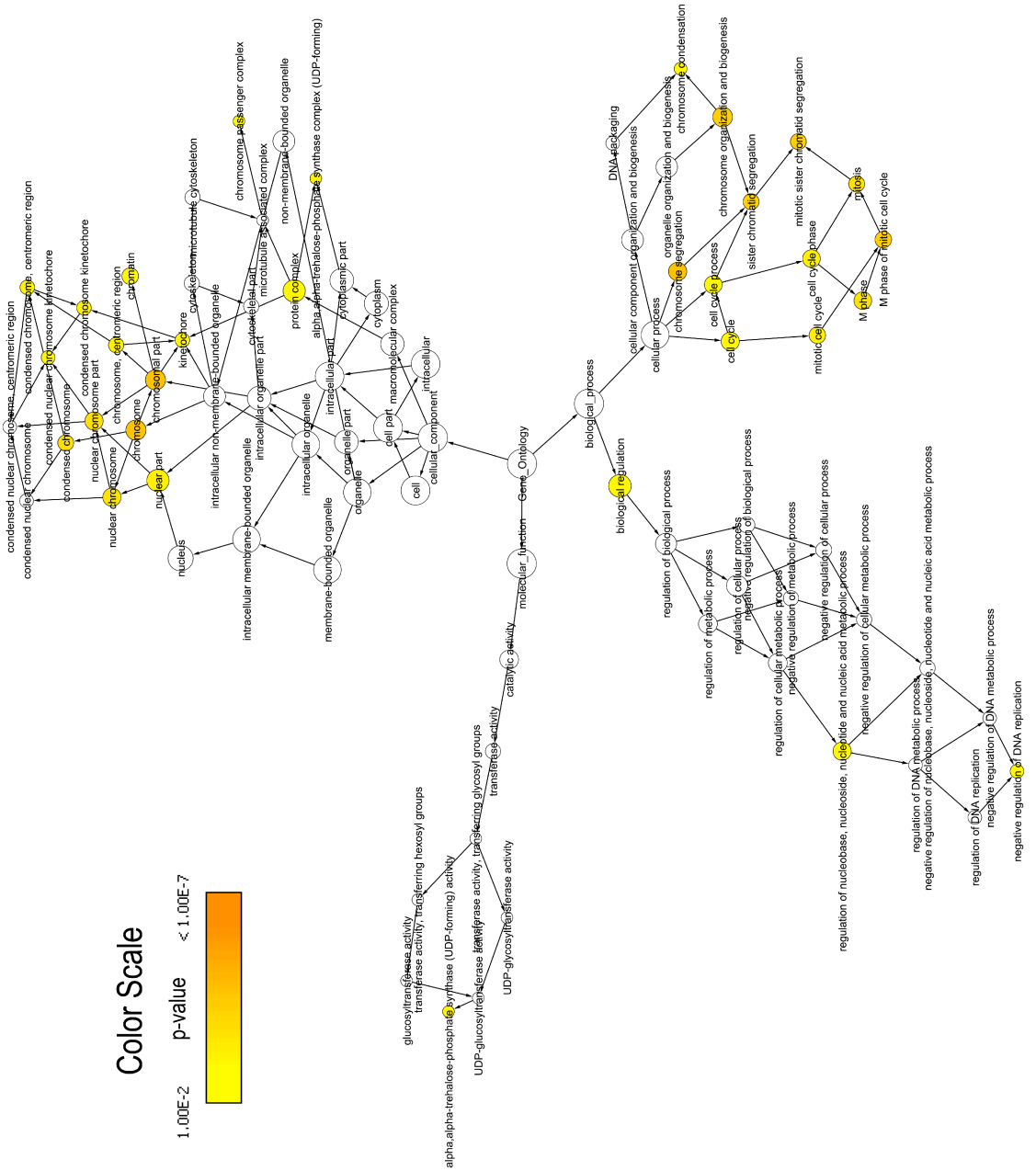


Fig. S7. GO term enrichment of proteins with class 1 and 2 phosphorylation sites. Significantly enriched GO terms are shown in a color range from yellow (p = 0.01) to orange (p = 1e-7).

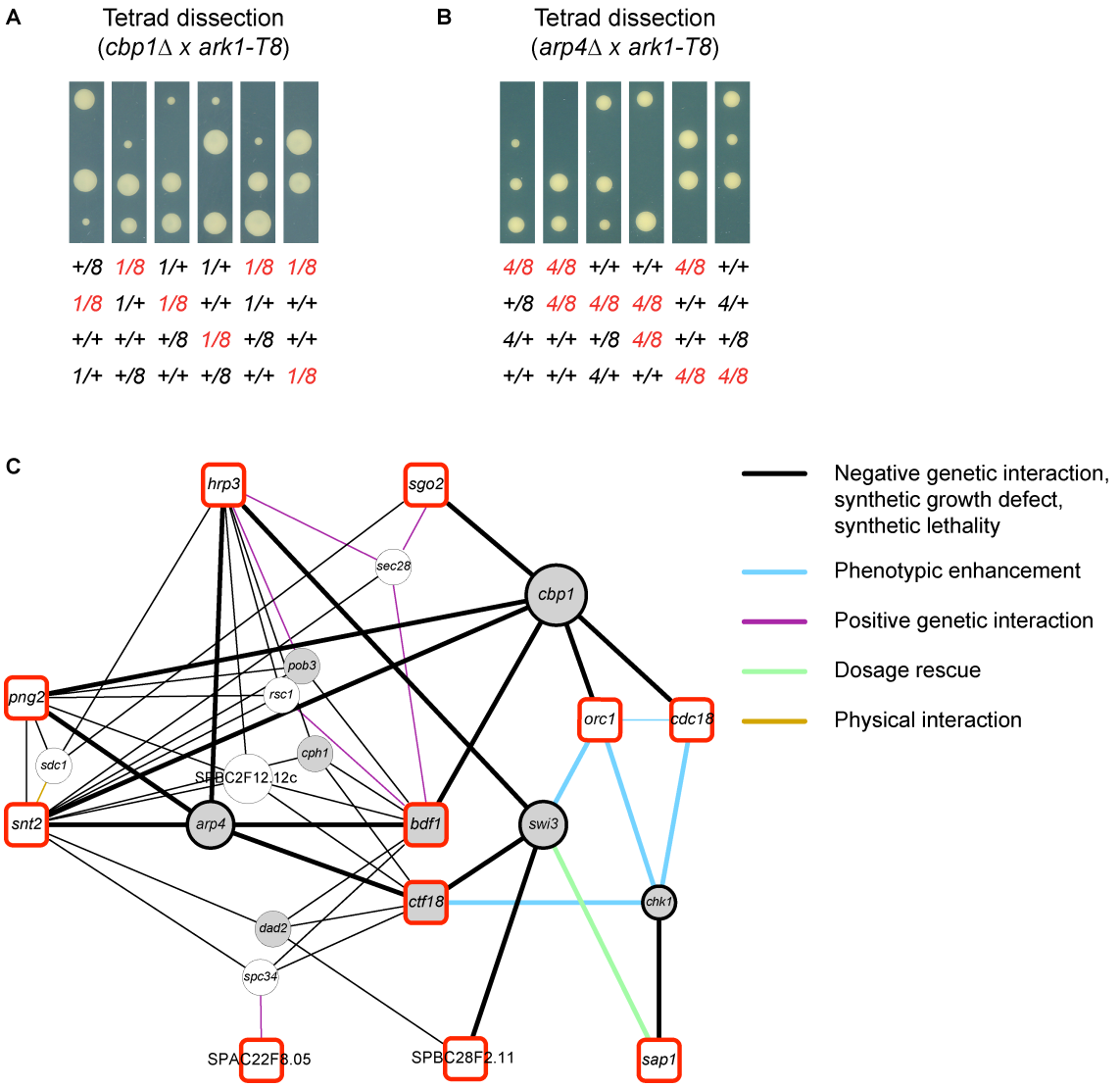


Fig. S8. Genetic interaction tests of *ark1* temperature-sensitive mutants and previously identified genetic interactions of fission yeast Aurora substrates. (**A**, **B**) Tetrads resulting from crossing a *cbp1*Δ and *ark1-T8* strain (panel A) or from crossing an *arp4*Δ and *ark1-T8* strain (panel B) were dissected on rich medium at 26.5°C. About one third (panel A) or half of the tetrads (panel B) were interpretable and examples of interpretable tetrads are shown. The genotype of the colonies as judged by their antibiotic resistance phenotype is indicated below. In panel A, '1' stands for *cbp1*Δ and '8' stands for *ark1-T8*. In panel B, '4' stands for *arp4*Δ and '8' stands for *ark1-T8*. (**C**) Shown are direct physical or genetic interactors (black frame) of identified Aurora substrates (bold red frame) (5). Only interactors connected to at least four different Aurora substrates are shown and the size of the node correlates to the number of substrates it interacts with. Genes that are known to be required for resistance towards DNA damaging insults are marked in gray. Genetic interactions that have been also depicted in Figure 5 are marked by thicker lines.

Table S1. Summary of MS data on all identified phosphorylation events. File is in Excel format and named 2001588_TableS1.xls. Columns represent the following:

Id: Unique identifier for each phosphorylation site

Position: Position of phosphorylation site in the protein sequence

Protein.Descriptions: Systematic ID, Primary name, Synonyms, and Protein product from the Sanger *S. pombe* GeneDB database (http://old.genedb.org/genedb/pombe/)

Pfam: Protein family identifier from http://www.sanger.ac.uk/resources/databases/pfam.html

Pfam.Names: Protein family names

Pfam.Type: Protein family type

GOCC: Gene ontology cellular component identifiers

GOCC.Names: Gene ontology cellular component names

GOMF: Gene ontology molecular function identifiers

GOMF.Names: Gene ontology molecular function names

GOBP: Gene ontology biological process

GOBP.Names: Gene ontology biological process names

Localization.Prob: Localization probability of the phosphorylation site

Number.of.Phospho..STY: Number of phosphorylations in the peptide

Amino.Acid: Phosphorylated residue

Sequence.Window: Sequence window around the phosphorylation site

Motifs: Matching kinase motifs

Best.Motif: Best matching kinase motif

PEP: Posterior error probability score

Mascot.Score: Mascot score of the identified peptide (not used for filtering the false positive thresholds)

PTM.Score: Posttranslational modification score

Modified.Sequence: Peptide sequence with modifications

Position.in.peptide: Position of phosphorylation site in the peptide sequence

Charge: Charge state of the peptide

m.z: Mass to charge ratio of the peptide

Mass.Error..ppm: Difference between measured and calculated mass in parts per million

Ratio.H.L.Normalized.Exp1: Normalized ratio between heavy (H) and light (L) phosphopeptide in Exp 1. The median of the total ratio population was shifted to 1.

Significance.B..Exp1.BH: Corrected (Benjamini & Hochberg) significance value of the corresponding ratio; Exp. 1

Ratio.H.L.Normalized.Exp2: Normalized ratio between heavy (H) and light (L) phosphopeptide in Exp. 2. The median of the total ratio population was shifted to 1.

Significance.B..Exp2.BH: Corrected (Benjamini & Hochberg) significance value of the corresponding ratio; Exp. 2

Ratio.H.L.Normalized.Exp3: Normalized ratio between heavy (H) and light (L) phosphopeptide in Exp. 3. The median of the total ratio population was shifted to 1.

Significance.B..Exp3.BH: Corrected (Benjamini & Hochberg) significance value of the corresponding ratio; Exp. 3

Exp1.BH: Direction of significant change (Exp. 1); -1, decreased H/L ratio; 0, no signifcant change; 1, increased H/L ratio; -, not quantified

Exp2.BH: Direction of significant change (Exp. 2); -1, decreased H/L ratio; 0, no signifcant change; 1, increased H/L ratio; -, not quantified

Exp3.BH: Direction of significant change (Exp. 3); -1, decreased H/L ratio; 0, no signifcant change; 1, increased H/L ratio; -, not quantified

Class.down.BH: Classification of sites with significantly decreased H/L ratio in Exp. 1 or 2 (Fig. 1C)

Nonspecific.Effect: +, sites with significantly decreased H/L ratio in Exp. 3 and with significantly decreased or undetermined H/L ratio in Exp. 1 and 2 (Fig. 2B)

[RK]x[ST][ILV].Consensus: 1, sites matching [RK]-X-[ST]-[ILV]; 0, no match (fig. S4)

Rx[ST].Consensus: 1, sites matching R-X-[ST]; 0, no match (Fig. 2B; fig. S6)

Kx[ST].Consensus: 1, sites matching K-X-[ST]; 0, no match (fig. S6)

Table S2. Number of phosphorylation sites within structured or unstructured regions. The number of phosphorylation sites within domains contained in the Pfam database (6), or in disordered regions predicted by DisEMBL v 1.4 (7) relative to all sites within a certain subset was determined.

		Sites within Pfam domains		**Sites within disordered regions according to DisEMBL**	
	Number of sites in this class	Number	% of sites in this class	Number	% of sites in this class
All localized sites	5812	182	3.13	4042	69.55
Class 1 – 4 sites	201	3	1.49	142	70.65
Class 1 – 2 sites	70	0	0.00	55	78.57*

*Analysis of the data by Holt *et al.* (8) using the DisEMBL algorithm finds 75.69% of the Cdk1-dependent phosphorylation sites (414 out of 547) to be in disordered regions.

Table S3. List of previously known yeast Aurora or metazoan Aurora B phosphorylation sites. Only sites that were phosphorylated by yeast Aurora or metazoan Aurora B in vitro and for which there was some evidence for their presence in vivo were considered. Typically, immunoblotting or staining with a phosphorylation site-specific antibody or identification by mass spectrometry was such evidence. Alternative names for a given protein are separated by slashes. There occasionally was disagreement between publications on the amino acid number of a certain phosphorylated residue because of different isoforms or because proteins from different species were used for different assays. Hence, the number listed under site may not match the number mentioned in all the relevant publications. In some cases, the discrepant amino acid numbers are given, separated by a slash. Some of the information was assembled with the help of the following databases:

Phosida (http://141.61.102.18/phosida/index.aspx)

PhosphoELM (http://phospho.elm.eu.org/)

PhosphoSitePlus (www.phosphosite.org)

PhosphoPep (http://www.phosphopep.org/index.php)

C.g, Cricetulus griseus; C.e., Caenhorhabditis elegans; H.s., Homo sapiens; R.n., Rattus norvegicus; S.c., Saccharomyces cerevisiae; X.l. Xenopus, laevis;

The literature on Aurora substrates is vast and we apologize should we have involuntarily omitted other published evidence.

Amino acid sequence	R-X-[ST]	K-X-[ST]	[RK]-X-[ST]-[ILV]	Species	Protein	Site	**References**
SRKRKISLLLQQQ	0	1	1	*S.c.*	Ask1	S200	(3)
PSLRRKTMCGTLD	1	0	0	*H.s.*	Aurora B	T232	(9, 10)
TKPRTVSNPTTAK	0	0	0	*S.c.*	Bim1	S139	(11)
TTAKRSSSTGTGS	1	0	0	*S.c.*	Bim1	S148	(11)
TAKRSSSTGTGSA	0	0	0	*S.c.*	Bim1	S149	(11)
GLATRHSSLGING	1	0	0	*S.c.*	Bim1	S165	(11)
LATRHSSLGINGS	0	0	0	*S.c.*	Bim1	S166	(11)
NGSRKTSVTQGQL	0	1	1	*S.c.*	Bim1	S176	(11)
GKGKRSSRANTVT	1	0	0	*H.s.*	Borealin	S165	(10, 12)
PVRRRHSSILKPP	1	0	0	*H.s.*	CASC5/KNL1/Blinkin	S24	(13)
KNSRRVSFADTIK	1	0	0	*H.s.*	CASC5/KNL1/Blinkin	S60	(13, 14)
MGPRRRSRKPEAP	1	0	0	*H.s.*	CENP-A	S7	(15, 16)
KRKRRVTWCLGKI	1	0	0	*H.s., X.l.*	CENP-E	T422	(17)
LTPVRRSRRLQEK	1	0	0	*H.s.*	CKAP2/TMAP	S627	(18)
ATEYRLSIGSAPT	1	0	1	*S.c.*	Dam1	S20	(3, 19, 20)

Amino acid sequence	R-X-[ST]	K-X-[ST]	[RK]-X-[ST]-[ILV]	Species	Protein	Site	**References**
SKLRRKSILHTIR	1	0	1	*S.c.*	Dam1	S257	(3, 20)
LHTIRNSIASGAD	1	0	1	*S.c.*	Dam1	S265	(3, 20)
SSSQRVSSYRRTF	1	0	0	*H.s.*	desmin	S11	(21)
VSSYRRTFGGAPG	1	0	0	*H.s.*	desmin	T16	(21)
YQVSRTSGGAGGL	1	0	0	*H.s.*	desmin	S59	(21)
DHQLESSLSPVEV	0	0	0	*H.s.*	DSN1/Mis13/KNL-3	S28	(13)
ERLQSKSLHLSPQ	0	0	0	*H.s.*	DSN1/Mis13/KNL-3	S76	(13)
ETNRRKSLHPIHQ	1	0	1	*H.s.*	DSN1/Mis13/KNL-3	S109	(13, 22)
QTSGRLSNVAPPC	1	0	0	*H.s.*	EB3/MAPRE3	S176	(23)
MERRRITSAARRS	1	0	0	*H.s.*	GFAP	T7	(21, 24)
TSAARRSYVSSGE	1	0	0	*H.s.*	GFAP	S13	(21, 24)
GPGTRLSLARMPP	1	0	1	*H.s.*	GFAP	S38	(21, 24)
TSERKVSLFGKRT	0	1	1	*H.s.*	Hec1	S55	(14, 25, 26)
LFGKRTSGHGSRN	1	0	0	*H.s.*	Hec1	S62	(14, 26)
GHGSRNSQLGIFS	1	0	0	*H.s.*	Hec1	S69	(25-27)
KQTARKSTGGKAP	1	0	0	*several*	histone H3	S10	(28-31)
TKVARKSAPATGG	1	0	0	*H.s.*	histone H3	S28	(10, 32, 33)
RYHKRTSSAVWNS	1	0	0	*H.s.*	INCENP	S893/S898	(10, 34)
YHKRTSSAVWNSP	0	0	0	*H.s.*	INCENP	S894/S899	(10, 34)
PENRRKTVCGTID	1	0	1	*S.c.*	Ipl1	T260	(3)
RLRHRRSRSAGDR	1	0	0	*H.s.*	KIF23/MKLP1/CHO1	S708	(35, 36)
GSRKRRSSTVAPA	1	0	0	*H.s.*	KIF23/MKLP1/CHO1	S911	(14, 37, 38)
RLQTRISEIVEES	1	0	0	*X.l.*	KIF2C/MCAK	S177	(39, 40)
NQGRRKSNIVKEM	1	0	0	*X.l.*	KIF2C/MCAK	S196	(39-41)
QKQKRRSVNSKIP	1	0	1	*H.s., C.g.*	KIF2C/MCAK	S92/S95	(42)
QNHKRKTISKIPA	1	0	1	*X.l.*	KIF2C/MCAK	T95	(40, 43)
LESLRVSLTSNPV	1	0	1	*H.s.*	mDia3	S196	(44)
KRPQRATSNVFAM	1	0	0	*H.s.*	MRLC	T19	(27, 45)

Amino acid sequence	R-X-[ST]	K-X-[ST]	[RK]-X-[ST]-[ILV]	Species	Protein	Site	**References**
RPQRATSNVFAMF	0	0	0	*H.s., R.n.*	MRLC	S20	(27, 45)
RKILRKSPHLEKF	1	0	0	*H.s.*	NSUN2	S139	(46)
TGLYRISGCDRTV	1	0	0	*H.s.*	RACGAP1/MgcRacGAP	S387	(47)
SVRRRTSFYLPKD	1	0	0	*H.s.*	RASSF1A	S203	(48)
SKASRQTFVIHKL	1	0	0	*H.s.*	Sgo2	T537	(49)
NNQRRKTIFVEDF	1	0	1	*S.c.*	Spc34	T199	(3)
NKIAKETNNKKKE	0	1	0	*H.s.*	survivin	T117	(50, 51)
RELKRASNESASQ	1	0	0	*C.e.*	TLK-1	S634	(52)
GTASRPSSSRSYV	1	0	0	*H.s.*	vimentin	S24	(27, 53, 54)
TSTRTYSLGSALR	0	0	0	*H.s.*	vimentin	S38	(27, 53)
GSALRPSTSRSLY	1	0	0	*H.s.*	vimentin	S46	(27, 53, 55)
VYATRSSAVRLRS	1	0	0	*H.s.*	vimentin	S65	(27, 53)
AVRLRSSVPGVRL	1	0	1	*H.s.*	vimentin	S72	(27, 53, 54, 56, 57)
QDSVDFSLADAIN	0	0	0	*H.s.*	vimentin	S86	(27, 53)
PKYQRRSKSASRL	1	0	0	*C.e.*	ZEN-4	S680	(35)

Table S4. List of GO terms and their associated proteins from class 1 and 2. Data was created with BiNGO (58) as described in the Materials and Methods section. Columns represent the following:

GO-ID: Gene Ontology identifier

P value: *P* value derived from hypergeometric test

corr. *P* value: *P* value corrected by the method of Benjamini and Hochberg

x: number of proteins in class 1 and 2 annotated with the respective GO-ID

n: number of proteins in the fission yeast proteome annotated with the respective GO-ID

X: number of proteins in class 1 and 2

N: number of proteins in the fission yeast proteome

Description: description of the GO-ID

Proteins from class 1 and 2: identifiers of proteins in class 1 and 2 that are annotated with the respective GO-ID.

