Regulation of Bcl-xL activity in apoptosis

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Abstract

Apoptosis is a complex process relying for its regulation on extra- and intracellular communication. In the extrinsic pathway, death ligand-receptors, e.g. CD95, regulate apoptosis. In the intrinsic pathway, several proteins, among them the Bcl-2 family, activate and regulate apoptosis. In both pathways protein abundance, protein – protein interactions and the presence of certain proteins or protein aggregates on cellular membranes play an important role in apoptosis regulation. Despite recent research efforts, the effects of the molecular mechanisms on the spatiotemporal organization and association between proteins in biological membranes leading to apoptosis are still not fully understood.

A part of this thesis is devoted to elucidating the role of CD95-eGFP receptor diffusion in the plasma membrane in healthy cells and in cells undergoing apoptosis. We could demonstrate that CD95 accumulates in the plasma membrane at the site of activation with a surface immobilized agonistic anti-CD95 antibody and that its mobility decreases upon activation. This effect was not detected at the receptors without direct contact with the surface immobilized antibody and therefore not activated.

The focus of the research of this thesis was to investigate the mechanisms in the regulation of Bcl-xL activity in MOMP and thus in apoptosis with an emphasis on the impact of posttranslational modifications on Bcl-xL stability. Bcl-xL has been shown to be crucial for HSPCs survival and we could prove that a transient overexpression and internalization of TAT-Bcl-xL inhibit apoptosis in LSK cells and recipient mice. We addressed the degradation mechanisms of Bcl-xL and could demonstrate that Bcl-xL in MEF cell lines is primarily controlled by proteasome degradation, followed by mitophagy and pro-apoptotic cleavage but not by calpain degradation.

A particular focus was placed on how phosphorylation of Bcl-xL affects its interaction / affinity with pro-apoptotic Bcl-2 family members Bax and tBid and on the ability to maintain the relative presence of both proteins in the OMM. We have generated eight phosphomimetic Bcl-xL variants: seven in its long unstructured domain and one in its structured domain. We have optimized the overexpression and purification of the full length wildtype and phosphomimetic proteins and investigated their anti-apoptotic activity in vitro, using a permeability assay based on the release of calcein from large unilamellar vesicles. We quantified the strength of the interactions of Bcl-xL wildtype or its phosphomimetic variants with cBid or Bax using SFCCS on membranes of single giant unilamellar vesicles and expanded tubeSFCCS to measure the interactions on the mitochondrial membrane in healthy cells. Furthermore we investigated the ability of Bcl-xL wildtype and the phosphomimetic variants to retro-translocate Bax and tBid from the mitochondria to the cytoplasm by FLIP analysis. We could demonstrate that phosphorylation at specific sites of Bcl-xL affects its interaction with tBid in terms of association strength as well as of regulation of tBid local presence at the OMM. Highly relevant is the observation that Bcl-xL associates stronger with tBid than with Bax and stabilizes tBid at the OMM.

Zusammenfassung

Apoptose ist ein komplexer Prozess, dessen Regulation auf extra- und intrazellulärer Kommunikation beruht. Im extrinsischen Weg haben die Todesligand-Rezeptoren, z.B. CD95, eine regulatorische Wirkung auf die Apoptose. Im intrinsischen Weg regulieren mehrere Proteine, darunter die Bcl-2 Familie, die Apoptose. In beiden Signalwegen spielen Protein-Protein-Wechselwirkungen oder die Inter-aktion von Proteinen mit der Zellmembranen eine wichtige Rolle. Trotz grossen Forschungsanstrengungen sind die molekularen Mechanismen der räumlich-zeitlichen Organisation und Assoziation von Proteinen in biologischen Membranen die zur Apoptose führen, noch nicht vollständig geklärt.

Ein Teil dieser Arbeit befasst sich mit der Aufklärung der Rolle der CD95-eGFP-Rezeptordiffusion in der Plasmamembran in gesunden und in apoptotischen Zellen. Wir konnten zeigen, dass sich CD95 an der Stelle der Aktivierung mit einem oberflächenimmobilisierten agonistischen Anti-CD95-Antikörper in der Plasma-membran anreichert und dass seine Mobilität bei Aktivierung abnimmt.