GO-ID	*P* value	corr. *P* value	x	n	X	N	Description	Proteins from class 1 and 2
44427	5.92E-08	2.22E-05	15	365	42	5230	chromosomal part	SPCC962.02C|SPCC1020.02|SPBC336.15|SPCC1672.02C|SPBC902.02C|SPCC5E4.06|SPCC306.03C|SPBC12C2.10C|SPBC947.11C|SPAC15A10.15|SPBC800.13|SPBC14C8.07C|SPAC3G6.01|SPAC3H1.12C|SPBC29A10.15
7059	1.00E-07	2.22E-05	11	180	42	5230	chromosome segregation	SPCC962.02C|SPBC947.11C|SPAC15A10.15|SPCC1020.02|SPBC336.15|SPCC1672.02C|SPBC800.13|SPBC902.02C|SPAC3G6.01|SPAC14C4.05C|SPCC306.03C
5694	1.09E-07	2.22E-05	15	382	42	5230	chromosome	SPCC962.02C|SPCC1020.02|SPBC336.15|SPCC1672.02C|SPBC902.02C|SPCC5E4.06|SPCC306.03C|SPBC12C2.10C|SPBC947.11C|SPAC15A10.15|SPBC800.13|SPBC14C8.07C|SPAC3G6.01|SPAC3H1.12C|SPBC29A10.15
16359	5.07E-07	7.66E-05	7	63	42	5230	mitotic sister chromatid segregation	SPCC962.02C|SPBC947.11C|SPAC15A10.15|SPBC336.15|SPAC3G6.01|SPAC14C4.05C|SPCC306.03C
87	7.35E-07	7.66E-05	8	97	42	5230	M phase of mitotic cell cycle	SPCC962.02C|SPBC947.11C|SPAC15A10.15|SPBC336.15|SPBC14C8.07C|SPAC3G6.01|SPAC14C4.05C|SPCC306.03C
7001	7.51E-07	7.66E-05	14	381	42	5230	chromosome organization and biogenesis	SPCC962.02C|SPBC336.15|SPCC1450.02|SPCC1672.02C|SPBC902.02C|SPAC14C4.05C|SPBC1709.11C|SPCC306.03C|SPBC12C2.10C|SPBC947.11C|SPAC15A10.15|SPBC800.13|SPAC3G6.01|SPAC3H1.12C
819	1.28E-06	1.12E-04	7	72	42	5230	sister chromatid segregation	SPCC962.02C|SPBC947.11C|SPAC15A10.15|SPBC336.15|SPAC3G6.01|SPAC14C4.05C|SPCC306.03C
793	6.77E-06	4.78E-04	7	92	42	5230	condensed chromosome	SPCC962.02C|SPAC15A10.15|SPCC1020.02|SPBC336.15|SPBC800.13|SPCC5E4.06|SPCC306.03C
44454	7.16E-06	4.78E-04	11	276	42	5230	nuclear chromosome part	SPCC962.02C|SPBC947.11C|SPAC15A10.15|SPCC1020.02|SPCC1672.02C|SPBC800.13|SPBC14C8.07C|SPBC902.02C|SPAC3G6.01|SPAC3H1.12C|SPBC29A10.15
7067	7.81E-06	4.78E-04	7	94	42	5230	mitosis	SPCC962.02C|SPBC947.11C|SPAC15A10.15|SPBC336.15|SPAC3G6.01|SPAC14C4.05C|SPCC306.03C
228	1.31E-05	7.30E-04	11	294	42	5230	nuclear chromosome	SPCC962.02C|SPBC947.11C|SPAC15A10.15|SPCC1020.02|SPCC1672.02C|SPBC800.13|SPBC14C8.07C|SPBC902.02C|SPAC3G6.01|SPAC3H1.12C|SPBC29A10.15
279	1.71E-05	8.74E-04	10	246	42	5230	M phase	SPCC962.02C|SPBC947.11C|SPAC15A10.15|SPBC336.15|SPCC1672.02C|SPBC14C8.07C|SPBC902.02C|SPAC3G6.01|SPAC14C4.05C|SPCC306.03C
278	6.47E-05	2.92E-03	8	177	42	5230	mitotic cell cycle	SPCC962.02C|SPBC947.11C|SPAC15A10.15|SPBC336.15|SPBC14C8.07C|SPAC3G6.01|SPAC14C4.05C|SPCC306.03C
22403	6.69E-05	2.92E-03	10	288	42	5230	cell cycle phase	SPCC962.02C|SPBC947.11C|SPAC15A10.15|SPBC336.15|SPCC1672.02C|SPBC14C8.07C|SPBC902.02C|SPAC3G6.01|SPAC14C4.05C|SPCC306.03C
775	8.42E-05	3.42E-03	6	93	42	5230	chromosome, centromeric region	SPCC962.02C|SPBC12C2.10C|SPAC15A10.15|SPCC1020.02|SPBC336.15|SPBC800.13
777	9.51E-05	3.42E-03	5	59	42	5230	condensed chromosome kinetochore	SPCC962.02C|SPAC15A10.15|SPCC1020.02|SPBC336.15|SPBC800.13

GO-ID	*P* value	corr. *P* value	x	n	X	N	Description	Proteins from class 1 and 2
44428	9.51E-05	3.42E-03	20	1093	42	5230	nuclear part	SPCC962.02C|SPCC1020.02|SPCC1450.02|SPCC1672.02C|SPBC902.02C|SPAC14C4.05C|SPCC1620.09C|SPBC1709.11C|SPBC12C2.10C|SPBC947.11C|SPAC15A10.15|SPAC3G9.01|SPAC18G6.07C|SPBC83.17|SPBC800.13|SPBC14C8.07C|SPAC3G6.01|SPAC3H1.12C|SPBC29A10.15|SPAC20G8.09C
5699	1.21E-04	4.10E-03	5	62	42	5230	kinetochore	SPCC962.02C|SPAC15A10.15|SPCC1020.02|SPBC336.15|SPBC800.13
779	1.75E-04	5.48E-03	5	67	42	5230	condensed chromosome, centromeric region	SPCC962.02C|SPAC15A10.15|SPCC1020.02|SPBC336.15|SPBC800.13
3825	1.88E-04	5.48E-03	2	3	42	5230	alpha,alpha-trehalose-phosphate synthase (UDP-forming) activity	SPACUNK4.16C|SPAC22F8.05
5946	1.88E-04	5.48E-03	2	3	42	5230	alpha,alpha-trehalose-phosphate synthase complex (UDP-forming)	SPACUNK4.16C|SPAC22F8.05
7049	2.28E-04	6.34E-03	11	401	42	5230	cell cycle	SPCC962.02C|SPBC947.11C|SPAC15A10.15|SPCC1020.02|SPBC336.15|SPCC1672.02C|SPBC14C8.07C|SPBC902.02C|SPAC3G6.01|SPAC14C4.05C|SPCC306.03C
65007	2.55E-04	6.79E-03	21	1269	42	5230	biological regulation	SPBC409.07C|SPCC962.02C|SPCC70.05C|SPCC1672.02C|SPBC28F2.11|SPCC306.05C|SPBC4F6.09|SPCC5E4.06|SPAC57A10.10C|SPAC22E12.17C|SPCC1620.09C|SPCC16C4.09|SPBC12C2.10C|SPBC947.11C|SPAC1F7.07C|SPBC83.17|SPBC14C8.07C|SPAC3G6.01|SPAC110.01|SPAC3H1.12C|SPBC29A10.15
5717	2.69E-04	6.85E-03	8	217	42	5230	chromatin	SPBC12C2.10C|SPBC947.11C|SPAC15A10.15|SPCC1672.02C|SPBC800.13|SPAC3G6.01|SPAC3H1.12C|SPBC29A10.15
22402	3.45E-04	8.33E-03	10	351	42	5230	cell cycle process	SPCC962.02C|SPBC947.11C|SPAC15A10.15|SPBC336.15|SPCC1672.02C|SPBC14C8.07C|SPBC902.02C|SPAC3G6.01|SPAC14C4.05C|SPCC306.03C
68	3.61E-04	8.33E-03	3	18	42	5230	chromosome condensation	SPCC962.02C|SPBC12C2.10C|SPCC306.03C
32133	3.74E-04	8.33E-03	2	4	42	5230	chromosome passenger complex	SPCC962.02C|SPBC336.15
778	3.87E-04	8.33E-03	4	44	42	5230	condensed nuclear chromosome kinetochore	SPCC962.02C|SPAC15A10.15|SPCC1020.02|SPBC800.13
19219	3.95E-04	8.33E-03	11	427	42	5230	regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolic process	SPBC12C2.10C|SPCC1672.02C|SPCC306.05C|SPBC28F2.11|SPBC83.17|SPBC14C8.07C|SPAC3G6.01|SPCC5E4.06|SPAC3H1.12C|SPBC29A10.15|SPCC1620.09C
8156	4.59E-04	9.36E-03	4	46	42	5230	negative regulation of DNA replication	SPCC1672.02C|SPBC14C8.07C|SPCC5E4.06|SPBC29A10.15
43234	4.74E-04	9.36E-03	20	1222	42	5230	protein complex	SPCC962.02C|SPCC1020.02|SPBC336.15|SPCC1450.02|SPCC306.05C|SPBC902.02C|SPCC5E4.06|SPCC306.03C|SPAC22E12.17C|SPBC1709.11C|SPCC1620.09C|SPAC1B1.04C|SPBC12C2.10C|SPACUNK4.16C|SPBC947.11C|SPAC15A10.15|SPAC22F8.05|SPBC800.13|SPAC3H1.12C|SPBC29A10.15

Table S5. List of proteins with class 1 or 2 sites tested for phosphorylation by fission yeast Aurora in vitro.

Protein	Site	Identified in vivo	Identified in vitro	[RK]-X-[ST]	Sequence	Class
Mra1 (SPAC18G6.07c)	S9	+	+	+	TYSKRK**S**RGSLEV	1
	S12	+	+	+	KRKSRG**S**LEVSEK	1
Ctf18 (SPBC902.02c)	S240	+	+	+	KNSQRV**S**LPFFSK	1
Elg1 (SPBC947.11c)	T34	+	+	+	PIGRRH**T**MSPVPA	1
	S36	+	-	-	PIGRRHTM**S**PVPA	1
Orc1 (SPBC29A10.15)	S6	+	+	+	_MPRRK**S**LRSQLL	1
	S9	+	+	-	RRKSLR**S**QLLING	2
	T440	-	+	+	LLDNRF**T**HASPNR	-
	S567	-	+	+	DICRRA**S**ELAENK	-
Spc7 (SPCC1020.02)	S4	+	-	-	_MPT**S**PRRNSI	1
	S9	+	+	+	TSPRRN**S**IATTDN	1
	S28	+ (Exp. 3 only)	+	-	SRKRPH**S**LGGPGA	-
RNA-binding pr. (SPCC31H12.03c)	S220	+	+	+	APSKRK**S**NILDDP	2
Mdb1 (SPACUNK4.14)	S156	-	+	-	PSSVSQ**S**LSGDPS	-
	S174	+	+	+	VFDRKQ**S**AEINSP	-
	S505	+	-	+	GQGRKR**S**SRSSWN	1
	S506	+	-	+	QGRKRS**S**RSSWNK	2
Sla1 (SPAC57A10.10c)	S286	+	-	+	FKKQRS**S**NASEEK	2
	S289	+	-	-	FKKQRSSNA**S**EEK	2
HMG-box (SPBC28F2.11)	T237	+	-	+	AQASRA**T**PDIKEQ	1
	S294	+	+	+	KKKRRK**S**SMSSSI	1
	S295	+	+	+	KKKRRKS**S**MSSSI	1
	T302	+	-	-	MSSSIT**T**PPTAKV	1
Nsk1 (SPAC3G9.01)	S6	-	+	+	_MAYKL**S**LSAVEP	-
	S342	+	-	-	KLPLES**S**LSPLDH	2
	S407	+	+	+	GNREKH**S**LNSTPE	-
Png2 (SPBC1709.11c)	S196	+	+	+	NAVPRK**S**SSPPLS	1
	S197	+	+	+	NAVPRKS**S**PPLS	1
	S198	+	-	-	NAVPRKSS**S**PPLS	1

Table S6. Recombinant proteins or protein fragments used for the in vitro kinase assays. Proteins or fragments were either histidine tagged (His) or glutathione-*S*-transferase tagged (GST) at the N terminus (N). aa-aa, amino acids; fl, full length protein

Protein	**aa – aa**	**tag**
Ark1 (SPCC320.13c)	fl	6 x His (N)
Pic1 (SPBC336.15)	941-1018	GST (N)
Mra1 (SPAC18G6.07c)	1 - 129	6 x His (N)
Orc1 (SPBC29A10.15)	fl	6 x His (N)
Mdb1 (SPACUNK4.14)	fl	6 x His (N)
RNA binding protein (SPCC31H12.03c)	fl	6 x His (N)
Elg1 (SPBC947.11c)	1 – 242	6 x His (N)
Ctf18 (SPBC902.02c)	1 – 270	6 x His (N)
Sla1 (SPAC57A10.10c)	fl	6 x His (N)
HMG box (SPBC28F2.11)	fl	6 x His (N)
Nsk1 (SPAC3G9.01)	fl	6 x His (N)
Png2 (SPBC1709.11c)	fl	6 x His (N)
Spc7 (SPCC1020.02)	1 – 401	6 x His (N)

Experimental Procedures

Yeast strains

Figure 1		
SK730’	*h-*	*lys1 plo1+-GFP<<kanR cdc25-22*
SI269	*h+*	*lys1 plo1+-GFP<<kanR cdc25-22 hygR<<ark1-as3*
Figure 4B		
JY333	*h-*	*leu1 ade6-M216*
SK715	*h+*	*hygR<<spc7+*
SK755	*h+*	*hygR<<spc7-S4A S9A*
SK719	*h-*	*leu1 ade6-M216 hygR<<spc7-S4D S9D*
SL588	*h-*	*leu1 ade6-M216 hygR<<spc7-S28A*
SL586	*h-*	*leu1 ade6-M216 hygR<<spc7-S28D*
SL589	*h-*	*leu1 ade6-M216 hygR<<spc7-S4A S9A S28A*
SL587	*h-*	*leu1 ade6-M216 hygR<<spc7-S4D S9D S28D*
Figure 4C		
SM146	*h?*	*leu1*
SM145	*h?*	*hygR<<spc7+*
SM143	*h?*	*leu1 ura4-D18 hygR<<spc7+ nuf2-2::ura4+*
SM144	*h+*	*ura4-D18 nuf2-2::ura4+*
SM138	*h?*	*ura4-D18*
SM137	*h?*	*leu1 ade6-M216 hygR<<spc7-S4A S9A S28A*
SM136	*h?*	*(ura4-D18?) leu1 ade6-M216 hygR<<spc7-S4A S9A S28A nuf2-2::ura4+*
SM135	*h?*	*(ura4-D18?) nuf2-2::ura4+*
SL847	*h?*	*leu1 ade6-M216*
SL848	*h?*	*ade6-M216 hygR<<spc7-S4D S9D S28D*
SL849	*h?*	*ura4-D18 hygR<<spc7-S4D S9D S28D nuf2-2::ura4+*
SL850	*h?*	*leu1 ura4-D18 nuf2-2::ura4+*
Figure 4E		
PY207	*h-*	*ade6-M216 rec8+-GFP<<kanR*
SL569	*h-*	*leu1 ade6-M216 rec8+-GFP<<kanR orc1-S6A S9A*
SL570	*h-*	*leu1 ade6-M216 rec8+-GFP<<kanR orc1-S6D S9D*
SH385	*h+*	*chk1Δ::ura4+*

Figure 4F

SM150	*h+*	*ade6-M216*
SM148	*h+*	*ade6-M216 rec8+-GFP<<kanR orc1+*
SM147	*h-*	*rec8+-GFP<<kanR orc1+ chk1Δ::ura4+*
SM149	*h-*	*chk1Δ::ura4+*
SM152	*h+*	*ade6-M216*
SM151	*h+*	*ade6-M216 rec8+-GFP<<kanR orc1-S6A S9A*
SM154	*h-*	*leu1 rec8+-GFP<<kanR orc1-S6A S9A chk1Δ::ura4+*
SM153	*h-*	*leu1 chkΔ1::ura4+*
SM157	*h+*	*ade6-M216*
SM156	*h-*	*leu1 ade6-M216 rec8+-GFP<<kanR orc1-S6D S9D*
SM155	*h-*	*leu1 rec8+-GFP<<kanR orc1-S6D S9D chk1Δ::ura4+*
SM158	*h+*	*chk1Δ::ura4+*

Figure 5A

SL809	*h+*	*leu1 ade6-M216*
SL810	*h+*	*leu1 ade6-M216 ark1-T8-GFP-flag-His<<kanR*
SL811	*h-*	*leu1 ade6-M216 arp4Δ::hygR ark1-T8-GFP-flag-His<<kanR*
SL812	*h-*	*leu1 ade6-M216 arp4Δ::hygR*
SL450	*h+*	
SL449	*h-*	*leu1 ark1-T8-GFP-flag-His<<kanR*
SL448	*h+*	*chk1Δ::ura4+ ark1-T8-GFP-flag-His<<kanR*
SL447	*h-*	*leu1 chk1Δ::ura4+*
SL494	*h+*	*leu1 ade6-M216*
SL495	*h+*	*leu1 ade6-M216 ark1-T8-GFP-flag-His<<kanR*
SL496	*h-*	*leu1 ade6-M216 swi3Δ::hygR ark1-T8-GFP-flag-His<<kanR*
SL497	*h-*	*leu1 ade6-M216 swi3Δ::hygR*

Figure 5B

JY333	*h-*	*leu1 ade6-M216*
SH372	*h+*	*ark1-T8-GFP-flag-His<<kanR*
SH380	*h-*	*leu1 ark1-T7<<kanR*
SH386	*h+*	*leu1 ark1-T11<<kanR*
Sp365	*h-*	*ctf18Δ::natMX6*

Figure S8

SL473'	*h-*	*leu1 ade6-M216 cbp1Δ::hygR*
SL476	*h-*	*leu1 ade6-M216 arp4Δ::hygR*

PX858 *h+ leu1 ade6-M216 ark1-T8-GFP-flag-His<<kanR*

The *ark1-T11* temperature-sensitive allele was generated by PCR-based random mutagenesis and integrated into the endogenous locus, replacing *ark1+*. Sequence analysis revealed that Gly^{281} of Ark1 is changed to Arg. Phosphorylation site mutants of *spc7* and *orc1* were generated by PCR-based mutagenesis and were integrated at the endogenous locus, replacing the wild type gene. For technical reasons, the *spc7* phosphorylation site mutant strains contain a hygromycin-resistance cassette (hygR) 540 bp upstream of the start codon. To ensure comparability, this cassette is also present in the strain that we indicate as *spc7+* (Fig. 4). For similar reasons, the *orc1* phosphorylation site mutant strains carry a green fluorescent protein (GFP)-tagged version of the *rec8+* gene, a gene that is directly adjacent to *orc1*. To ensure comparability, *rec8+-GFP* is also present in the strain that we indicate as *orc1+* (Fig. 4). All other mutant alleles have been described previously (59-70).

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2.2 A Chemical Genetic Approach for Covalent Inhibition of Analogue-Sensitive Aurora Kinase

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Published in: **ACS Chemical Biology**, Volume 7, Issue 4, pp 723–731 (2012)

Author contributions:

I carried out the creation of the various *ark1* mutants, purification of recombinant proteins used for all *in vitro* assays, *in vitro* kinase and *in vitro* binding assays (Fig. 2A,D-F, Fig. S1), characterization of inhibitor effects on various *ark1* alleles by flow cytometry (Fig. 3A, Fig. S3) and microscopy (Fig. 3B,C), western blot analysis with phosphospecific antibodies (Fig. 4A,B), SAC activity assays (Fig. 4C,D) as well as inhibitor washout experiments in S phase arrested cells (Fig. 4E,F). In addition I contributed to the writing of the manuscript together with Haridas B. Rode, Silke Hauf and Daniel Rauh.

Haridas B. Rode synthesized and performed quality control of the chemical compounds **1**, **2**, **3**, **4**, **5** and **6** (Fig. 1A, Fig. 2C and Fig. S5 – S7).

André Richters determined the IC_{50} values of the chemical compounds (Fig. 2B and Table S1), conducted *in vitro* binding assays analyzed by fluorescence emission (Fig. S2), and determined the ATP- and substrate-Km of recombinant Ark1 and Ark1-as3-cys (Table S2).

Daniel Rauh supervised Haridas B. Rode and André Richters during the study.

Silke Hauf supervised the study.

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A Chemical Genetic Approach for Covalent Inhibition of Analogue-Sensitive Aurora Kinase

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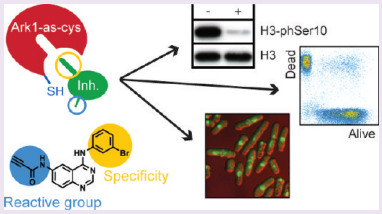
Supporting Information

ABSTRACT: The perturbation of protein kinases with small organic molecules is a powerful approach to dissect kinase function in complex biological systems. Covalent kinase inhibitors that target thiols in the ATP binding pocket of the kinase domain proved to be ideal reagents for the investigation of highly dynamic cellular processes. However, due to the covalent inhibitors' possible off-target reactivities, it is required that the overall shape of the inhibitor as well as the intrinsic reactivity of the electrophile are precisely tuned to favor the reaction with only the desired cysteine. Here we report on the design and biological characterization of covalent anilinoquinazolines as potent inhibitors of genetically engineered Aurora kinase in fission yeast.



Protein kinases catalyze the transfer of phosphate groups from ATP to substrate proteins and are involved in nearly every signaling pathway.[1,2] A powerful approach used to unravel the complex nature of kinase biology is to perturb kinase function by small molecule inhibitors and to compare differences between perturbed and unperturbed states.[3] However, the limited selectivity of most inhibitors makes perturbation of a single kinase difficult.[4] Chemical genomics techniques such as the "bump-and-hole approach" allow for the acute chemical knock-down of target protein kinase function with precision and selectivity not possible with traditional genetic approaches.[5] Irreversible inhibitors are particularly powerful tools by covalently and thereby permanently perturbing kinase function at the cellular level and are moving to the forefront of kinase inhibitor research both in medicinal chemistry and chemical biology.[6] Compared to reversible inhibitors, such probes often remain bound throughout the lifetime of the target kinase, and the duration of their action is therefore a function of the rate of enzyme turnover.[7] Enhanced potency and efficacy of covalent kinase inhibitors may result in increased specificity and less susceptibility to ATP competitive effects, which often are bottlenecks in the development of specific kinase inhibitors.[6] Irreversible inhibitors can be designed to specifically target particular Cysteine (Cys) residues that are located in the vicinity of the ATP-binding pocket.[8,9] Cys797 in EGFR (epidermal growth factor receptor) continues to serve as a prototypic nucleophile for covalent kinase inhibition.[10] This Cys is located in a conserved α-helix at the lip of the ATP-binding pocket, which is naturally present only in a few eukaryotic kinases but can be introduced into other kinases of interest and be targeted by inhibitors carrying an electrophile.[11,12] The ideal covalent modifier is typically poorly reactive under physiological conditions with solution nucleophiles such as glutathione or solvent-exposed Cys and will react with the desired nucleophile only upon appropriate positioning within the binding pocket of the target kinase. In order to achieve this and to prevent the electrophile to indiscriminately form adducts with thiols of other proteins, it is necessary to tailor the reactivity of covalent inhibitors for the selected kinase. To further investigate the structural and chemical requirements for covalent inhibition of mutant kinase targets for chemical biology research, we set out to equip 4-anilinoquinazolines with various electrophiles, which complement for selectivity filters incorporated into the kinase domain of Aurora (Ark1) from fission yeast (*Schizosaccharomyces pombe*). The serine-threonine protein kinases of the Aurora family are essential for the proper execution of mitosis in eukaryotes.[13] The single Aurora in yeast (known as Ipl1 in budding yeast and Ark1 in fission yeast, in metazoans known as Aurora B) ensures that chromosomes become properly segregated by controlling chromosome compaction and chromosome attachment to the mitotic spindle, as well as cytokinesis and abscission.[14] We show that inhibition of Aurora in yeast is specific for the chosen selectivity filters in biochemical and cellular assays and is dependent on the nature of the selected electrophile.

Received: November 12, 2011
Accepted: January 20, 2012
Published: January 20, 2012

RESULTS AND DISCUSSION

Inhibitor and Mutant Kinase Design. We based the design of our probe molecules on a general kinase inhibitor scaffold that can be readily equipped with different electrophiles. The 4-anilinoquinazoline PD168393 (**1**) is one of the most studied covalent inhibitors of EGFR[15] and carries an acrylamide at the 6-position of the quinazoline core. In order to test for the electrophile best suited to inhibit mutant kinase activity, we selected propiolamide (**2**), *N*,*N*-dimethylamino-2-butenamide (**3**), and but-2-ynamide (**4**) as additional reactive groups (Figure 1A).

In order to develop a chemical genetic system for covalent inhibition of Aurora in fission yeast, we modified the ATP-binding site of Ark1 (i) to create analogue-sensitive (as) kinase alleles and (ii) to allow for covalent inhibition by 4-anilinoquinazolines. As a first selectivity filter, we exchanged leucine (Leu) 166, the gatekeeper residue in the hinge region of Ark1 (Figure 1B–D), to a smaller alanine residue in order to accommodate the bulky bromophenyl moiety of the inhibitors. Additional suppressor mutations, resulting in Ark1-as3, were needed to rescue the functionality of the kinase.[16] As a second selectivity filter glutamate (Glu) 173, located in a short helix following the hinge region of the kinase domain of Ark1 and isostructural to Cys797 in EGFR, was exchanged to Cys (Ark1-cys) (Figure 1B–D). We speculated that this Cys residue could serve as anchor point for covalent inhibition, thus further increasing compound specificity and potency.

Biochemical Validation of Inhibitors. We initially tested the inhibitory effect of **1**–**4** on recombinant Ark1 protein in various biochemical assays, and compared it to the outcomes seen with 1NM-PP1, a commonly used reversible ATP competitive inhibitor targeting gatekeeper-mutated analogue-sensitive kinase alleles.[17] As expected, 1NM-PP1 was able to inhibit Ark1-as3 and Ark1-as3-cys but did not have any effect on Ark1-cys or wild type Ark1 (Figure 2A, Supplementary Figure 1A). The IC_{50} for inhibition of Ark1-as3-cys was in the low nanomolar range (Figure 2B, Supplementary Table 1 and 2). In an end point assay using histone H3 as a substrate, compounds **1**–**4** were more efficient in inhibiting Ark1-as3-cys compared to Ark1-as3 and did not inhibit Ark1-cys (Figure 2A). The IC_{50} determinations showed that these molecules are potent, nanomolar inhibitors of Ark1-as3-cys, but not of Ark1 (Figure 2B, Supplementary Table 1). A reversible counterpart of **1** and **2** (**7**) inhibited Ark1-as3-cys with an IC_{50} considerably higher than that of **1** or **2** (272 ± 171 nM, Supplementary Table 1) and did not inhibit wild type Ark1, further highlighting that efficient inhibition by **1**–**4** may depend on covalent binding. In order to confirm that the engineered cysteine in Ark1-as3-cys can be covalently modified by the electrophilic compounds, we applied a fluorescence-based binding assay recently developed in our lab.[18] The assay exploits the fact that covalent bond formation of **1** and **3** with the introduced Cys173 in Ark1-as3-cys leads to an increase in fluorescence emission of the inhibitor. We observed fast covalent bond formation with Ark1-as3-cys but not with wild type Ark1 (Supplementary Figure 2). Covalent binding of **2** and **4** results in a different addition product when compared to **1** and **3** and leaves a double bond, which does not change fluorescence emission of the inhibitor. However, binding of **2** and **4** could be detected indirectly, since preincubation with these compounds blocked binding of **1** and **3** (Supplementary Figure 2B). To further analyze binding of **2** and **4**, we derivatized these compounds by attaching the fluorophore BODIPY *via* a poly(ethylene glycol) linker to the 7-position of the quinazoline core to generate functional probes **5** and **6** (Figure 2C). Previous experiments had demonstrated that the 7-position of the quinazoline scaffold is likely to be solvent -exposed even after binding to the target kinase.[11,19] Probe **6** bound in an SDS-resistant and therefore covalent manner to

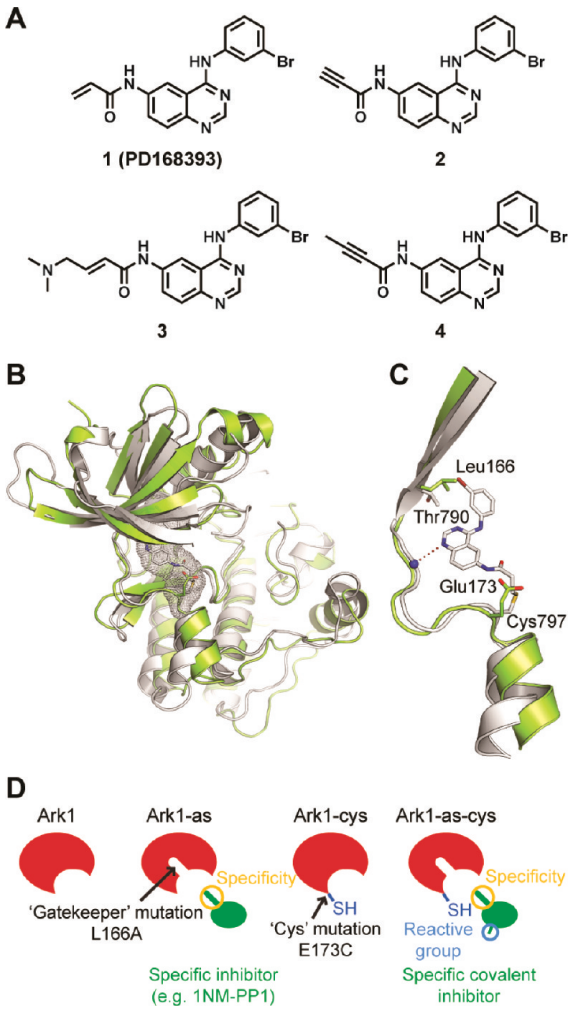


Figure 1. Covalent inhibitors and design of genetically engineered Ark1 kinase versions. (A) Chemical structures of compounds **1**–**4**. (B) Structural alignment of EGFR (gray) (in complex with the irreversible inhibitor **1**, PDB code: 2J5F) and a homology model of apo Ark1 from fission yeast (green) based on the crystal structure of *Xenopus* Aurora B (PDB code: 2BFY). (C) Detailed view of structural alignment highlighting the hinge region of the kinase domain with the gatekeeper position (Leu166, Thr790) and the short α-helix (Glu173, Cys797). Quinazoline-based inhibitors form key hydrogen bonds to the hinge region of the kinase domain (red dotted line). Cys797 of EGFR is covalently modified by **1**, and Glu173 in Ark1 is iso-structural to Cys797 in EGFR and was mutated to Cys to allow for covalent modification. The larger gatekeeper in Ark1 (Leu166) had to be replaced by a smaller amino acid to accommodate the bulky *m*-bromophenyl moiety of the inhibitor. (D) Schematic representation of wild type and mutant Ark1 kinase alleles. The Ark1-as version used in this study (Ark1-as3) has the additional mutations Q28R, Q176R, and S229A,[16] which rescue functionality of the kinase in which the gatekeeper is mutated (L166A).

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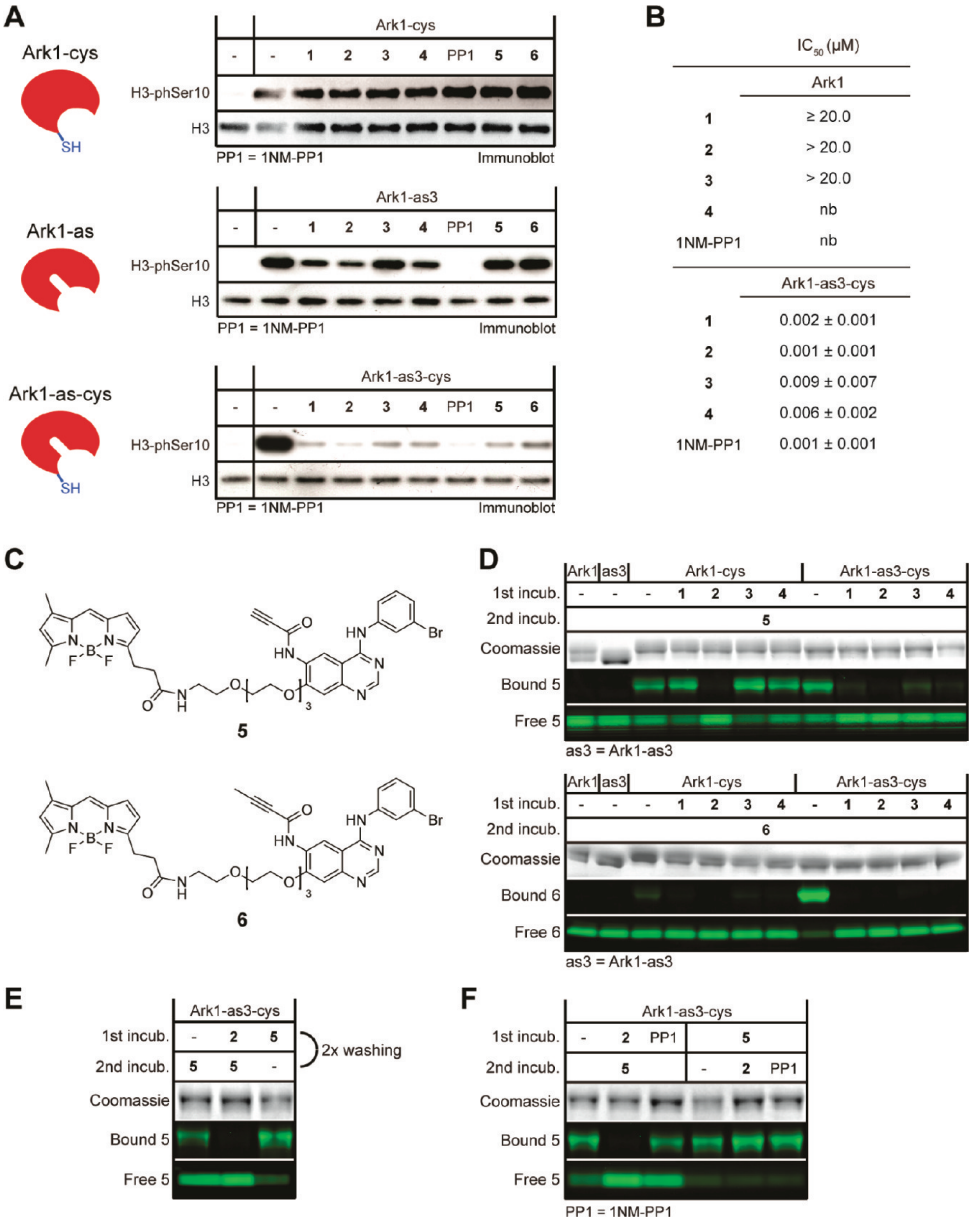

Figure 2. Compounds **1**−**4** specifically inhibit Ark1-as3-cys in biochemical assays. (A) Kinase assays with recombinant Ark1-cys (upper panel), Ark1-as3 (middle panel), and Ark1-as3-cys (lower panel), each incubated with the indicated compounds at 5 μM concentration prior to the addition of the substrate (histone H3). The reaction mixtures were separated by SDS-PAGE and immunoblotted with a phospho-specific antibody against Ser10 in histone H3 (H3-phSer10). Immunoblotting for histone H3 served as loading control. (B) IC_{50} values for the indicated compounds with wild type Ark1 or Ark1-as3-cys (see Supplementary Table 1 for full information). nb, non binding (no inhibitory effect at 50 μM). (C) Chemical structures of compounds **5** and **6**. (D) Recombinantly produced versions of Ark1 were preincubated with inhibitors for 5 min (1st incub.) followed by incubation with probes **5** (upper panel) or **6** (lower panel) for 5 min (2nd incub.). Reaction mixtures were separated by SDS-PAGE. Ark1 was detected by Coomassie staining. The amount of fluorescent compound comigrating with Ark1 ("Bound") or present in the running front of the SDS gel ("Free"), respectively, are shown. (E) Recombinantly produced Ark1-as3-cys was bound to beads, incubated for 5 min with **2** or **5** (1st incub.), washed twice, and incubated with **5**, as indicated, for another 5 min (2nd incub.). The reaction mixtures were analyzed as in panel D. (F) Recombinantly produced Ark1-as3-cys was incubated with the indicated compounds and analyzed as in panel D.