Der Fokus dieser Arbeit lag auf der Untersuchung der Regulationsmechanismen der Bcl-xL-Aktivität in MOMP, insbesondere dem Einfluss posttranslationaler Modifikationen auf die Bcl-xL-Stabilität. Es wurde gezeigt, dass Bcl-xL entscheidend für das Überleben von HSPCs ist, und dass eine vorübergehende Überexpression und Internalisierung von TAT-Bcl-xL die Apoptose in LSK-Zellen und Empfänger-mäusen hemmt. Ebenfalls konnten wir zeigen, dass Bcl-xL in MEF-Zelllinien haupt-sächlich durch Proteasom-Abbau, gefolgt von Mitophagie und pro-apoptotischer Spaltung, aber nicht durch Calpain-Abbau kontrolliert wird.

Eine zentrale Frage war wie die Phosphorylierung von Bcl-xL die Wechselwirkung / Affinität mit den pro-apoptotischen Bcl-2-Familienmitgliedern Bax und tBid beein-flusst. Wir haben acht phosphomimetische Bcl-xL-Varianten erzeugt, sieben in der langen unstrukturierten und eine in der strukturierten Domäne. Nach optimierter Überexpression und Aufreinigung der Full-Length Wildtyp- und phosphomimetischen Proteine wurde ihre anti-apoptotische Aktivität in vitro mit einem Permeabilitätstests, basierend auf der Calcein Freisetzung aus GUVs untersucht. Wir haben die Stärke der Wechselwirkungen von Bcl-xL-Wildtyp oder seinen phosphomimetischen Varianten mit cBid oder Bax mithilfe von SFCCS an Membranen einzelner riesiger unilamellarer Vesikel quantifiziert und tubeSFCCS erweitert, um die Wechsel-wirkungen auf der Mitochondrienmembran in gesunden Zellen zu messen. Dar-über hinaus untersuchten wir durch FLIP-Analyse die Fähigkeit des Bcl-xL-Wildtyps und der phosphomimetischen Varianten, Bax und tBid von den Mitochondrien in das Zytoplasma zu diffundieren. Wir konnten zeigen, dass die Phosphorylierung an bestimmten Stellen von Bcl-xL die Wechselwirkung mit tBid in Bezug auf die Assoziationsstärke sowie die Regulation der lokalen Präsenz von tBid im OMM beeinflusst. Sehr relevant ist die Beobachtung, dass Bcl-xL mit tBid stärker assoziiert als mit Bax und tBid auf der OMM stabilisiert.

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List of Abbreviations

MOMP	Mitochondrial Outer Membrane Permeabilization
Bad	Bcl-2-associated death promoter
Bak	Bcl-2 homologous antagonist killer
Bax	Bcl-2-associated x
Bcl-2	B-cell lymphoma 2
Bcl-xL	B-cell lymphoma-extra-Large
BH	Bcl-2 Homology
Bid	BH3-interacting domain death agonist
Bik	Bcl-2 interacting killer
Bim	Bcl-2-interacting mediator of cell death
Bmf	Bcl-2-modifying factor
Bok	Bcl-2-related ovarian killer
CAD	Caspase-Activated DNAse
CARD	Caspase Recruitment Domain
DD	Death Domain
DISC	Death-Inducing Signaling Complex
DR	Death Receptor
DSB	Double Strand Breaks
ER	Endoplasmic Reticulum
ERAD	ER-Associated Degradation
FADD	Fas-Associated Death Domain
Fas	Fatty Acid Synthetase
FCCS	Fluorescence Cross-Correlation Spectroscopy
FCS	Fluorescence Correlation Spectroscopy