Ark1-as3-cys and to a much weaker extent to Ark1-cys, but not to Ark1 or Ark1-as3 (Figure 2D), highlighting the requirement of orthogonal filters for efficient targeting by the probe molecules. Binding of **6** to Ark1-as3-cys could be competed for by preincubation with **1**−**4**, indicating that all of these compounds specifically bind the engineered Cys173. In contrast to **6**, **5** showed equally strong binding to Ark1-cys and Ark1-as3-cys, and binding to Ark1-cys could be competed for by **2**, but not by **1**, **3**, or **4** (Figure 2D). This indicates that the most active compound **2**, which carries a propiolamide as the reactive group, covalently binds Ark1-cys in addition to Ark1-as3-cys. However, neither **2** nor **5** inhibited Ark1-cys in our biochemical assay (Figure 2A), confirming that presence of only one of the selectivity filters is insufficient for functionality

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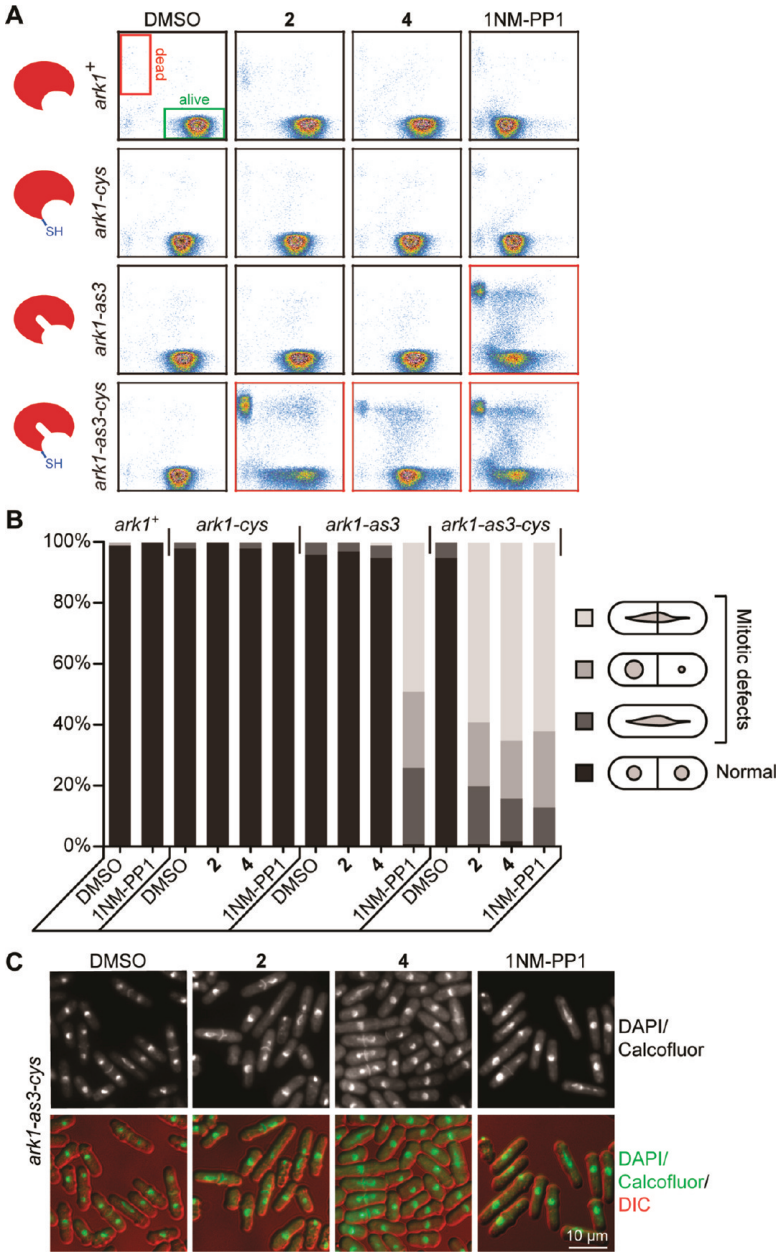

Figure 3. Compound **2** and **4** impair growth of *ark1-as3-cys*-expressing cells. (A) Cells in logarithmic growth phase expressing *ark1+*, *ark1-cys*, *ark1-as3*, or *ark1-as3-cys* were grown for 4 h in the presence of the indicated compounds at 5 μM concentration and stained with "Yeast viability kit" (Partec), and 50,000 cells of each treatment were measured in a flow cytometer. Viable cells cluster in the lower right (green box), and dead cells in the upper left (red box) of the scatter plot. Combinations of strain and compound that showed an increase in dead cells are marked with a red box. (B) Cells in logarithmic growth phase expressing *ark1+*, *ark1-cys*, *ark1-as3*, or *ark1-as3-cys* were grown for 2 h in the presence of the indicated compounds at 5 μM concentration, fixed with methanol, and stained with DAPI to visualize DNA and Calcofluor to visualize the cell wall. DNA segregation defects of late mitotic cells were scored as shown in the legend. Around 100 mitotic cells were analyzed for each condition. (C) Example pictures of *ark1-as3-cys*-expressing cells treated with the indicated inhibitors from the experiment shown in B.

of the probe. To exclude that reversible, rather than irreversible, binding of the nonfluorescent compounds lead to the reduced binding of **5** or **6**, we performed the assay with Ark1-as3-cys coupled to beads and stringently washed the beads after addition of **2** and before addition of **5** (Figure 2E). Compound **2** still competed for binding of **5** in this assay, indicating that not only **5** but also **2** bound covalently. In addition, unlike preincubation with **2**, preincubation of Ark1-as3-cys with the reversible inhibitor 1NM-PP1 did not prevent binding of **5** (Figure 2F). When **5** was added before **2**, compound **2** was unable to compete out **5** (Figure 2F). A similar result was obtained for **6** (Supplementary Figure 1B). We conclude that compounds **1**–**6** covalently bind the engineered Cys173 and that efficient binding depends on both selectivity filters.

Cellular Characterization of Functional Probes. In order to test the effect of these probes in cells, homologous

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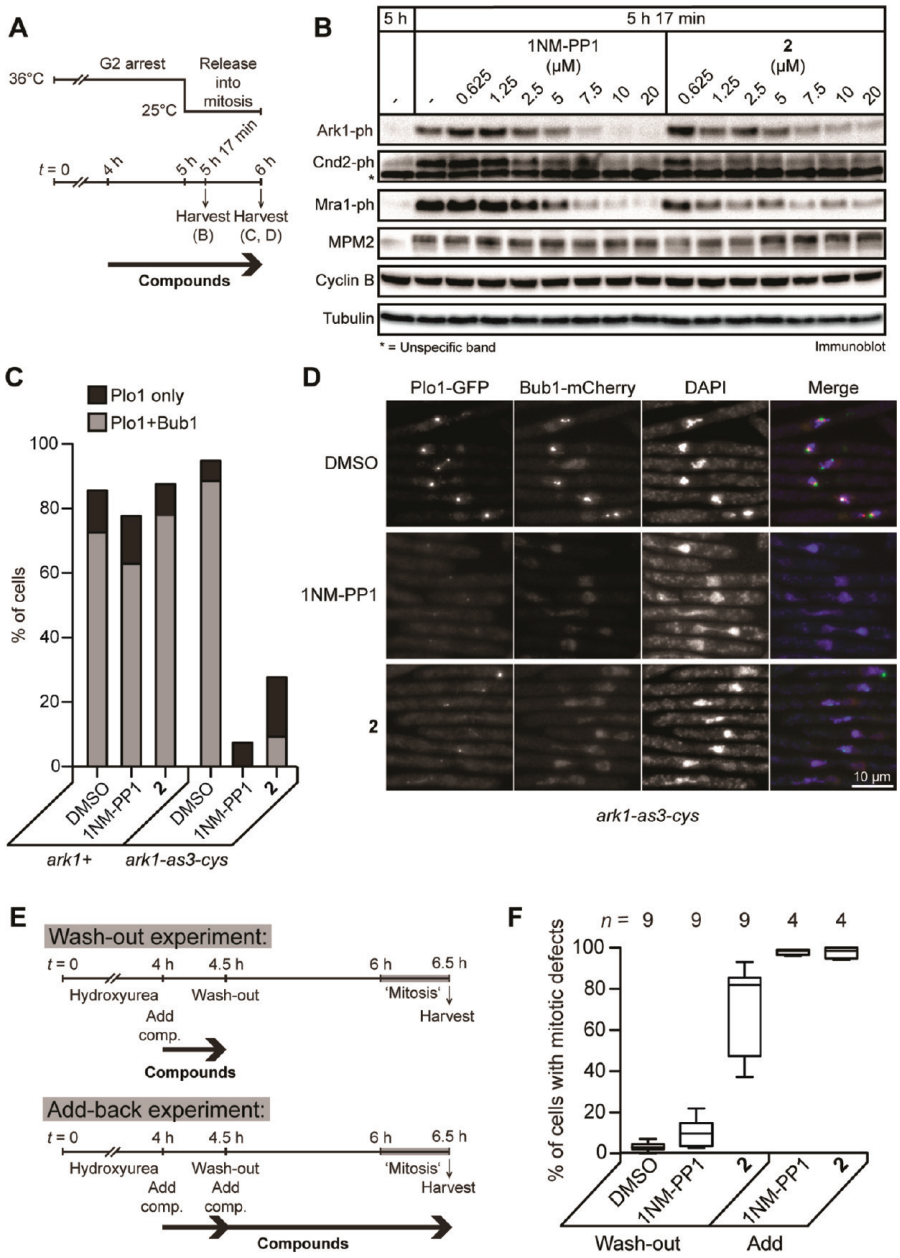

Figure 4. Compound **2** impairs the activity of Ark1-as3-cys in cells in an irreversible manner. (A) Schematic of the experiments shown in panels B, C, and D. Cells with the temperature-sensitive *cdc25-22* mutation were arrested prior to mitosis (in G2 phase) by culturing them for 5 h at 36 °C (restrictive temperature). During the last 1 h of the arrest, 1NM-PP1, **2**, or the solvent DMSO was added, and the cells were released synchronously into mitosis by shifting the culture to 25 °C. At the indicated points in time, cells were harvested for immunoblotting (B) or microscopic analysis (C, D). (B) Immunoblot of whole cell extracts at the time point of release into mitosis (5 h) and 17 min after the release (5 h 17 min), when cells had reached mitosis. Shown are the Ark1-specific phosphorylations on Ark1-Thr244 (Ark1-ph[21,22]), condensin subunit Cnd2-S5 (Cnd2-ph[23,24]), and Mra1-S9 (Mra1-ph[23]). The asterisk marks a cross-reacting band in the Cnd2 immunoblot. Presence of the MPM2 phosphoepitope[26] and cyclin B (fission yeast Cdc13[25]) indicates that the inhibitor-treated cells were in mitosis. Tubulin serves as loading control. (C) Bub1-mCherry and Plo1-GFP in *cdc25-22 cut7-446* cells were visualized by microscopy in cells harvested 1 h after release of the culture into mitosis. Bub1 is a spindle assembly checkpoint protein that localizes to kinetochores when the checkpoint is active.[36] Plo1 localizes to spindle pole bodies during early mitosis.[30] Pictures were scored for the presence of a localized Plo1-signal only or of both a localized Plo1- and Bub1-signal. In one set of experiments, cells expressed wild type *ark1+*, in the other *ark1-as3-cys*. More than 100 cells were analyzed for each condition. (D) Example pictures of *ark1-as3-cys* cells scored in panel C. Shown are the Plo1-GFP signal (green in the merged picture), the Bub1-mCherry signal (red in the merged picture), and DNA stained with DAPI (blue in the merged picture). (E) Schematic of wash-out (upper part) and add-back experiments (lower part) shown in panel F. Cells expressing *ark1-as3-cys* were arrested in S phase by culturing them for 5 h in hydroxyurea-containing medium. In the last 30 min of the incubation, 1NM-PP1, **2**, or DMSO was added and afterward washed out together with the hydroxyurea. Cells were then cultured either in the presence (add-back) or absence (wash-out) of 1NM-PP1 and **2** until harvesting shortly after mitosis. (F) The experiments outlined in E were performed multiple (*n*) times, and mitotic defects were scored as in Figure 3B. More than 100 cells were analyzed in each experiment for each condition. The lines in the

dx.doi.org/10.1021/cb200465c | *ACS Chem. Biol.* 2012, 7, 723–731

Figure 4. continued

box-whisker-plot indicate the minimum and maximum value for the percentage of cells with mitotic defects obtained in any of the *n* experiments, as well as the median and 25th and 75th quartile.

recombination was used to replace endogenous fission yeast Aurora (Ark1) with the modified alleles of the kinase. Similar to Ark1-as3,[16] both Ark1-cys and Ark1-as3-cys were able to functionally replace wild type Ark1, as cells expressing these kinase versions did not exhibit any overt defects during mitosis in the absence of inhibitor (Figure 3A,B). We incubated yeast cells expressing the different variants of Ark1 with **1**−**4** or 1NM-PP1 and assessed cell viability by flow cytometry (Figure 3A, Supplementary Figure 3). Ark1 is essential for cell viability,[20] and inhibition of the kinase therefore results in an accumulation of dead cells. As expected, treatment of *ark1-as3* cells with 5 μM 1NM-PP1 caused cell death, and a similar outcome was observed when using *ark1-as3-cys* cells. Interestingly, treatment with **2** or **4** at 5 μM specifically impaired viability of *ark1-as3-cys* cells, but not of *ark1+*, *ark1-as3*, or *ark1-cys* cells, indicating that both selectivity filters are needed for efficient inhibition by these molecules in cells. Compounds **1** and **3** at 5 μM did not affect viability of *ark1-as3-cys* cells (Supplementary Figure 3A). Compound **4** was less efficient in this assay when compared with **2** (Figure 3A), but its inhibitory effect on *ark1-as3-cys* cells could be enhanced by increasing the concentration to 50 μM (Supplementary Figure 3B). This indicates that the nature of the electrophile is important for efficient Ark1-as3-cys inhibition in cells. Whereas incubation of wild type yeast cells with a high concentration (50 μM) of 1NM-PP1 caused toxicity, **2** and **4** were tolerated at concentrations up to 50 μM (Supplementary Figure 3B). Hence, the range between efficient inhibition in cells and onset of toxicity is larger for **2** than for 1NM-PP1, indicating that the irreversible inhibitor may be more specific. In order to confirm that cell death was caused by inhibition of Ark1, we assessed the cellular phenotypes after treatment with the compounds. Inhibition of Ark1 leads to severe DNA segregation defects in mitosis, as exemplified by the phenotype of *ark1-as3* cells treated with 1NM-PP1 (Figure 3B,C).[16,20] Similar defects were observed in *ark1-as3-cys* cells upon treatment with **2** or **4**. In contrast, *ark1+*, *ark1-cys*, and *ark1-as3* cells did not show any obvious phenotype when treated with **2** or **4**, highlighting the selective inhibition of Ark1-as3-cys (Figure 3B,C).

To demonstrate Ark1 inhibition on the molecular level and to compare the potency of **2** and 1NM-PP1 in cells, we arrested *ark1-as3-cys* cells just prior to mitosis, added **2** or 1NM-PP1, and released cells into mitosis in the presence of these compounds (Figure 4A). Ark1-dependent phosphorylation sites in Ark1 itself[21,22] as well as in Cnd2[23,24] and Mra1[23] were phosphorylated in control-treated mitotic cells, but phosphorylation was strongly reduced in a concentration-dependent manner in cells treated with 1NM-PP1 or **2**, with both compounds showing a similar effect (Figure 4B). The presence of cyclin B (fission yeast Cdc13[25]) and of the MPM2 phosphoepitope[26] (Figure 4B) confirmed that the inhibitor-treated cells were still in mitosis and excluded that loss of phosphorylation on Ark1, Cnd2, and Mra1 was a secondary consequence of mitotic exit. This confirms that **2** inhibits the enzymatic activity of Ark1-as3-cys in cells. To further assess functional impairment of Ark1, we assayed the activity of the spindle assembly checkpoint (SAC), a cellular surveillance pathway that requires Ark1 activity.[27] The SAC was activated by a mutation in Kinesin-5 (*cut7-446*[28,29]) and the percentage of cells in mitosis was analyzed by localization of Plo1 (Figure 4C,D), which targets to spindle pole bodies specifically during mitosis.[30] Both 1NM-PP1 and **2** shortened the SAC-mediated mitotic delay in *ark1-as3-cys*-expressing cells, but not in cells expressing wild type *ark1* (Figure 4C,D), indicating that the compounds induced a SAC failure by inhibiting Ark1-as3-cys rather than through an unexpected off-target effect. Localization of the spindle assembly checkpoint protein Bub1 was lost after treatment of *ark1-as3-cys* cells with 1NM-PP1 or **2** (Figure 4C,D), presumably as a consequence of exit from mitosis.[27] The Bub1 signal persisted in *ark1+* cells treated with 1NM-PP1 or **2** (Figure 4C, Supplementary Figure 4), corroborating that these compounds do not show unexpected effects in mitosis.

Covalent inhibitors often bind irreversibly. To test whether inhibition of Ark1-as3-cys by **2** in cells is irreversible, we performed a wash-out experiment: *ark1-as3-cys* cells were arrested in S phase using hydroxyurea and treated with **2** or 1NM-PP1 for 30 min. Hydroxyurea and compound were then washed out, and cells were allowed to undergo G2 phase and enter mitosis in compound-free medium (Figure 4E). As a control, the compound was readded just after the wash-out (Figure 4E). DNA segregation defects in late mitosis were scored to assess the degree of Ark1 inhibition (Figure 4F). Whereas transient treatment with 1NM-PP1 during S phase caused hardly any DNA segregation defect in mitosis, transient treatment with **2** led to considerable defects in DNA segregation. This response was somewhat variable, and the number of cells with DNA segregation defects was not quite as high as after add-back of 1NM-PP1 or **2** (Figure 4F). Possible causes for this variable response could be (i) incomplete inhibition because the 30 min incubation was not long enough for covalent binding to all Ark1-as3-cys present in the cells, (ii) resynthesis of Ark1-as3-cys after the inhibitors had been washed out, or (iii) dissociation of the inhibitor with a slow off-rate in the considerable time span between inhibitor treatment and mitosis (1.5−2 h). At present, we cannot distinguish between these possibilities. Nevertheless, the more pronounced mitotic defects seen after transient treatment with **2** compared to transient treatment with 1NM-PP1 (Figure 4F) suggest that a considerable fraction of Ark1-as3-cys had become inhibited irreversibly.

Conclusion. Kinase inhibitors have proven powerful tools to dissect kinase function in cellular systems. Most kinase inhibitors however target the ATP binding site of the catalytic domain, which is highly conserved across all protein kinases, thus making it difficult to achieve high levels of selectivity needed for chemical biology research. Here we report on the use of a chemical-genetic approach, which is based on two selectivity filters for generating an orthogonal covalent kinase inhibitor pair for the Aurora kinase Ark1 in fission yeast. The inhibition of analogue-sensitive Ark1-as3-cys in cells was dependent on the nature of the electrophile present at the 6-position of the probes' quinazoline core. In many applications covalent inhibitors are superior in potency and ligand-target residence times when compared to reversible binders.[7] In order to achieve suitably high selectivity for covalent binders in biologically relevant systems, the reactivity and spatial position-

ing of the electrophiles must be tailored to the corresponding nucleophile in the target protein of interest. Among the tested compounds equipped with various electrophiles, **2** was the most potent entity and showed efficient and largely irreversible inhibition in cells while exhibiting no detectable toxicity, even at very high concentrations. Our results indicate that the combination of two selectivity filters increases specificity and highlights the importance for a given covalent probe to properly bind to the desired target in an initially reversible step to allow for residence time that is sufficient for covalent bond formation. Based on our and previous work,[7,11,18,31] we expect that this targeted design strategy can be extended to other kinases or to Aurora kinases from different organisms to make them susceptible to irreversible inhibitors. Such inhibitors can also act as scaffolds to develop activity-based probes,[32] which provide additional functionality.