FRAP	Fluorescence Recovery After Photobleaching
HCT 116	Human Colon Cancer cell line
Hrk	activator of apoptosis Hara-kiri
HSC	Hematopoietic Stem Cell
HSPC	Hematopoietic Stem and Progenitor Cell
IMS	Mitochondrial Intermembrane Space
LUD	Long Unordered Domain
LUV	Large Unilamellar Vesicle
Mcl-1	Myeloid cell leukaemia differentiation protein
MEF	Mouse Embryonic Fibroblast cell line
NHEJ	Non-Homologous End Joining
NMR	Nuclear Magnetic Resonance
OMM	Outer Mitochondrial Membrane
PCR	Polymerase Chain Reaction
Puma	P53-upregulated modulator of apoptosis
SFCCS	Scanning Fluorescence Cross-Correlation Spectroscopy
SFCS	Scanning Fluorescence Correlation Spectroscopy
SLB	Supported Lipid Bilayers
tBid	truncated Bid
TALEN	Transcription Activator-Like Effector Nucleases
Tet	Tetracycline
TM	transmembrane domain
TNF	Tumor Necrosis Factor
TRAIL	TNF-Related Apoptosis-Inducing Ligand
VDAC2	Voltage-Dependent Anion-selective Channel protein 2
ZFN	Zinc-Finger Nuclease

1. Introduction

Cells die for various reasons and are swiftly cleared by other cells acting as morticians. When the body is shaped during embryogenesis, many cells are overproduced, and the excessive or harmful cells are developmentally programmed to undergo cell death (Fuchs and Steller, 2011; Nagata, 2018). Different cell types have different lifespans (e.g. neutrophils, less than 10 h). Thus, several hundred billion cells die daily and are replaced by newly generated cells. How they die depends on their cell type. Cells infected by bacteria or viruses also die. The cell death that occurs under physiological conditions mainly proceeds by apoptosis, which is a non inflammatory, or silent, process, while pathogen infection induces necroptosis or pyroptosis, which activates the immune system and causes inflammation (Nagata, 2018).

This thesis is focused on Apoptosis. Until recently, apoptosis was the only known Regulated Cell Death (RCD) mechanism. In the last few years, other RCD mechanisms were discovered and described, namely, Necroptosis, Ferroptosis and Pyroptosis. They differ in their initiation of cell death, induction of the signaling pathway and activation of genetically-encoded molecular machinery regulating cell death. Despite progress made in apoptosis research, there are still unanswered questions related to the molecular mechanisms of apoptosis.

1.1 Apoptosis

Apoptosis, the archetype of programmed cell death, is an essential and evolutionarily-conserved mechanism that regulates homeostasis and defense against invasion of pathogens and genotoxic stress.

Activation of apoptosis occurs through either intrinsic or extrinsic pathways. They are independently-activated by different stimuli, however cross-talk exists through the activation of caspase-8 and the subsequent cleavage of Bid whenever tighter regulation and quicker response are required (Figure 1.1).

Caspases are a family of cysteine proteases that initiate and execute apoptotic cell death. Based on their role in apoptosis or inflammation, caspases are subdivided into initiator and effector (executioner) caspases. Cellular signals, such as death ligands or Mitochondrial Outer Membrane Permeabilization (MOMP), activate the initiator caspases (e.g. caspase-2, -8, -9 and -10) which either cleave and activate executioner caspases (e.g. caspase-3,-6,-7), or cleave other proteins that transmit the apoptotic signal to the executioner caspases. Executioner caspases are responsible for cleaving a myriad of substrates, some of which mediate the apoptotic phenotype in dying cells. About 1000 caspase substrates with functions that encompass many cellular processes have been identified (McStay and Green, 2014).