METHODS

Compounds and Chemicals. Compounds **1**−**4** were prepared as described previously.[33] The scheme for synthesis of **5** and **6** is shown in Supplementary Figure 5. Anhydrous solvents were purchased from Acros Organics and Fluka. Other chemicals were purchased from Alfa Aesar, Fluka, and Sigma Aldrich and were used as received.

Analytical Data. ^{1}H and ^{13}C NMR spectra were recorded on a Varian Mercury 400, Bruker DRX 500 or Varian Inova 600 spectrometer. The spectra refer to the residual solvent signals: dimethylsulfoxide-d_6 (2.50 ppm) for ^{1}H and (39.52 ppm) for ^{13}C. Chemical shifts (δ) are given in parts per million (ppm), and the coupling constants (J) are reported in Hz. The following abbreviations are used: s = singlet, d = doublet, dd = doublet of doublet, ddd = doublet of doublet of doublet, t = triplet, td = triplet of doublets, dt = doublet of triplets, q = quartet, qd = quartet of doublet, qn = quintet, bs = broad singlet, m = multiplet. LC−MS spectra were obtained on a LTQ Orbitrap (high resolution mass spectrometer from Thermo Electron) coupled to an "Accela" HPLC System (consisting of Accela pump, Accela autosampler and Accela PDA detector) supplied with a "Hypersil GOLD" column (50 mm × 1 mm, 1.9 μm particle size) from Thermo Electron. Analytical TLC was carried out on Merck 60 F_{245} aluminum-backed silica gel plates. Preparative HPLC was carried out on the final compounds using a Varian Prostar with UV-detector (Model 340) and a VP 25-21 Nucleodur (C18 Gravity, 5 μm) column (serial no. 2105150).

*3-(1-((6-Amino-4-((3-bromophenyl)amino)quinazolin-7-yl)oxy)-12-oxo-3,6,9-trioxa-11-azatetradecan-14-yl)-5,5-difluoro-7,9-dimethyl-5H-dipyrrolo[1,2-c:2′,1′-f][1,3,2]diazaborinin-4-ium-5-uide (**vi**).* 7-(2-(2-(2-(2-Aminoethoxy)ethoxy)ethoxy)ethoxy)-N^4-(3-bromophenyl)quinazoline-4,6-diamine (**v**) (prepared as previously described[11]) (0.050 g, 0.09 mmol), 4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-*s*-indacene-3-propionic acid, succinimidyl ester (0.038 g, 0.09 mmol), and diisopropylethylamine (20 μL, 0.11 mmol) were added in DMF (2 mL) and stirred at RT for 2 h. The reaction mixture was poured into water and extracted with EtOAc (4 × 100 mL). The organic phase was dried with Na_2SO_4 and concentrated under high vacuum. The crude product was purified by preparative HPLC to furnish 0.054 g (54%) of **vi** as an orange solid. ^{1}H NMR (400 MHz, DMSO-d_6): 9.45 (br s, 1H), 8.40 (s, 1H), 8.22 (s, 1H), 8.03 (t, J = 5.5 Hz, 1H), 7.86 (d, J = 8.2 Hz, 1H), 7.67 (s, 1H), 7.46 (s, 1H), 7.30 (t, J = 8.1 Hz, 1H), 7.21 (d, J = 7.9 Hz, 1H), 7.12 (s, 1H), 7.07 (d, J = 4.0 Hz, 1H), 6.34 (d, J = 4.0 Hz, 1H), 6.28 (s, 1H), 5.35 (br s, 2H), 4.28 (t, J = 4.0 Hz, 2H), 3.86 (t, J = 4.3 Hz, 2H), 3.67−3.62 (m, 2H), 3.59−3.49 (m, 6H), 3.38−3.29 (m, 2H), 3.41 (t, J = 5.8 Hz, 2H), 3.26−3.17 (m, 2H), 3.07 (t, J = 7.7 Hz, 2H), 2.45 (s, 3H), 2.24 (s, 3H). ^{13}C NMR (101 MHz, DMSO-d_6): 170.95, 162.39, 159.11, 157.74, 155.11, 151.91, 150.15, 144.07, 142.00, 138.57, 134.43, 132.97, 130.29, 128.93, 125.35, 124.93, 123.28, 121.20, 120.25, 119.90, 116.60, 110.59, 106.43, 101.13, 70.03, 69.86, 69.77, 69.61, 69.13, 68.70, 68.04, 38.63, 33.66, 24.01, 14.53, 11.02. HRMS (ESI-MS): calcd 780.24864 and 782.24660 for $C_{36}H_{42}{}^{79}BrBN_7O_5F_2$ [M + H$^+$] and $C_{36}H_{42}{}^{81}BrBN_7O_5F_2$ [M + H$^+$]; found 780.25022 and 782.24804.

*3-(1-((4-((3-Bromophenyl)amino)-6-propiolamidoquinazolin-7-yl)oxy)-12-oxo-3,6,9-trioxa-11-azatetradecan-14-yl)-5,5-difluoro-7,9-dimethyl-5H-dipyrrolo[1,2-c:2′,1′-f][1,3,2]diazaborinin-4-ium-5-uide (**5**).* Propiolic acid (6.7 μL, 0.10 mmol) and EDCI·HCl (0.020 g, 0.10 mmol) were stirred in DMF (4 mL) at 0 °C for 0.5 h. 3-(1-((6-Amino-4-((3-bromophenyl)amino)quinazolin-7-yl)oxy)-12-oxo-3,6,9-trioxa-11-azatetradecan-14-yl)-5,5-difluoro-7,9-dimethyl-5*H*-dipyrrolo-[1,2-*c*:2′,1′-*f*][1,3,2]diazaborinin-4-ium-5-uide (0.028 g, 0.04 mmol) was added, and the reaction mixture was stirred overnight at RT. The reaction mixture was then concentrated under high vacuum to 1 mL and poured into water. The resulted solution was then extracted with EtOAc (2 × 200 mL), and the EtOAc layer was washed with brine and dried over Na_2SO_4. Solvent was removed under high vacuum, and the resulted crude product was purified by preparative HPLC to give 0.002 g (7%) of **5** as an orange solid. HRMS (ESI-MS): calcd 832.24356 and 834.24151 for $C_{39}H_{42}{}^{79}BrBN_7O_6F_2$ [M + H$^+$] and $C_{39}H_{42}{}^{81}BrBN_7O_6F_2$ [M + H$^+$]; found 832.24455 and 834.2461.

*3-(1-((4-((3-Bromophenyl)amino)-6-(but-2-ynamido)quinazolin-7-yl)oxy)-12-oxo-3,6,9-trioxa-11-azatetradecan-14-yl)-5,5-difluoro-7,9-dimethyl-5H-dipyrrolo[1,2-c:2′,1′-f][1,3,2]diazaborinin-4-ium-5-uide (**6**).* 2-Butynoic acid (0.018 g, 0.21 mmol) and EDCI·HCl (0.034 g, 0.18 mmol) were stirred in DMF (1 mL) at 0 °C for 0.5 h. 3-(1-((6-Amino-4-((3-bromophenyl)amino)quinazolin-7-yl)oxy)-12-oxo-3,6,9-trioxa-11-azatetradecan-14-yl)-5,5-difluoro-7,9-dimethyl-5*H*-dipyrrolo-[1,2-*c*:2′,1′-*f*][1,3,2]diazaborinin-4-ium-5-uide (0.008 g, 0.01 mmol) was added, and the reaction mixture was stirred overnight at RT. The reaction mixture was concentrated under high vacuum, poured into water, and extracted with EtOAc (2 × 100 mL). The EtOAc layer was washed with brine and dried over Na_2SO_4. The solvent was removed under high vacuum, and the resulted crude product was purified by preparative HPLC to yield 0.001 g (12%) of **6** as an orange solid. HRMS (ESI-MS): calcd 846.25921 and 848.25716 for $C_{40}H_{44}{}^{79}BrBN_7O_6F_2$ [M + H$^+$] and $C_{40}H_{44}{}^{81}BrBN_7O_6F_2$ [M + H$^+$]; found 846.25991 and 848.25806.

Yeast Strains. Figure 3: *JY333 h- leu1 ade6-M216*; *SI205 h- leu1 ade6-M216 hygR≪ark1-cys(E173C)*; *SI239 h- leu1 ade6-M216 hygR≪ark1-as3*; *SI240 h- leu1 ade6-M216 hygR≪ark1-as3-cys*. Figure 4B: SI284″ *h- hygR≪ark1-as3-cys plo1+-GFP≪kanR cdc25-22*. Figure 4C,D: SM676 *h? hygR≪ark1-as3-cys plo1+-GFP≪kanR bub1+-mCherry≪natR cdc25-22 cut7-446*; *SP017 h? ade6-M216 plo1+-GFP≪kanR bub1+-mCherry≪natR cdc25-22 cut7−446*. Figure 4E,F: SI240 *h- leu1 ade6-M216 hygR≪ark1-as3-cys*.

The *ark1-cys and ark1-as3-cys* alleles were generated by PCR-based mutagenesis of Glu173 to Cys and reintegration into the endogenous locus. Sequence analysis revealed that ARG135 of *ark1-as3-cys* was in addition inadvertently changed to GLN. All other mutant alleles have been described previously.[16,28,34]

Cell Growth. *Schizosaccharomyces pombe* cells were cultured in rich medium (YEA) at 30 °C (Figures 3 and 4E,F) or in case of strains containing the temperature-sensitive *cdc25-22* allele at 25 °C (permissive temperature) and 36 °C (restrictive temperature) (Figure 4A−D).

Antibodies. The following antibodies were used: mouse monoclonal against histone H3 (05-499, Upstate Biotechnology), rabbit polyclonal against histone H3-S10ph (06-570, Upstate Biotechnology), rabbit monoclonal against Ark1-T244ph (2914S, Cell Signaling/NEB), mouse monoclonal against α-tubulin (T5168, Sigma), cyclin B (Cdc13) (NB200-576, Novus/Acris), and MPM2 (GTX14581, Acris/GeneTex). Anti-Cnd2-S5ph was raised in rabbit against the following peptide MKRA(Sph)LGGHAPEDC, and the serum was affinity purified. Anti-Mra1-S9ph was raised in rabbit against the following peptide TYSKRK(Sph)RGSLEVSC, and the serum was affinity purified. As secondary antibodies HRPO-coupled goat anti-mouse IgG (115-035-003, Dianova) and goat anti-rabbit IgG (111-035-003, Dianova) were used.

Protein Extraction. Cell pellets from 5 × 10^7 cells were resuspended in 1 mL of ice-cold 20% (v/v) TCA and pelleted by centrifugation (1000*g*, 2 min, RT). Cells were washed with 1 mL of 1

M Tris and pelleted again by centrifugation. The cell pellet was resuspended in 200 μL of 2x SDS sample buffer (125 mM Tris pH 6.8, 4% (w/v) SDS, 20% (v/v) glycerol, 200 mM DTT, 0.02% (w/v) bromophenol blue) and boiled at 95 °C for 10 min. Glass beads (500 μm) were added, and the mixture was subjected three times to 40 s of shaking in a FastPrep machine (FP120, Qbiogene). A hole was made in the bottom of the tube, and the cell extract was eluted into a new reaction tube by centrifugation (1000*g*, 1 min, RT) thereby separating the extract from the glass beads. The resulting samples were boiled again at 95 °C for 10 min and centrifuged (16000*g*, 10 min, RT) in order to pellet the cell debris. Samples were stored at −20 °C until loading on SDS-polyacrylamide gels.

Fixation and Microscopy of *Schizosaccharomyces pombe* Cells. An aliquot of the cultures was fixed in −20 °C methanol. After washing once with PEM (100 mM PIPES pH 6.9, 1 mM EGTA, and 1 mM $MgSO_4$)/methanol and once with PEM, DNA was stained with 1 μg/mL DAPI (4′,6-diamidino-2-phenylindole; Sigma-Aldrich). Images were acquired on a Zeiss AxioImager microscope coupled to a CCD (charged-coupled device) camera and processed with MetaMorph 7.6.0 software (Molecular Devices Corporation). Typically, a Z-stack of about 3 μm thickness, with single planes spaced by 0.3 μm, was acquired and subsequently projected to a single image. Shown in Figure 3C are color-combined pictures of single planes of the DAPI Z-stack (blue channel) and the single picture taken with differential interference contrast (DIC) (red channel). In Figure 4 maximum intensity projections of the GFP Z-stack (green channel) and the mCherry Z-stack (red channel) with single plains of the DAPI Z-stack (blue channel) are shown.

Cell Synchronization by *cdc25-22* Arrest-Release. Cells were arrested prior to mitosis by shifting the culture to 36 °C (restrictive temperature of the *cdc25-22* mutant) for 4 h and then treated for 60 min either with 10 μM 1NM-PP1 ((4-amino-1-*tert*-butyl-3-(1′-naphthylmethyl)pyrazolo[3,4-*d*]pyrimidine; Toronto Research Chemicals), 10 μM **2**, or the same amount of DMSO at 36 °C. Cells were released from the *cdc25-22* arrest by reducing the temperature back to 25 °C in an ice−water bath and were grown subsequently at 25 °C until cell harvest. At the indicated points in time 5×10^7 cells (protein extraction) and 1×10^7 cells (methanol fixation) were harvested by centrifugation (3000*g*, 1 min, RT).

Flow Cytometry. Cells were cultured at 30 °C for 4 h in the presence of the indicated compounds. About 4×10^6 cells were stained with "Cell Viability Kit" (Partec) and measured in a flow cytometer (CyFlow SL, Partec).

Hydroxyurea Wash-out Experiment. Cells were grown in 10 mL of YEA to a concentration of 4×10^6 cells/mL, and 12 mM hydroxyurea (Sigma) was added to the culture. After 4 h, 5 or 10 μM 1NM-PP1, 10 μM **2**, or an equal amount of the solvent DMSO was added for 30 min. To wash out hydroxyurea, cells were pelleted (1000*g*, 3 min, RT) and washed with 50 mL of fresh YEA for 2 min with thorough mixing. Washing was repeated twice. After the three washing steps, the cells were taken up in 10 mL of YEA and grown either in the presence or absence of 5 μM 1NM-PP1 or **2**. Two hours after washing out hydroxyurea, the cells were fixed with methanol for microscopic analysis.

Kinase Assay Using Histone H3 as Substrate. Ark1 wild type and mutant versions were purified as previously described,[23] and 100 ng of the different versions of Ark1/Pic1 was incubated for 10 min at 32 °C with the indicated compounds at 5 μM in a buffer containing 50 mM Tris-HCl pH 7.5, 100 mM NaCl, 20 mM $MgCl_2$, 1 mM DTT, 1 mM ATP. After this incubation, 1 μg of histone H3 protein (calf thymus, Roche) was added, and the mixture was incubated for 15 min at 32 °C before addition of SDS-sample buffer and boiling at 95 °C for 10 min.

Determination of IC_{50} and K_m. Experiments were carried out using a homogeneous time-resolved FRET (HTRF) assay (KinEase-STK from Cisbio) according to the manufacturer's instructions and as published previously.[35] Concentrations of ATP and substrate were set around their respective K_m values (Supplementary Table 2) for each tested kinase and mutant variant. Each determination was replicated at least three times and in duplicate.

Binding Assay with BODIPY-Labeled Compounds. Five hundred nanograms of wild type Ark1 or mutant versions (purified together with Pic1) was incubated with the indicated compounds at 5 μM in a buffer containing 50 mM Tris-HCl pH 6.8, 100 mM NaCl, 1 mM DTT, 20 mM $MgCl_2$ for 5 min at 32 °C; a second compound at 5 μM was added, and the mixture was incubated for an additional 5 min at 32 °C before addition of SDS-sample buffer and boiling at 95 °C for 10 min. Samples were separated on a 10% SDS-polyacrylamide gel, and fluorescence of **5** and **6** was detected by scanning the gel in a VersaDoc imaging system (BioRad; 530 BP filter and blue laser). As a loading control for Ark1, the gel was stained with Coomassie.

For the binding assay with wash-out of compounds, 4 μg of Ark1-as3-cys was bound to Ni-NTA agarose beads (Qiagen). After the first incubation with the indicated compounds at 5 μM, the Ni-NTA-coupled kinase was washed twice with a 10 times excess of buffer (50 mM Tris-HCl pH 6.8, 100 mM NaCl, 1 mM DTT, 20 mM $MgCl_2$) and then incubated with **5** at 5 μM concentration.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

This material is available free of charge *via* the Internet at http://pubs.acs.org.

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■ ACKNOWLEDGMENTS

We thank E. Illgen for excellent technical support, S. Graf for help with immunoblotting, and J. Kamenz for generating the *bub1+-mCherry* strain. The work was supported by the German Federal Ministry for Education and Research through the German National Genome Research Network-Plus (NGFN-Plus) (Grant No. BMBF 01GS08104 to D.R.) and by the Max Planck Society. MSD, Bayer HealthCare, Merck-Serono and Bayer CropScience are thanked for financial support.