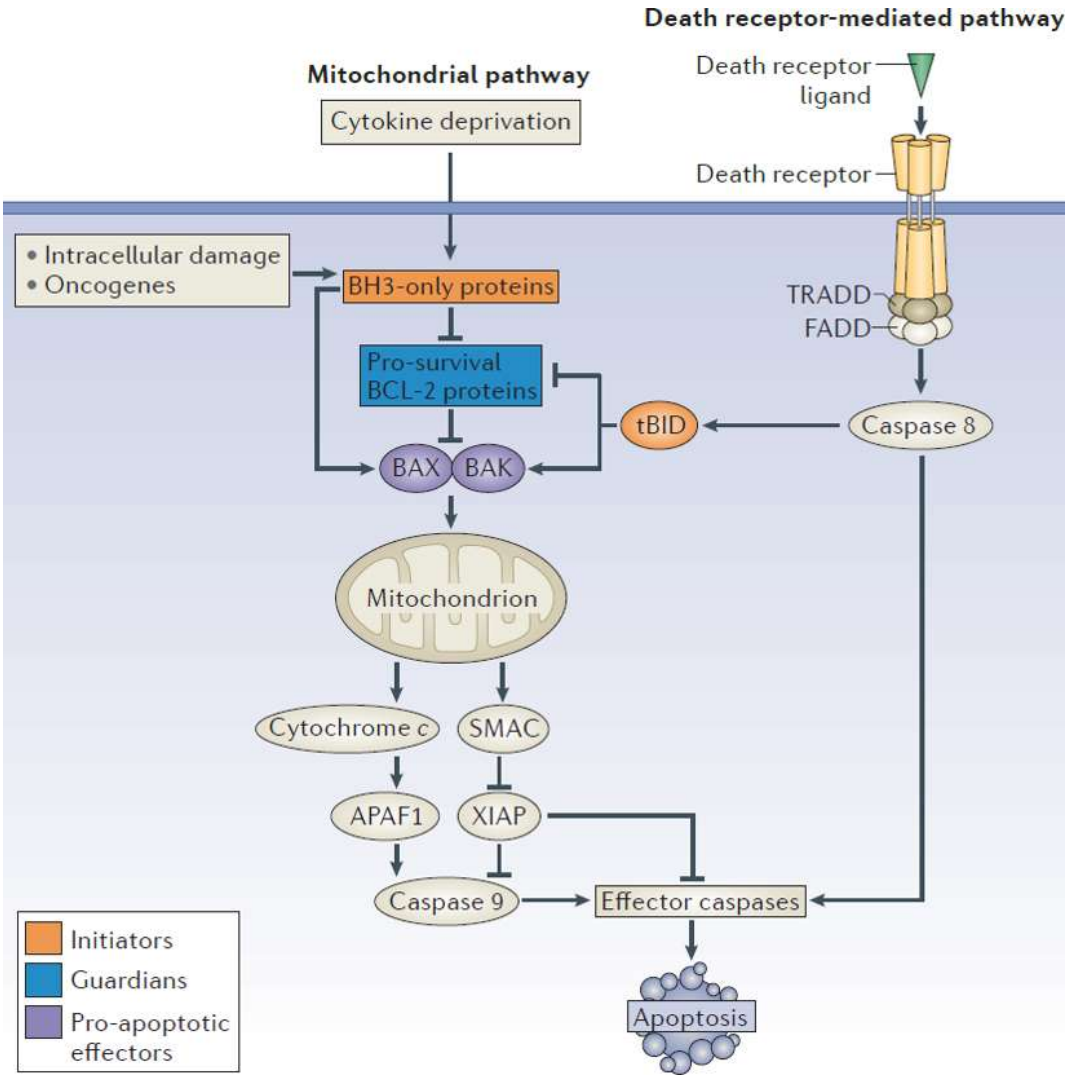


Figure 1.1 Interactive pathways of apoptosis. The mitochondrial apoptosis pathway is activated by growth factor deprivation and intracellular disturbances comprising DNA damage and oxidative stress. The main regulators are the Bcl-2 proteins, consisting of pro-apoptotic initiators, pro-apoptotic activators and pro-survival guardians. Depending on the presence or absence of stress signals they interact differently. Upon stress signals the BH3 only proteins disturb the interactions between the pro-survival guardians and pro-apoptotic effectors by binding and inhibiting the pro-survival proteins, thus leading - through multiple steps - to apoptosis: the permeabilization of the OMM, followed by the release of pro-apoptotic factors such as Cytochrom c and SMAC, the formation of the cytoplasmic apoptosome, the activation of the effector caspases 3, 6 and 7. The death receptor-mediated pathway is activated by extracellular stress-ligands binding to and activating the death receptor. This leads to its trimerization, followed by the association of FADD, the recruitment and activation of procaspase-8, the activation of a caspase cascade and - similar to the mitochondrial pathway- the activation of effector caspases 3, 6 and 7. Both pathways are connected via the activation step of Bid to tBid. (from Czabotar et al., 2014)