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Supplementary Information

A chemical genetic approach for covalent inhibition of analog-sensitive Aurora kinase

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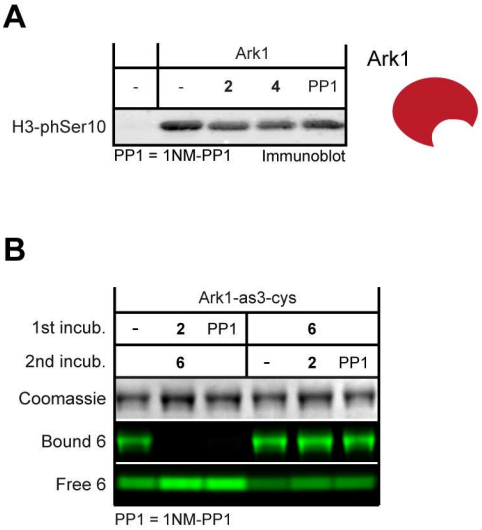
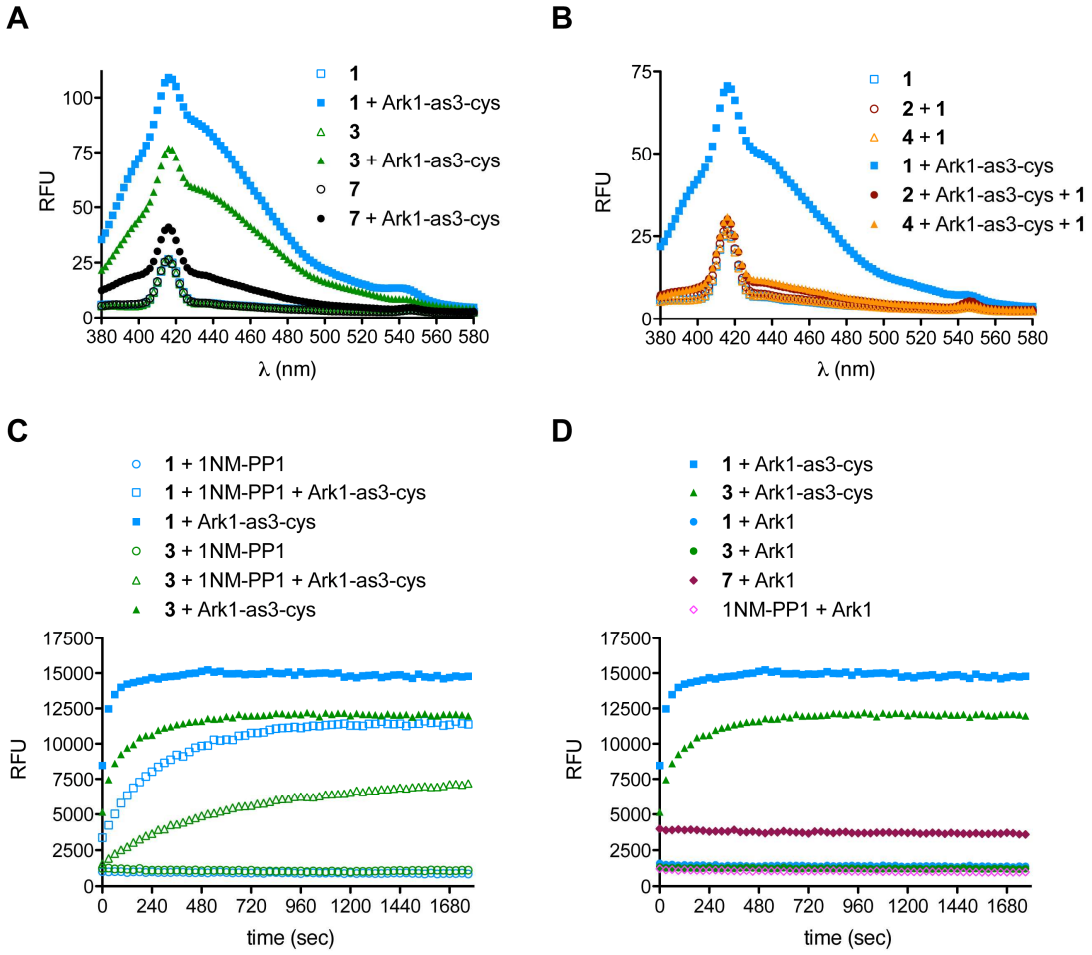
Supplementary Information



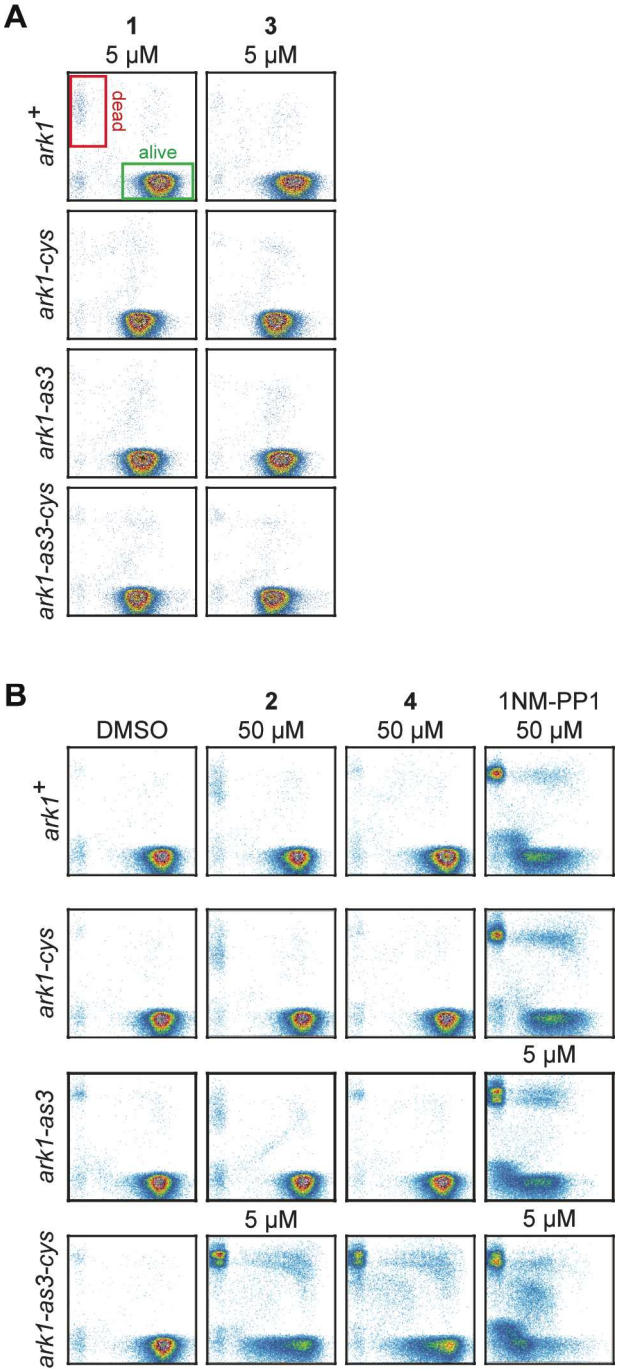
Supplementary Figure 1 Additional characterization of compounds **2**, **4**, and **6**.

(A) Compounds **2** and **4** do not inhibit the kinase activity of wild type Ark1 in a biochemical assay. Kinase assays were performed with recombinant Ark1 that was incubated with 5 µM of the indicated compounds prior to the addition of the substrate (histone H3). The reaction mixtures were separated by SDS-PAGE and immunoblotted with a phospho-specific antibody against Ser10 in histone H3 (H3-phSer10). **(B)** Compound **2** competes for binding of **6** when added before **6**, but not when added after **6**. Recombinantly produced Ark1-as3-cys was pre-incubated with **2**, **6**, or 1NM-PP1 for 5 minutes (1st incub.) followed by incubation with **2**, **6**, or 1NM-PP1 for 5 minutes as indicated (2nd incub.). Reaction mixtures were separated by SDS-PAGE. Ark1 was detected by Coomassie staining. The amount of fluorescent compound **6** co-migrating with Ark1 ('Bound') or present in the running front of the SDS-gel ('Free') respectively are shown. Unlike with **5**, 1NM-PP1 is able to compete for binding of **6**, but only when added before, not when added after **6**. This likely reflects the requirement of **6** to access both selectivity filters for efficient binding.



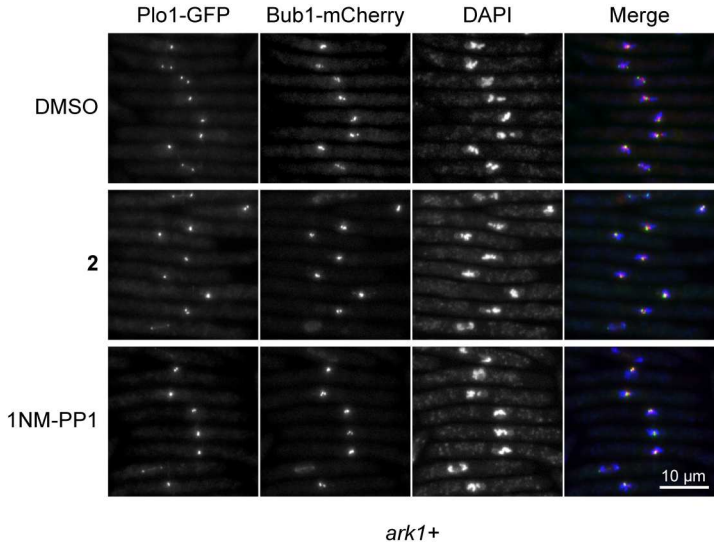
Supplementary Figure 2 Binding of compound **1–4** to Ark1-as3-cys analyzed by fluorescence emission. **(A)** Emission spectra of the quinazoline scaffold of **1** and **3** were measured at 380-580 nm. Emission intensity was significantly increased only when the irreversible inhibitor formed a covalent bond with the Cys anchor point in the ATP-binding site. For **7**, the reversible counterpart of **1** (Supplementary Figure 5), the slight increase in the emission spectra was not due to covalent bond formation since this amplification was also observed when treating Ark1 WT with **7** (data not shown; and see (D)). RFU, random fluorescent units. **(B)** The covalent bond formation of **2** and **4** could not be monitored by fluorescence emission, since these compounds do not change their fluorescent properties upon binding to the protein. To investigate whether these compounds bind to Ark1-as3-cys as proposed, we pre-incubated the kinase with **2** or **4** for 30 min and subsequently added **1**. After another 60 min the change in fluorescence emission was monitored, revealing that **1** alone led to increased fluorescence intensity whereas in combination with **2** or **4** no significant increase was observed providing clear evidence for covalent binding of **2** and **4** to Ark1-as3-cys.

(C) Monitoring fluorescence emission at 420 nm over time allowed for the real-time kinetic analysis of the irreversible inhibition of the Ark1-as3-cys by **1** and **3**. Since the covalent binding event was too fast, competition experiments were performed with 1NM-PP1 to slow down the initial reaction rate. **(D)** Non-specific Cys labeling of Ark1 by **1** and **3** was assessed by monitoring covalent bond formation in real-time over a period of 30 min. Covalent bond formation of **1** and **3** with Cys sulfhydryl of Ark1-as3-cys was detected by an increase in fluorescence emission at 420 nm. Addition of **1**, **3**, **7** or 1NM-PP1 to Ark1 did not result in a time-dependent fluorescence change, suggesting that these do not react non-specifically with solvent-exposed Cys residues.



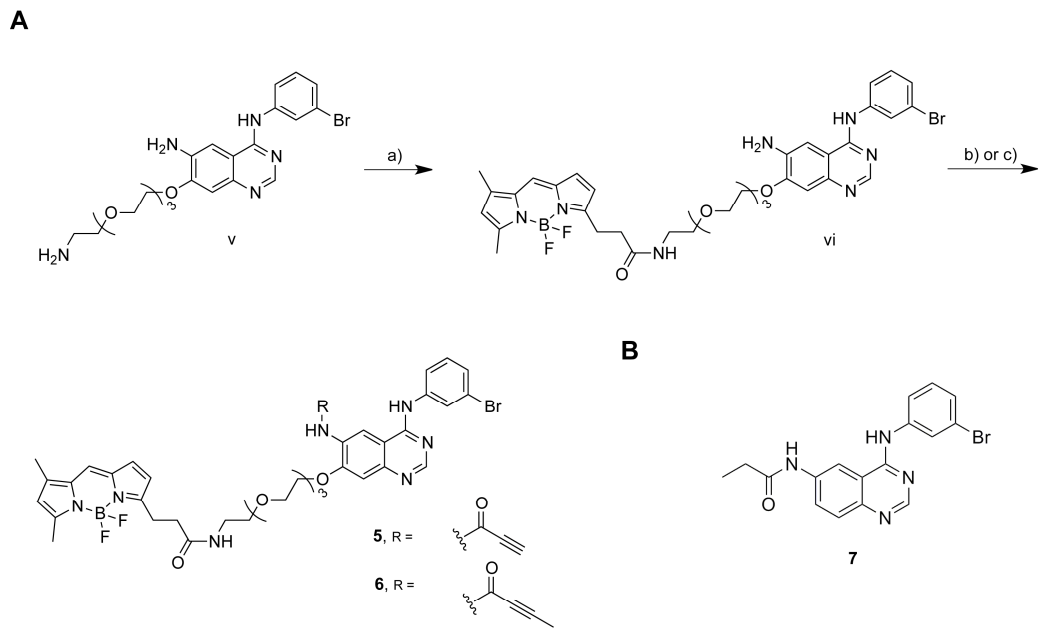
Supplementary Figure 3 Effect of different concentrations of compounds **1** – **4** on cellular viability.

(A, B) Cells in logarithmic growth phase expressing *ark1+*, *ark1-cys*, *ark1-as3* or *ark1-as3-cys* were grown for 4 hours in presence of 5 µM or 50 µM of the indicated compounds, stained with 'Yeast viability kit' (Partec) and 50,000 cells of each treatment were measured in a flow cytometer. Viable cells cluster in the lower right (green box), and dead cells in the upper left (red box) of the scatter plot.



Supplementary Figure 4 Representative pictures of *ark1+* cells from the experiment shown in Figure 4C.

Shown are the Plo1-GFP signal (green in the merged picture), the Bub1-mCherry signal (red in the merged picture) and DNA stained with DAPI (blue in the merged picture).

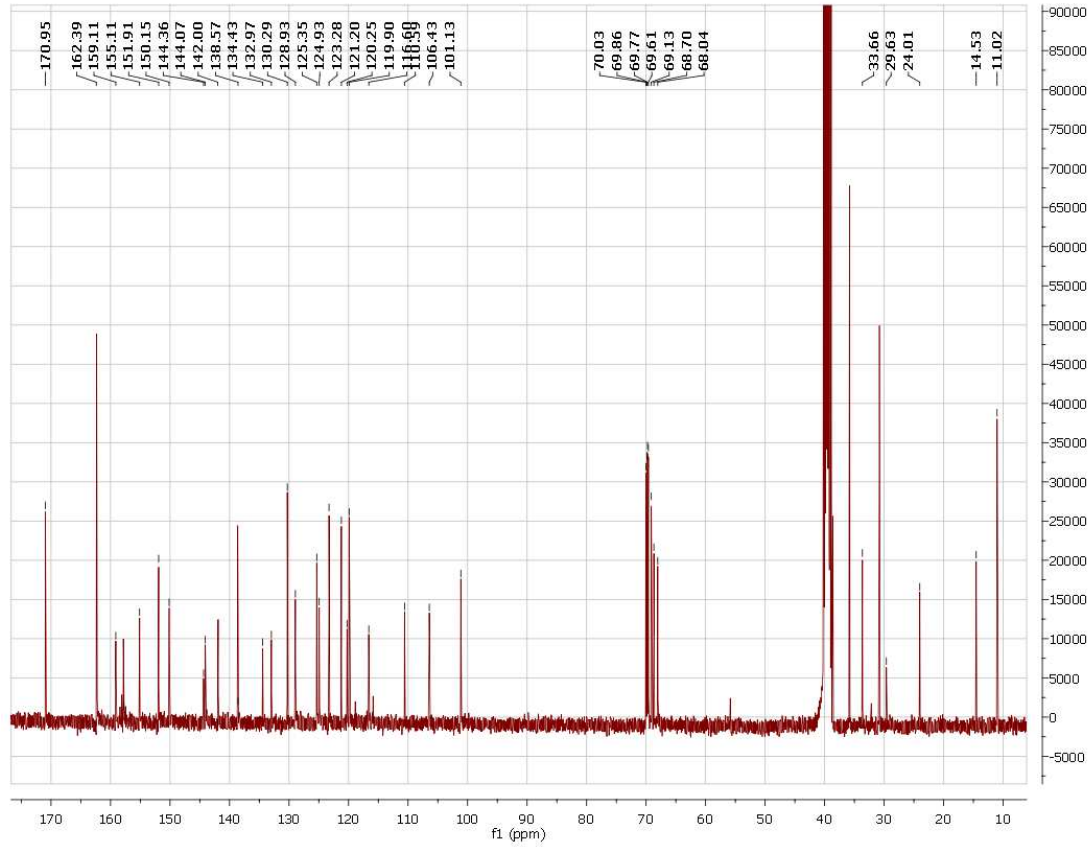


Reagents and conditions: a) 4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-propionic acid, succinimidyl ester, DIPEA, DMF, rt; b) Propiolic acid, EDCI.HCl, 0 °C, DMF, rt; c) 2-Butynoic acid, EDCI.HCl, 0 °C, DMF, rt.

Supplementary Figure 5 Synthesis of fluorescent probes (**5**, **6**), shown in (A), and structure of compound **7**, shown in (B).

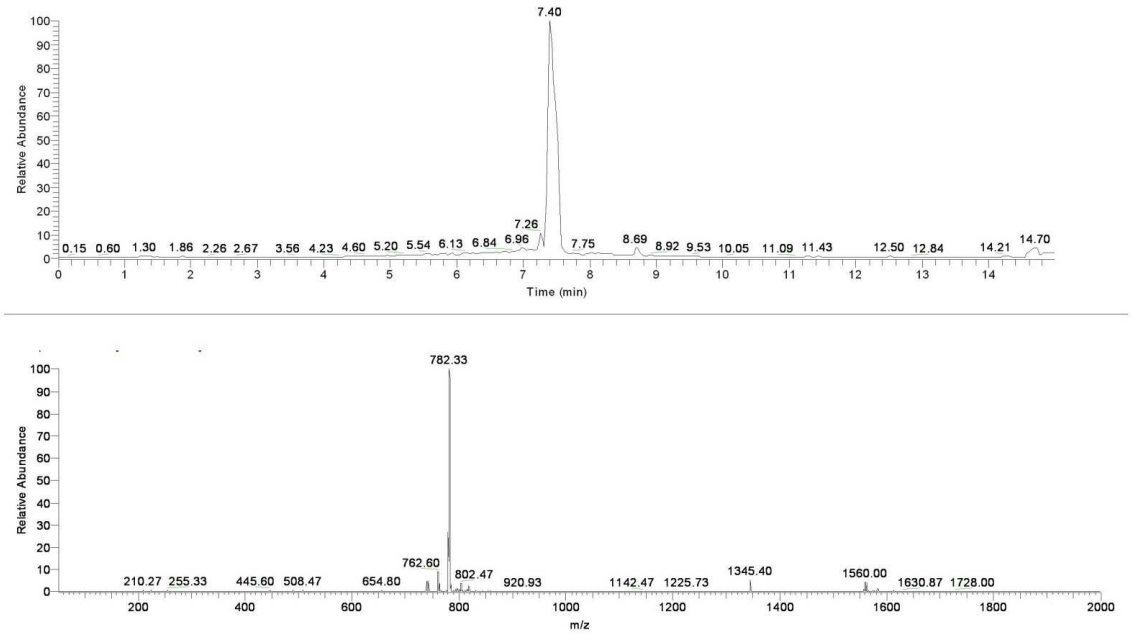
A

B



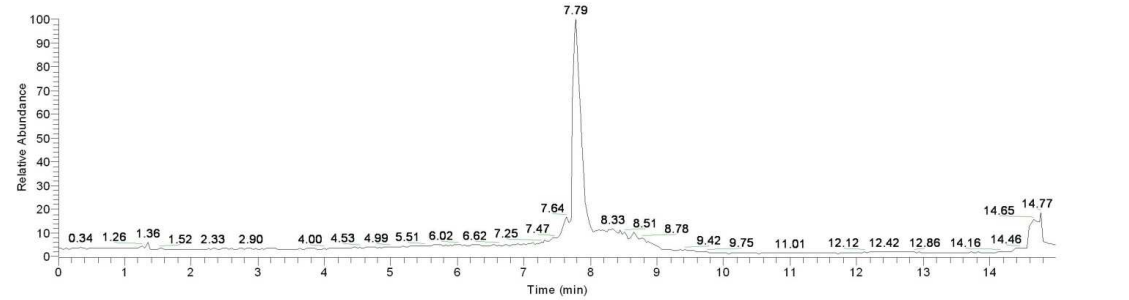
Supplementary Figure 6 (A) ^{1}H-NMR and (B) ^{13}C-NMR data of **vi**.