1.1.2 Extrinsic pathway of apoptosis

Extracellular stress signals can activate the extrinsic or so-called death receptor-mediated pathway (Flusberg et al., 2015) by binding to Death Receptors (DRs) located at the plasma membrane. DRs include the Tumor Necrosis Factor (TNF)-Related Apoptosis-Inducing Ligand (TRAIL) receptors (TRAILR1, TRAILR2) (Aggarwal et al., 2012) and the cell surface death receptor Fatty Acid Synthetase

(Fas) (also known as CD95 and APO1) (Wajant, 2002). Once released, the ligands of Fas and TRAIL bind to their respective receptors. This induces and stabilizes their homo-trimerization at distinct domains of the plasma membrane. The homo-trimer undergoes a conformational change in its intracellular domain that enables the Death Domain (DD)-dependent association of the Fas-Associated Death Domain (FADD) (Boldin et al., 1995; Walczak and Krammer, 2000; Grassmé et al., 2001; Siegel et al., 2004; Scott et al., 2009; Fu et al., 2016). FADD recruits the initiator procaspase-8 to the activated death receptor to form the Death-Inducing Signaling Complex (DISC) (Kischkel et al., 1995; Majkut et al., 2014). Binding of procaspase-8 causes its homo-dimerization and self-activation by auto-proteolytic cleavage, the activation of a caspase-cascade and to cell death (Oberst et al., 2010; Dickens et al., 2012) (Figure 1.1). Cellular FLICE / caspase 8-Like Inhibitory Protein (cFLIP) is an important regulator of caspase activation. It is structurally similar to procaspases 8 and 10 and competes for FADD binding, but cFLIP lacks the procaspase 8 / 10 catalytic sites, preventing procaspase 8 / 10 homo-dimerization and activation (Verbrugge et al., 2010).Essential for induction of the death receptor-mediated apoptosis is a direct cell-to-cell contact between the Fas ligand of a cytotoxic T lymphocyte or Natural Killer (NK) cells and the CD95 receptor of the infected cell; otherwise known as the cytotoxic immunological synapse. In many cell types Fas ligand is not cytotoxic but rather promotes cell proliferation, migration and cytokine generation (Wajant et al., 2003; Barnhart et al., 2004). It has been shown that the Fas ligand activates the ERK signaling for neuronal regeneration (Desbarats et al., 2003; Guicciardi and Gores, 2009).

1.1.3 Intrinsic pathway of apoptosis

Intracellular disturbances, such as growth factor withdrawal, DNA damage, Endoplasmic Reticulum (ER) stress, replication stress, microtubular alterations or mitotic defects (Czabotar et al., 2014; Roos et al., 2016; Pihan et al., 2017) can initiate the intrinsic or, so-called, mitochondrial apoptotic pathway. The B-cell lymphoma 2 (Bcl-2) protein family is the main player in the intrinsic apoptotic pathway. Internal stimuli converge in the activation of the pro-apoptotic effectors Bax and Bak, which are members of the Bcl-2 protein family. Bax and Bak undergo structural changes leading to the translocation of Bax from the cytoplasm to the Outer Mitochondrial Membrane (OMM), the activation and oligomerization of Bax and Bak at the OMM and to MOMP. MOMP is considered a point of no return because it typically leads to cell death even in the absence of caspase activity (Tait and Green, 2013). MOMP is followed by the release of apoptotic factors into the cytosol, such as cytochrome c from the Mitochondrial Intermembrane Space (IMS). Released cytochrome c binds the key caspase adaptor molecule Apaf-1. In the absence of an apoptotic stimulus, Apaf-1 exists in a monomeric form. Apaf-1 possesses a N-terminal Caspase Recruitment Domain (CARD), to which caspase-9 is recruited and a C-terminal domain, to which cytochrome c can bind. In response to an apoptotic stimulus cytochrome c is released from the mitochondrial IMS and binds to the C-terminus of the Apaf-1 molecule. Therefore Apaf-1 undergoes extensive