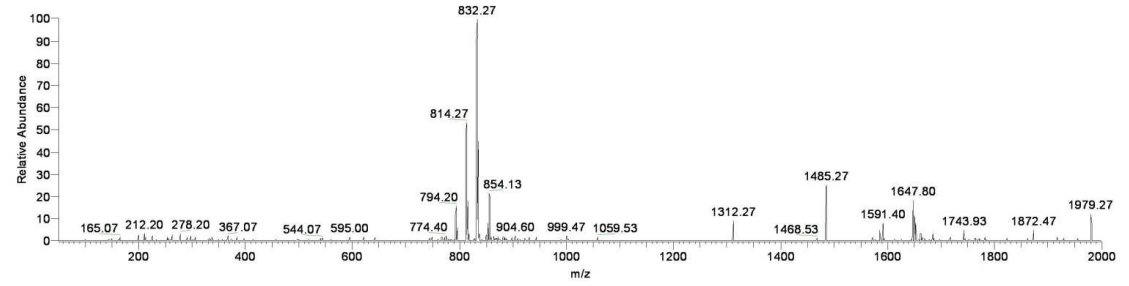
A


Relative Abundance
Time (min)
7.40
7.26
6.96
0.15 0.60 1.30 1.86 2.26 2.67 3.56 4.23 4.60 5.20 5.54 6.13 6.84 7.75 8.69 8.92 9.53 10.05 11.09 11.43 12.50 12.84 14.21 14.70

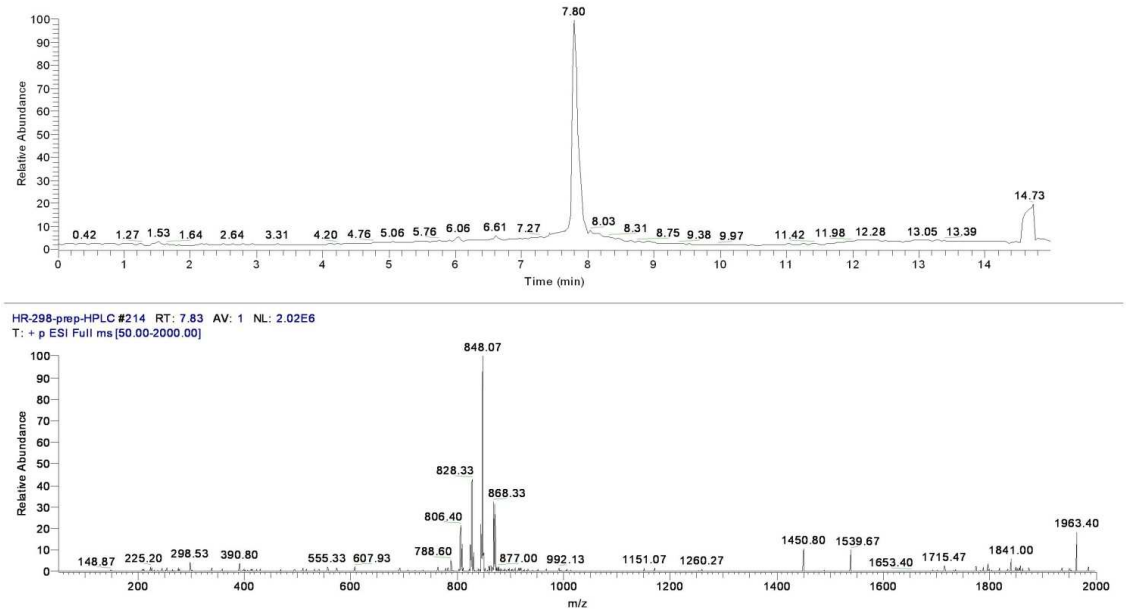
Relative Abundance
m/z
782.33
762.60
802.47
210.27 255.33 445.60 508.47 654.80 920.93 1142.47 1225.73 1345.40 1560.00 1630.87 1728.00

B


Relative Abundance
Time (min)
7.79
7.64
7.47
14.65
14.77
0.34 1.26 1.36 1.52 2.33 2.90 4.00 4.53 4.99 5.51 6.02 6.62 7.25 8.33 8.51 8.78 9.42 9.75 11.01 12.12 12.42 12.86 14.16 14.46


Relative Abundance
m/z
832.27
814.27
854.13
794.20
1485.27
1647.80
1312.27
1591.40
1979.27
165.07 212.20 278.20 367.07 544.07 595.00 774.40 904.60 999.47 1059.53 1468.53 1743.93 1872.47

C



Supplementary Figure 7 LCMS data of **vi** (A), **5** (B) and **6** (C).

Supplementary Table 1 IC_{50} values of investigated compounds.

	IC_{50} (μM)			
	Ark1	**Ark1 + Pic1**	**Ark1-as3-cys**	**Ark1-as3-cys + Pic1**
1	≥ 20.0[a]	≥ 20.0[a]	0.004 ± 0.003	0.002 ± 0.001
2	> 20.0	> 20.0	0.010 ± 0.008	0.001 ± 0.001
3	15.3 ± 1.15	> 20.0	0.012 ± 0.008	0.009 ± 0.007
4	nb[b]	nb[b]	0.004 ± 0.003	0.006 ± 0.002
7	> 20.0	nb[b]	0.272 ± 0.171	0.349 ± 0.147
1NM-PP1	nb[b]	nb[b]	0.006 ± 0.004	0.001 ± 0.001

[a] precipitation of compound at concentrations > 20 µM

[b] nb = non binding: no inhibitory effect at 50 µM

Supplementary Table 2 ATP- and substrate-K_m of Ark1 and Ark1-as3-cys.

	ATP-K_m (μM)	**substrate-K_m (μM)**
Ark1	9.43 ± 0.58	0.103 ± 0.014
Ark1 + Pic1	28.2 ± 4.58	0.054 ± 0.018
Ark1-as3-cys	12.8 ± 1.89	0.207 ± 0.014
Ark1-as3-cys + Pic1	13.7 ± 1.37	0.147 ± 0.018

Supplementary Methods

Measurement of fluorescence emission spectra. Emission spectra were measured on a JASCO FP-6500 fluorescence instrument using 4.5 mL cuvettes (4 clear sides; Carl Roth) containing a suspension of compound (0.15 µM) in the presence and absence of kinase (0.1 µM) in buffer containing 50 mM Tris, pH 7.5, 150 mM NaCl, 0.005% Triton X-100. Inhibitors were pre-incubated with kinase for 60 min to ensure equilibrium was reached.

Real-time detection of covalent bond formation in microtiter plates. The assay was carried out in black 384-well small volume plates and fluorescence emission was measured over time using a TECAN Infinite M1000 microtiter plate reader. Sample wells were excited at 368 nm (slits = 5 nm) and the changing emission intensity was monitored at 420 nm (slits = 20 nm). The reaction was monitored immediately after combining equal volumes (7 µL) of Ark1-as3-cys (1 µM) and inhibitor (1 µM) solution in each well. Competition experiments were carried out by incubating the respective kinase with 1 µM 1NM-PP1 for 15 min prior to adding the inhibitor of interest. In all cases, kinase and inhibitor solutions were prepared using the same buffer described above for measuring fluorescence emission spectra in cuvettes.

3 Discussion

In the presented studies I (i) identified new substrates of the fission yeast Aurora kinase Ark1 and (ii) engineered the kinase to make it susceptible to a covalent inhibitor.

3.1 SILAC and chemical genetics are suited for the identification of Aurora substrates in fission yeast

To identify mitotic Aurora-dependent phosphorylation sites, we compared the mitotic phosphoproteome in the presence and absence of Aurora activity (Part 2.1). Overall we identified 8254 phosphorylation events on a total of 1544 fission yeast proteins, which is to date the most comprehensive fission yeast phosphoproteome dataset available (Part 2.1, Fig. S1) (Wilson-Grady et al., 2008; Beltrao et al., 2009). By direct quantitative comparison using SILAC, we identified 70 probable Aurora phosphorylation sites on 42 proteins. Among these were the known fission yeast Aurora targets histone H3, Cnd2, and Bir1 (Petersen et al., 2001; Nakazawa et al., 2011; Leverson et al., 2002). Among the 42 proteins were also Pic1 (INCENP) and Sgo2, which are both known as interaction partners of Aurora in fission yeast (Leverson et al., 2002; Vanoosthuyse et al., 2007) and have been reported as substrates in other organisms (Bishop and Schumacher, 2002; Tanno et al., 2010). The fact that we found these known targets shows the practicality of our approach.

On the basis of work in other organisms, Aurora phosphorylates several conserved outer kinetochore proteins (Cheeseman et al., 2002; DeLuca et al., 2006; Welburn et al., 2010), and of these we identified Spc7 (human KNL1/Blinkin). Ndc80/Hec1 is an Aurora substrate in budding yeast and humans (DeLuca et al., 2006; Akiyoshi et al., 2009) with six potential Aurora target sites (based on the Aurora phosphorylation motif [RK]-X-[ST][ILV] (Cheeseman et al., 2002)) in the conserved N-terminal region, but Ndc80 was absent in our list of phosphoproteins. Aurora is known to act in the spindle assembly checkpoint (SAC) (Hauf et al., 2003; Kallio et al., 2002; Musacchio, 2011), but we could not detect any Aurora-dependent phosphorylation site that may explain its role.

The absence of expected targets could be due to interspecies differences, i.e. Aurora does not phosphorylate the same set of proteins in fission yeast as in other organisms, or the failure to identify known or expected targets could be due to experimental and/or technical constraints, which will be discussed in the following sections (see 3.2 – 3.5).

On the other hand, our study identified a number of Aurora substrates that were unexpected. Although chromosome condensation and chromosome segregation defects are the dominant phenotypes of Aurora inhibition in mitosis, only a fraction of the identified substrates are known or expected to play a role in these processes. This raises the question whether our study falsely identified substrates, or whether Aurora has previously unknown roles in mitosis, which is also discussed below (see 3.6 – 3.7).

3.2 Could the experimental setup of the SILAC study have been improved?

Aurora has many different functions during mitosis and many of its substrates will only be modified under certain conditions or for a certain period of time during mitosis. Hence, the stage at which cells are harvested determines the spectrum of substrates that can be identified. Since our analysis was performed with early mitotic cells, we could not expect to find substrates for Aurora's potential function during late mitosis (Norden et al., 2006; Carmena, 2008; Steigemann et al., 2009; Carlton et al., 2012). Even during early mitosis, some substrates may only be transiently phosphorylated. For example: in order to regulate kinetochore-microtubule attachment, Aurora phosphorylates kinetochore proteins, but only as long as chromosomes have not yet achieved amphitelic attachment (Liu et al., 2009). These phosphorylations become removed by counteracting phosphatases once chromosomes have achieved proper amphitelic attachment (reviewed in (Kelly and Funabiki, 2009)). Hence, one would be unable to identify the relevant substrates if one uses cells, where chromosomes have already become properly attached. In one of the experimental conditions that we used (Part 2.1, Fig. 1A, Exp.1, unperturbed mitosis), this likely was the case for many cells. We therefore included a second experimental condition (Part 2.1, Fig. 1A, Exp. 2, addition of the microtubule-depolymerizing drug MBC), which prevented kinetochore-microtubule attachment. This results in a reduced physical distance of Aurora and its

substrates and we expected to identify kinetochore proteins implicated in microtubule attachment as Aurora substrates in this experiment. This was not the case and several reasons could explain such a result: (i) there are no such targets present in fission yeast, (ii) the targets were not identified due to technical constraints (discussed in later section), or (iii) the microtubule depolymerisation leads to a reduced physical distance of Aurora and its substrates but not to an increased phosphorylation. It might be possible that Aurora substrate specificity on several targets depends on the presence of microtubules or factors that are transported by microtubules to the kinetochore. In *Xenopus* the inner centromere protein telophase disc-60kD (TD-60) and microtubules are required for the activation of Aurora (Rosasco-Nitcher et al., 2008). So far it is speculative if Aurora phosphorylates specific substrates only in the presence of microtubules. The fact that Aurora inhibition in the presence of microtubule-depolymerizing drugs or microtubule mutants still lead to a phosphatase dependent mitotic exit indicates that not all substrates are influenced by the presence of microtubules (Hauf et al., 2003; Vanoosthuyse and Hardwick, 2009). So far no ortholog of TD-60 is known in fission yeast.

As an alternative to our approach it may have been beneficial to use an additional condition in order to achieve attachment of microtubules without the physical separation of Aurora and its targets. This would have the advantage that microtubule-associated proteins can still localize properly to microtubules instead of being delocalized when microtubules are absent. One possibility to do so is the formation of monopolar attachments. In human cells monopolar attachments are achieved by adding the mitotic kinesin Eg5 inhibitor monastrol (Kapoor et al., 2000). In fission yeast, monastrol does not have the same potential but it was reported that a temperature sensitive allele of the Eg5 ortholog *cut7+* (*cut7-446*) led to monopolar spindle formation when grown at the restrictive temperature (Hagan and Yanagida, 1990). Due to the low mitotic index in *cut7-446* cells, which is a crucial parameter for the experiment, we did not consider using this allele. In our second study (Part 2.2), however, we showed that the combination of the *cdc25-22* allele together with the *cut7-446* allele resulted in cells with monopolar attachments and a high mitotic index (Part 2.2, Fig. 4C,D). It would be interesting to use this strategy in the SILAC approach. If this yields in the identification of

more Aurora targets is speculative, but it may be beneficial over a complete microtubule disruption.

To summarize, it would be optimal to sample different time points during mitosis or to perturb cell physiology in multiple ways, but the number of experiments is limited by the expenditure for these experiments.

3.3 Could the Aurora as-allele have been improved?

A frequent phenomenon of gatekeeper mutations in analogue-sensitive kinases is the reduction in kinase activity compared to their unmodified counterparts (Zhang et al., 2005; Cipak et al., 2011). Although replacing wild type *ark1+* with *ark1-as3* did not reduce the viability of cells, the activity of the engineered kinase in biochemical studies was reduced compared to the wild type kinase (Part 2.2; Suppl. Table 2). Because of the reduced kinase activity of Ark1-as3, some substrates may already be less phosphorylated even without addition of the inhibitor. This potentially decreases the total abundance of a phosphorylation and the difference between the 'kinase-active' and 'kinase-inactive' state. In particular low abundant phosphorylation sites may therefore be difficult to detect. Although we were performing a phosphopeptide enrichment step, some phosphopeptide species might still be too low in quantity so that they would never be 'chosen' for sequencing in the mass spectrometer. Hence, it may have been beneficial to increase the Aurora activity of the *ark1-as3* allele by introducing additional suppressor mutations (Zhang et al., 2005). This is not always successful (Zhang et al., 2005), and an alternative therefore would have been to use a different experimental setup, where *ark1+* and *ark1-as3* cells are directly compared with each other in the presence of 1NM-PP1. In such an experimental setup phosphorylations due to inhibitor side effects would be reduced in both samples and therefore not perturb the Aurora substrate identification. Experiment 3 (Part 2.1, Fig. 1A) would have become dispensable, but it would not have been possible to tell the identity of 1NM-PP1 off-targets. However, it is unlikely that this experimental design would have substantially increased the amount of Aurora-dependent phosphorylation sites identified, since both the number of phosphorylation sites and the percentage of these sites with an R-X-[ST] motif was

similar between Experiment 1 or 2 (with *ark1-as3*) and Experiment 3 (*ark1+*) (Fig. 5.1; Part 2.1, Fig 1B).

3.4 Could the phosphoproteome coverage have been improved?

3.4.1 Could the phosphoproteome coverage have been improved by using a different protease?

The identification of proteins and their PTMs by MS is mainly influenced by the characteristics of the peptides that result from protein digestion. The ionization of peptides, which is crucial for the detection in the mass spectrometer, is influenced by their physicochemical properties, such as weight, secondary structure, charge, propensity and hydrophobicity (Kawashima et al., 1999). Bioinformatic analyses of several MS proteome experiments led to the identification of so called proteotypic peptides, that are, experimentally observable peptides that uniquely identify a specific protein or protein isoform (Kuster et al., 2005). Such proteotypic peptides have properties that favor their detection in MS and are repeatedly identified in different MS runs when using the same protease for protein digestion.

The most commonly used proteases for protein digestion prior to MS are trypsin and Lys-C. Trypsin cleaves peptide chains at the carboxyl side of the amino acids lysine or arginine, except when either is followed by proline. Thus, tryptic peptides include either an arginine or a lysine residue at the C-terminus (except for the very C-terminal fragment of a protein). In the case of Lys-C, which cleaves peptide chains at the carboxyl side of lysine residues, digested peptides include a lysine residue at the C-terminus (except for the very C-terminal fragment of a protein). In a SILAC approach these characteristic cleavage properties are used to ensure that the digested peptides contain the isotope-labelled amino acids arginine and/or lysine. Digestion with trypsin or Lys-C can result in similar peptides when cleaved after lysine, whereas the cleavage of trypsin after arginine results in unique peptides. The peptides cleaved after arginine exhibit different physicochemical properties that may favor their detection in the mass spectrometer and thus result in a different subset of identified peptides and proteins. Coon and co-workers showed in a proteome MS study with budding yeast that it could indeed be beneficial to use more than one protease for protein digestion. The authors were

able to increase the total number of identified budding yeast proteins from 3030 (with Lys-C) to 3908 by using four additional proteases for protein digestion (Swaney et al., 2010). In addition to the increase in total number of identified proteins, the authors could also increase the overall proteome coverage from 10.5% (Lys-C) to 25.5% (all 4 proteases) (Swaney et al., 2010). In another budding yeast study, Mann and co-workers compared quantitative changes of the haploid versus the diploid proteome in a SILAC approach. They used either Lys-C or trypsin for digestion in an otherwise similar experimental setup. They identified 4399 proteins (combined data from Lys-C and trypsin), which was an increase of 10% and 18% when using only Lys-C (3987 proteins) or trypsin (3639 proteins), respectively (de Godoy et al., 2008). These studies showed that it is beneficial to use different enzymes in order to increase proteome and protein coverage. In our experiment, this would have doubled the amount of labelling experiments as well as MS runs. The possible gain in Aurora phosphorylation sites is accompanied by increased material costs and experimental time for sample preparation and measurement.

Using trypsin as protease would have required labelling with heavy isotope-labelled arginine, which would have brought up an additional problem. In several organisms it has been observed that $^{13}C_6$-arginine is metabolically converted to $^{13}C_5$-proline, resulting in a 5 Da heavier satellite peptide cluster in proline-containing peptides when measured by MS (Ong et al., 2003). This ‘arginine-to-proline conversion’ is prominent in fission yeast and also extends to glutamate, glutamine, and lysine (Bicho et al., 2010). This conversion can jeopardize the outcome of a SILAC experiment, as the heavy label cannot be reliably assigned to lysines anymore. Sawin and co-workers could almost eliminate this conversion problem in fission yeast by deletion of enzymes involved in arginine catabolism (Bicho et al., 2010). As these strains exhibit reduced cell viability, we did not consider using these strains.

3.4.2 Could the phosphoproteome coverage have been improved by using a different phosphopeptide enrichment strategy?

The success of a phosphoproteomic approach also highly depends on the ability to efficiently enrich for phosphopeptides in order to separate them from the unmodified peptide pool. The most frequently used strategies for phosphopeptide

enrichment are affinity-based, including immobilized metal affinity chromatography (IMAC), metal oxide affinity chromatography (MOAC), and strong cation exchange chromatography (SCX). Aebersold and co-workers compared the different enrichment methods and showed that they differed in their specificity and, notably, in the set of phosphopeptides isolated. This indicated that the three methods detect different, partially overlapping segments of the phosphoproteome (Bodenmiller et al., 2007). The same study also showed that technical replicates could increase the amount of detected phosphorylation sites by up to 25% per replicate (Bodenmiller et al., 2007). Nonetheless this depends on the complexity of the starting material and might reach a plateau after several measurements.