conformational changes, leading to its oligomerization and the formation of a heptameric wheel-like structure in which the CARD is exposed and binds the initiator procaspase-9, forming the apoptosome, and the initiation of a cascade of events culminating in cell death (Tait and Green, 2010; 2013) (Figure 1.1).

Bcl-2 proteins share one to four homology regions known as the Bcl-2 Homology (BH) domains BH1, BH2, BH3 and BH4 (Shamas-Din et al., 2013; Czabotar et al., 2014). Bcl-2 proteins can be classified into three categories depending on their function and number of homology motifs (Figure 1.2).

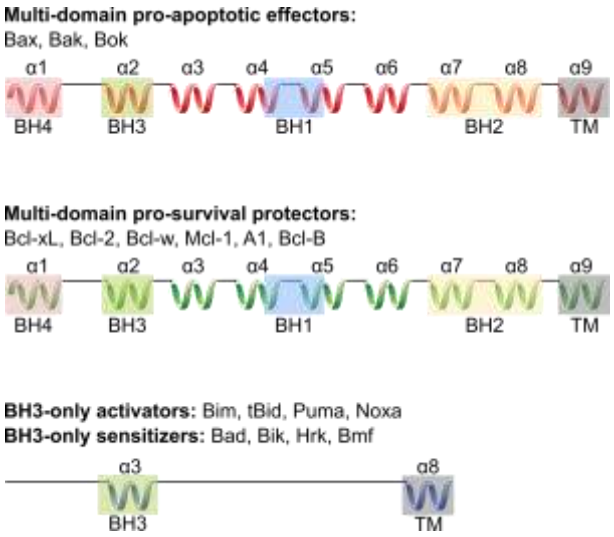


Figure 1.2 Sequence- and structure- homology of the Bcl-2 protein family. Due to their pro- and anti-apoptotic activity and the Bcl-2 Homology (BH)-domains, the Bcl-2 proteins are divided into 3 groups. Additionally they have a Transmembrane (TM)-domain for insertion in cellular membranes.

The first group belongs to the pro-apoptotic proteins. Multi-domain pro-apoptotic effector proteins Bcl-2-associated x (Bax), Bcl-2 homologous antagonist killer (Bak) and Bcl-2-related ovarian killer (Bok) contain four BH domains and a transmembrane domain (TM). They directly mediate the permeabilization of the Outer Mitochondrial Membrane and other cellular membranes (Aouacheria A, et al., 2013; Llambi et al., 2016). The function of Bok remains controversial. In some reports, Bok was found to act in a pro-apoptotic fashion similar to Bax and Bak (see below) (Bartholomeusz et al., 2006; Rodriguez et al., 2006). In some others, Bok was found to associate with the ER membrane and act in a pseudo pro-apoptotic manner to cause ER-Associated Degradation (ERAD) (Einsele-Scholz et al., 2016; Llambi et al., 2016). The rest of the literature has shown that Bok acts as an anti-apoptotic protein during Calcium-mediated neuronal injury and ER stress (Echeverry et al., 2013; Fernandez-Marrero et al., 2016). The function of Bax and Bak is more obvious: they are pro-apoptotic. In a healthy cell, Bax is a monomeric, mainly cytosolic protein with a globular structure consisting of nine α-helices and commutes between the cytosol and the OMM (Suzuki et al., 2000; Schellenberg et al., 2013). Structurally, Bax has a central amphipathic hairpin motif formed by the α-helix 5, surrounded by the other α-helices (Muchmore et al., 1996). These lead to a hydrophobic groove delineated by α-helices 2 – 8 (BH 1 – 3 domains) (Suzuki et al., 2000). α-9 is the transmembrane

(TM) domain, which is kept engaged in the hydrophobic groove through the binding of α-1 to α-9, keeping Bax monomeric in the cytosol. Upon an apoptotic stimulus, the BH3-only activators e.g. tBid bind Bax (Lovell et al., 2008; Edwards et al., 2013), thus leading to the exposure of Bax α-1, followed by the disengagement of α-9 from the hydrophobic groove (Suzuki et al., 2000; Bleicken and Zeth, 2009; Kim et al., 2009) and the insertion of α-9 into the OMM. These mechanisms represent the first step in the Bax activation. Unlike Bax, Bak is an integral mitochondrial membrane protein and therefore does not require such an activation step. This explains its faster mode of action compared to the one of Bax. At the OMM, the pro-apoptotic activity of Bak is suppressed by the interaction with the Voltage-Dependent Anion-selective Channel protein 2 (VDAC2) (Cheng et al., 2003; Kim et al., 2009; Lazarou et al., 2010). Upon induction of apoptosis, the BH3-only activators promote the activation of Bax and its translocation to the mitochondria and / or the release of Bak from VDAC2. This is followed by conformational changes in Bax and Bak leading to the formation of a symmetric dimer interface (canonical binding groove / core domain) comprising α-helices 2 – 5 with an exposed BH3-domain (α-2) and a latch domain α-helices 6 – 8. Bax as well as Bak homo-dimerize via the BH3-domain of one monomer with the canonical binding groove of another activated monomer, forming the BH3-in-groove dimers (Bleicken et al., 2010; 2014; Dewson et al., 2012; Czabotar et al., 2013). While the α-helices 1 and 9 play an important role in the initial activation step of Bax, the pore formation of Bax in the membrane is dominated by the partial opening of the α-helices 5 and 6 hairpin leading to a clamp-like structure, which is essential for the stabilization of the membrane pore (Bleicken et al., 2014).

Besides the anti-apoptotic Bcl-xL and Bcl-2 proteins, Bcl-w, also known as Bcl-2-like protein 2 (Bcl2L2), Myeloid cell leukemia differentiation protein (Mcl-1), Bcl-2-related protein A1 (Bcl2A1, also known as Bfl-1) and Bcl-2-like protein 10 (Bcl2L10, also known as Bcl-B) belong to this group. The listed anti-apoptotic proteins contain four BH domains and a membrane anchor that is inserted into the OMM and into the ER membrane (Kvansakul et al., 2008; Shamas-Din et al., 2013; Czabotar et al., 2014). It was shown that anti-apoptotic proteins inhibit the action of the pro-apoptotic Bax protein by binding to it and preventing its oligomerization at the OMM (Yin et al., 1994; Cheng et al., 2001). The canonical binding groove in anti-apoptotic proteins binds the BH3-domains of the pro-apoptotic effectors Bax and Bak and the BH3-only activator proteins, inhibiting their function by sequestering them (Kale et al., 2018).

This thesis is focused on Bcl-xL. The cytosolic and soluble form of Bcl-xL has eight α-helices and a C-terminal transmembrane α-helix (Petros, Olejniczak and Fesik, 2004) and is similarly structured to Bax. Most of the Nuclear Magnetic Resonance (NMR) and X-ray crystal structure analysis of Bcl-xL have been performed with the inactive form of Bcl-xL, which is lacking its transmembrane domain. Like Bax, the hydrophobic transmembrane domain of Bcl-xL is supposedly hidden in its hydrophobic groove, occupying the groove and hindering the interaction of its groove pocket with the BH3 domain of pro-apoptotic Bcl-2 proteins. On the OMM the