We used a combination of SCX followed by MOAC (TiO_2) for phosphopeptide enrichment and performed three technical replicates for each of the three biological experiments. We found one-third of the fission yeast proteome being phosphorylated. Given that 30% of a eukaryotic proteome was estimated to be phosphorylated (Cohen, 2000) shows the success of our approach. Nevertheless, it would have been beneficial to use IMAC in addition to MOAC, as both showed a phosphorylation site overlap of only about 35% in comparative studies (Bodenmiller et al., 2007; Jensen and Larsen, 2007). Again, it was a matter of time, biological material and material costs that made us decide for a single method that has been used successfully in prior phosphoproteomic studies (Olsen et al., 2006; Macek et al., 2008; Beltrao et al., 2009; Bodenmiller et al., 2010).

3.5 Concluding remarks about technical and experimental constraints

As discussed above, many decisions have to be made that all influence the success of a phosphoproteomic study. In summary, the combination of various proteases, phosphopeptide enrichment strategies, and biological experiments would increase the number of identified phosphorylation sites. As much as we would have liked to use a combination of all these methods, decisions had to be made in order to perform these experiments within a reasonable time-scale and with practicable costs. Advances and developments on methods and MS techniques will probably help to increase the amount of detected phosphopeptides in the near future. Nevertheless, a comprehensive catalogue of the total phosphoproteome of an organism might still be an unreachable goal (Meyer et al.,

2011), as factors that lie in the nature of the proteins themselves such as their abundance or their physicochemical properties might always hinder detection by MS.

3.6 Newly identified targets

Apart from already known substrates such as histone H3, Bir1, Pic1, Sgo2 and the recently reported targets Cnd2 and Spc7, we identified a high number of unexpected targets. Examples are Orc1, Ctf18, Elg1, Mra1, Png2, SPBC28F2.11 (Part 2.1, Fig. 3). Among the new candidate substrates are heterochromatin proteins and nucleolar proteins, as well as proteins implicated in the posttranslational modification of histones, in DNA replication, in DNA damage signalling, in RNA processing, and in nucleocytoplasmic transport (Part 2.1, Fig. 3). These processes have previously not been linked to Aurora (Vader and Lens, 2008). Nevertheless, we have reasons to believe that these proteins are indeed targets of Aurora in fission yeast, as we (i) have repeatedly detected these phosphorylation sites in cells with active Aurora, (ii) have repeatedly found less of the phosphorylated peptide in Aurora-inhibited cells, and (iii) did not find significant changes in the abundance of the phosphorylation of these sites when testing for nonspecific effects of the inhibitor (Part 2.1, Fig. 1, Exp. 3). It is still possible that some of these proteins are not direct Aurora substrates, but secondary targets. However, we were able to identify a common motif for most of these sites (R-X-[ST]; Part 2.1, Fig. 2), and most of the substrates that we tested (8 of 11) were phosphorylated by Aurora *in vitro* on the same sites identified *in vivo* (Part 2.1, Fig. 3 and table S5). In addition two out of three candidate targets whose *in vivo* sites we could not confirm *in vitro*, were phosphorylated on different sites that matched the consensus motif. These data indicate that at least a subset of the identified sites that exhibited reduced phosphorylation upon fission yeast Aurora inhibition are directly targeted by Aurora.

The spectrum of candidate substrates that we identified suggests that Aurora influences chromatin dynamics during mitosis more broadly than previously thought. Given the generally high conservation in function between yeast Aurora and metazoan Aurora B, we speculate that such influence on chromatin may also occur in metazoans. Indeed, two other recently published studies on Aurora

substrate identification showed comparable results. In a similar inhibitor-based quantitative phosphoproteomic study, Gerber and co-workers (Kettenbach et al., 2011) found several new Aurora substrates in mitotic HeLa cells that are implicated in processes such as DNA damage repair and RNA processing. In another study, Lens and co-workers identified numerous potential Aurora substrates with roles in DNA damage response, DNA repair, transcription, RNA processing and translation (Hengeveld et al., 2012). Hence, these two studies identified potential Aurora kinase substrates that are involved in similar cellular processes as those we have found (Part 2.1, Fig. 3). Although this indicates that Aurora in fission yeast and human cells are involved in regulating similar processes or pathways, the number of orthologous targets is low. One of these targets might be the HMG (HMG = high mobility group) protein (SPBC28F2.11), which we validated as a target of Aurora in fission yeast in our screen (Part 2.1, Fig. 3 and Table S5). Lens and co-workers for example validated a chromatin-associated protein HMGN2 and HMGN5 as a target of Aurora B (Hengeveld et al., 2012). However, if the phosphorylation of these three proteins by Aurora fulfils the same cellular function is yet unknown. That phosphorylation sites do not have to be necessarily conserved was shown in a comparative phosphoproteomic study from Krogan and co-workers (Beltrao et al., 2009). They investigated the conservation of phosphorylation sites in the three yeast species *S. pombe, S. cerevisiae and C. albicans*. The results in this study indicated that although specific phosphorylation sites on orthologous proteins often diverge, the overall phosphorylation status of a protein complex or proteins implicated in the same pathway stays constant (Beltrao et al., 2009). The low direct conservation of phosphorylation sites might be caused by the fact that phosphorylation sites are enriched in disordered protein regions, which, in general, are less conserved than ordered regions (Gnad et al., 2007; Jimenez et al., 2007). Nevertheless, other studies have shown that there might indeed be an evolutionary conservation of phosphorylation sites (pSer/pThr/pTyr), which is higher than for their non-modified partners (Ser/Thr/Tyr) (Boekhorst et al., 2008; Malik et al., 2008; Ba and Moses, 2010; Chen et al., 2010). The ever-rising number of phosphoproteomic data (Lemeer and Heck, 2009) might allow more comprehensive studies and will help to shed light onto the evolutionary conservation of phosphorylation sites. This in turn may be helpful to elucidate the evolution of different Aurora targets. An important

step in the future will be first to test if Aurora indeed is implicated in the proposed functions in different organisms, and second, if the targets are the same or have been evolved to other proteins in the same cellular process or pathway.

3.7 Why have the new Aurora functions previously been unknown?

Although we identified new Aurora targets and showed that Aurora mutants have an increased DNA damage sensitivity (Part 2.1, Fig. 5A), it is not yet clear which precise functional consequence the newly identified phosphorylations have. Aurora inhibition leads to severe chromosome condensation defects, missegregation of chromosomes, and cytokinesis failures (Vader and Lens, 2008). However, several of the new targets (e.g. Orc1, Elg1, Ctf18) that we identified are implicated in DNA replication. It is surprising to see that the Aurora kinase, which is specifically active during mitosis (Bischoff et al., 1998; Petersen et al., 2001), may influence this process. For a better understanding of the function of the Aurora-dependent phosphorylations it will be important to know when these phosphorylations occur and when they are removed. The generation of phospho-specific antibodies or MS analysis (e.g. immunoprecipitation or phosphoproteomic studies at different cell cycle stages) are possibilities to address the dynamic state of protein phosphorylation. The data in our study allowed us to make the statement that the phosphorylation of these proteins was present during mitosis. Since Aurora only becomes active during mitosis, it is unlikely that its targets are already phosphorylated in G2. Indeed a new phosphoproteomic study from our lab revealed that none of the detected Aurora phosphorylation sites (including histone H3, Orc1, Ctf18, Elg1) was phosphorylated during G2 (Graf, 2011). The auto-phosphorylation of Ark1 on Thr244 (Walter et al., 2000) that is necessary for its activity was shown to persist into S phase (Graf, 2011). This is not surprising as Aurora activity is important for proper cytokinesis and in fission yeast cytokinesis happens after S phase has started (Bostock, 1970). Indeed, several Aurora-dependent target phosphorylation sites remained phosphorylated until S phase (Graf, 2011). If the persistence of these phosphorylations was due to lack of counteracting phosphatase activity, or due to permanent activity of Aurora on the sites needs to be elucidated. Immunoprecipitated Aurora from G1, S, or G2 phase

was, for example, not able to phosphorylate histone H3 (Petersen et al., 2001). Indeed, the phosphorylation of histone H3 was mitosis-specific and this could indicate that substrate specificity of Aurora might change from mitosis to G1 and S phase (Graf, 2011).

The circumstance that the newly suggested Aurora functions have not been reported before could have several reasons. The inability to phosphorylate the targets (i) causes the same phenotype as was already known for Aurora inhibition, (ii) leads to defects in later stages of mitosis, or the cell cycle, that are covered by effects early in mitosis, or (iii) has only a mild or no detectable phenotype at all. Phosphorylation of the targets by Aurora may influence the localization, the affinity to other proteins or structures, or the enzyme function of a protein. The mitotic phosphorylation of three Aurora sites in Cnd2, for example, ensures the localization of the condensin complex to mitotic chromosomes (Nakazawa et al., 2011). Mutation of these phosphorylation sites to alanine exhibited the same condensation phenotype as Aurora inhibition (Nakazawa et al., 2011). If these phosphorylations disrupt condensin complex formation or influence Cnd2 function in a different way has not been addressed. It is possible that the phosphorylation of proteins involved in DNA replication (Orc1, Ctf18, Elg1) during mitosis is also important for their localization. Loading these proteins onto DNA during mitosis might be a prerequisite for the following S phase after sister chromatids have segregated. Apart from loading, Aurora could also influence the function of these proteins and might regulate their temporal activity. Hence Aurora might be important for proper start or execution of DNA replication. The problem investigating these functions is that mitotic Aurora inhibition leads to severe chromosome condensation and segregation defects. In fission yeast the majority of cells exhibit a ‘cut’ (cell untimely torn) phenotype after Aurora inhibition (Part 2.2, Fig. 3B,C) (Leverson et al., 2002; Petersen and Hagan, 2003). Since S phase usually starts after sister chromatids have been segregated, the severe defects and resulting cell death after the cells have divided prematurely make it difficult or impossible to judge DNA replication in these cells.

Mutational analysis of Aurora phosphorylation sites in the newly identified substrates implicated in DNA replication may be helpful in order to investigate the effect Aurora has on DNA replication. The phosphomimicking mutants of Aurora phosphorylation sites in Cnd2 largely rescued the condensation defect seen after

an Aurora inhibition (Nakazawa et al., 2011; Tada et al., 2011) and showed a reduced amount of cells exhibiting a ‘cut’ phenotype. The *cnd2* phosphomutant cells might allow monitoring the effects of Aurora inhibition on S phase. Given that the *cnd2* mutants showed a hypercondensation of mitotic chromosomes could nonetheless complicate the interpretation of the result (Nakazawa et al., 2011; Tada et al., 2011).

Instead of localizing the proteins to chromatin, Aurora-dependent phosphorylations on DNA replication and chromatin proteins may also have the opposite effect. Phosphorylation could destabilize the binding of the proteins to chromatin and therefore ‘strip off’ protein complexes from DNA so that they do not hinder the segregation of sister chromatids. Such a ‘cleansing’ role has been predicted for the condensin and cohesin complex (Yanagida, 2009) and might be influenced by Aurora. If DNA replication factors indeed have to be ‘cleansed’ from DNA prior to chromosome segregation, an inability to do so could cause missegregation defects as have been reported for Aurora inhibition. Mutational analysis of phosphorylation sites on the target proteins and determination of missegregation defects could clarify if the inability of removing these proteins from chromatin could impair chromosome segregation.

So far only a few phosphorylation site mutants are available and therefore it remains speculative what the cellular function of the phosphorylations is. The genetic interaction approach we have taken might be promising in order to elucidate the cellular role of phosphorylation events (Part 2.1, Fig. 4 and Fig. 5A). In the case of Orc1, the presence of the phosphomutants alone exhibited no obvious phenotype when we investigated DNA damage sensitivity (Part 2.1, Fig. 4D). This might lead to the conclusion that these phosphorylation sites are of no functional importance. However, in combination with a gene deletion of the important DNA damage sensor protein Chk1 (Chen and Sanchez, 2004), the phosphomutants of *orc1* exhibited enhanced sensitivity against DNA damaging reagents (Part 2.1, Fig. 4D). This indicates that effects of the phosphomutants are not detectable at first glance, but need specialized assays (e.g. combination of mutants; drug sensitivity tests) in order to elucidate their cellular function.

The increased DNA damage sensitivity of Aurora mutants (Part 2.1, Fig. 5) has not been reported before. A temperature-sensitive allele of the CPC member Bir1 (*cut17-275*), however, exhibited increased sensitivity to hydroxyurea (DNA

replication inhibitor) and UV-irradiation (causing DNA damage) even when cultured at permissive temperature (Morishita et al., 2001). Yanagida and co-workers showed that DNA repair in *cut17-275* cells was defective, as thymine dimers resulting from UV-irradiation were not removed efficiently compared to wild type cells. Besides this DNA repair defect, *cut17-275* cells exhibited condensation defects and impairment of late anaphase spindle extension when grown at restrictive temperature. In addition to these phenotypes, Ark1 localization was also abolished in *cut17-275* cells. This is not surprising as knockdown of any member of the CPC by RNAi delocalizes the other subunits and results in similar mitotic phenotypes (Honda et al., 2003). These data suggest that there is indeed a role of Aurora and the CPC in DNA damage response. Additional experiments have to show if this effect is due to delocalization of the CPC *per se* or due to missing phosphorylations on target proteins as a consequence of Aurora delocalization. Mutational analysis of target phosphorylation sites in combination with DNA damage sensitivity assays will help to clarify Aurora's role in the DNA damage response in the future.

3.8 Covalent inhibition as a way to increase inhibitor specificity

The development of small molecule inhibitors targeting kinases has made a significant contribution in understanding their cellular function. However, for the majority of kinases no specific small molecule inhibitor has been found. In other cases, inhibitors are not specifically targeting a single kinase, but target similar kinases of the same family or even unrelated kinases (Bain et al., 2003). Shokat and co-workers solved both problems by mutating the kinase of interest at a residue called the gatekeeper (Bishop et al., 2000). This made the engineered kinase, also called analogue-sensitive (as) kinase, susceptible for 1NM-PP1 or similar derivatives of the kinase inhibitor PP1 (Hanke et al., 1996). I have used this method to create an as allele of the fission yeast Aurora kinase (Ark1-as). Although only cells harbouring the *ark1-as3* allele were sensitive to treatment with 5 µM 1NM-PP1 and although none of the 109 kinases in fission yeast harbours a small-gatekeeper such as alanine or glycine, a control experiment to identify possible side effects (targets) of the inhibitor on wild type cells was performed. We could indeed identify phosphorylation sites that were downregulated after inhibitor

treatment in control cells (Part 2.1, Fig. 1B). This clearly shows the necessity of such a control experiment, as without this we would have falsely assigned such phosphorylation sites to be dependent on Aurora inhibition. Although not being the first study to use the combination of an as-kinase and 1NM-PP1 in order to identify kinase targets (Holt et al., 2009), this was the first study controlling for the side effects of the inhibitor. In general, proteomic studies with small molecule inhibitors should always be performed with control experiments to rule out side effects.

To make inhibition of Aurora yet more specific and thus reducing side effects of potential inhibitors, we decided to develop a covalent inhibitor, which requires two determinants for binding and thus may be more specific. This approach has been applied before to the tyrosine kinase EGFR (Blair et al., 2007), but not to a Ser/Thr kinase such as Aurora.

In general the approach is based on two selectivity filters, the gatekeeper mutation and a cysteine residue in a conserved α-helix at the lip of the ATP-binding pocket (Part 2.2, Fig. 1C-D). As this was not present in Ark1, we had to engineer Ark1-as3 by mutating the analogous residue to cysteine. The engineered kinase (Ark1-as3-cys) was functional as shown by viability assays and showed phosphorylation of endogenous targets both *in vitro* and *in vivo* (Part 2.2, Fig. 2A and Fig. 4B). Nevertheless activity measurements revealed that Ark1-as3 as well as Ark1-as3-cys exhibits reduced activity, as has been often reported for analogue-sensitive kinases (Zhang et al., 2005). Given that untreated Ark1-as3 and Ark1-as3-cys cells exhibited normal growth and no defective mitotic phenotype, suggests that a reduced kinase activity does not reduce general viability of the cells (Part 2.2, Fig. 3A,B). We showed that Ark1-as3-cys is specifically targeted and inhibited by the 4-anilinoquinazoline equipped with propiolamide at the 6-position of the quinazoline core (**2**) (Part 2.2, Fig. 1A). This was dependent on both selectivity filters, such that the single mutants did not exhibit any kinase inhibition (Part 2.2, Fig. 3A,B).

Whereas 50 µM of 1NM-PP1 reduced viability of wild type cells, 50 µM of the covalent inhibitor **2** did not (Part 2.2, Suppl. Fig. 3B). This suggests that the covalent inhibitor might have fewer side effects. However, 1NM-PP1 acts faster and may be more potent (Part 2.2, Fig. 4B-D). Screening of more 4-anilinoquinazolines or other scaffolds equipped with a greater variety of reactive groups could potentially identify an optimized covalent inhibitor.

Although **2** and 1NM-PP1 inhibitors are shown to inhibit the engineered kinase and show the same inhibition phenotype it will be interesting to see if they indeed have different off target effects. It was not the aim of the study to repeat the phosphoproteome experiment with a different set of as-kinase and inhibitor but nevertheless it would be interesting to see if they give the same outcome. I would expect the same outcome for Aurora targets, but different side effects. It is possible that **2** also has detectable side effects. The difference in the high dose (50 µM) effect of **2** and 1NM-PP1 only shows that no essential protein is hit efficiently by **2**.

I showed that 4-anilinoquinazolines could also covalently target a Ser/Thr kinase family member. We expect that this targeted design strategy can be extended to other kinases to make them susceptible to covalent inhibitors. I suggest it inhibits the engineered kinase with increased specificity and could be helpful in decreasing inhibitor side effects that could interfere with proper substrate identification and interpretation of function.

An additional feature of such inhibitors is their possibility to act as scaffolds to develop activity based probes (Nomura et al., 2010). The inhibitors could be equipped with fluorophores or chemical crosslinkers. In the latter case, the engineered kinases with a covalently bound inhibitor that is equipped with a reactive group would come into close vicinity to its substrates and interaction partners. The crosslinker could then potentially connect them with each other. Analysis of the purified kinase complex by MS may reveal new substrates or binding partners.

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5 Supplemental data

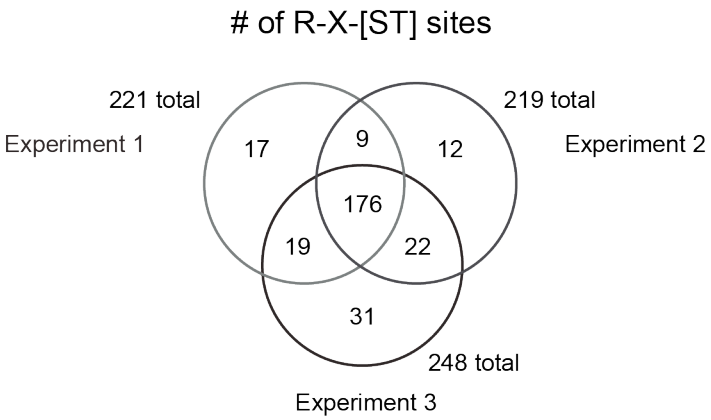

Figure 5.4 Comparison of the R-X-[ST]-sites that were identified and quantified in the three independent experiments

The Venn diagram shows the overlap of quantified and localized phosphorylation sites that match the identified Aurora phosphorylation motif R-X-[ST] (Part 2.1, Fig. 2 and Table S1).

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