hydrophobic transmembrane anchor is bound to the membrane, hence the hydrophobic groove is free (Czabotar et al., 2014). Bcl-xL comprises both, an ordered region and a long unstructured domain (LUD), which is located between its α-helices 1 and 2 (Youle and Strasser, 2008; Shamas-Din et al., 2013). Bcl-xL is known to use dual mechanisms to regulate apoptosis. First, the hydrophobic groove in Bcl-xL binds to the BH3 domain of pro-apoptotic regulators, inhibiting apoptosis. Second, a site distal to the hydrophobic groove in Bcl-xL binds cytosolic p53, inhibiting p53-dependent activation of Bax (Petros et al., 2000; Follis et al., 2014; Follis et al., 2018). Bcl-xL has been found post translationally modified in different cancer types (Violette et al., 2002; Adams and Cory, 2007; Millimouno et al., 2014) and interestingly most of them are located in its LUD. Recent studies have shown that the LUD of Bcl-xL is highly flexible. Upon phosphorylation of Bcl-xL in its LUD, the LUD binds to the surface of the Bcl-xL ordered domain and triggers a structural rearrangement, leading to reduce the affinity for BH3-domains of pro-apoptotic proteins (Follis et al., 2018).

The third and last group belongs to BH3-only proteins. Members of this group share only the BH3 domain and are activated by post-translational activation or transcriptional upregulation. They are further sub-grouped into direct activators and sensitizers. Direct activators include the Bcl-2-interacting mediator of cell death (Bim), truncated BH3-interacting domain death agonist (tBid) and the P53-upregulated modulator of apoptosis (Puma). Sensitizers include the Bcl-2-associated death promoter (Bad), Bcl-2 interacting killer (Bik), activator of apoptosis Hara-kiri (Hrk), Bcl-2-modifying factor (Bmf) and phorbol-12-myristate-13-acetateinduced protein 1 (Noxa). Upon apoptotic stimulus, activators promote MOMP by interacting with Bax or Bak, whereas sensitizers by binding and inhibiting anti-apoptotic proteins.

Pro- and anti-apoptotic multi-domain Bcl-2 proteins share a similar globular structure consisting of nine α-helices with a central amphipathic hairpin formed by helices α5 and 6 (Muchmore et al., 1996). This structure leads to a hydrophobic groove delineated by α-helices 2, 3, 4 and 5. The hydrophobic groove is illustrated as the interface for interactions with the BH3 domain of other Bcl-2 proteins. Important for the interaction of Bcl-2 proteins is the presence of a cellular membrane. In aqueous environments, Bcl-2 proteins assume a structure where its transmembrane domain folds inside its groove. Occupation of the groove, as such, hinders the interaction of Bcl-2 proteins together. On the OMM, the transmembrane anchor binds to the membrane freeing the groove (Czabotar et al., 2014).

Unlike the multi domain Bcl-2 proteins, the BH3 only proteins are structurally disordered with the exception of Bid. Bid is - due to its structure - intrinsically inactive with a hidden BH3 domain. Caspase 8 only converts Bid into active tBid with an exposed BH3 domain (Hinds et al., 2007; Czabotar et al., 2014) (Figure 1.3).

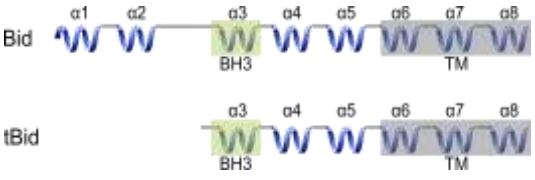


Figure 1.3 Secondary structure and processing of Bid. Compared to other BH3-only protein members, Bid

is structurally similar to the multi-domain proteins. Bid is inactive due to its structurally hidden BH3-domain. Caspase-8 activates Bid to the truncated form tBid.

The interplay between members of the Bcl-2 family is of importance to MOMP and to the fate of a cell. Several models were proposed to explain how members of the family interact to orchestrate life and death decisions. The direct and indirect activation, embedded together, unified and integrated models are briefly explained below (Letai et al., 2002; Kuwana et al., 2005; Willis et al., 2007; Shamas-Din et al., 2011; Bleicken et al., 2017) (Figure 1.4).

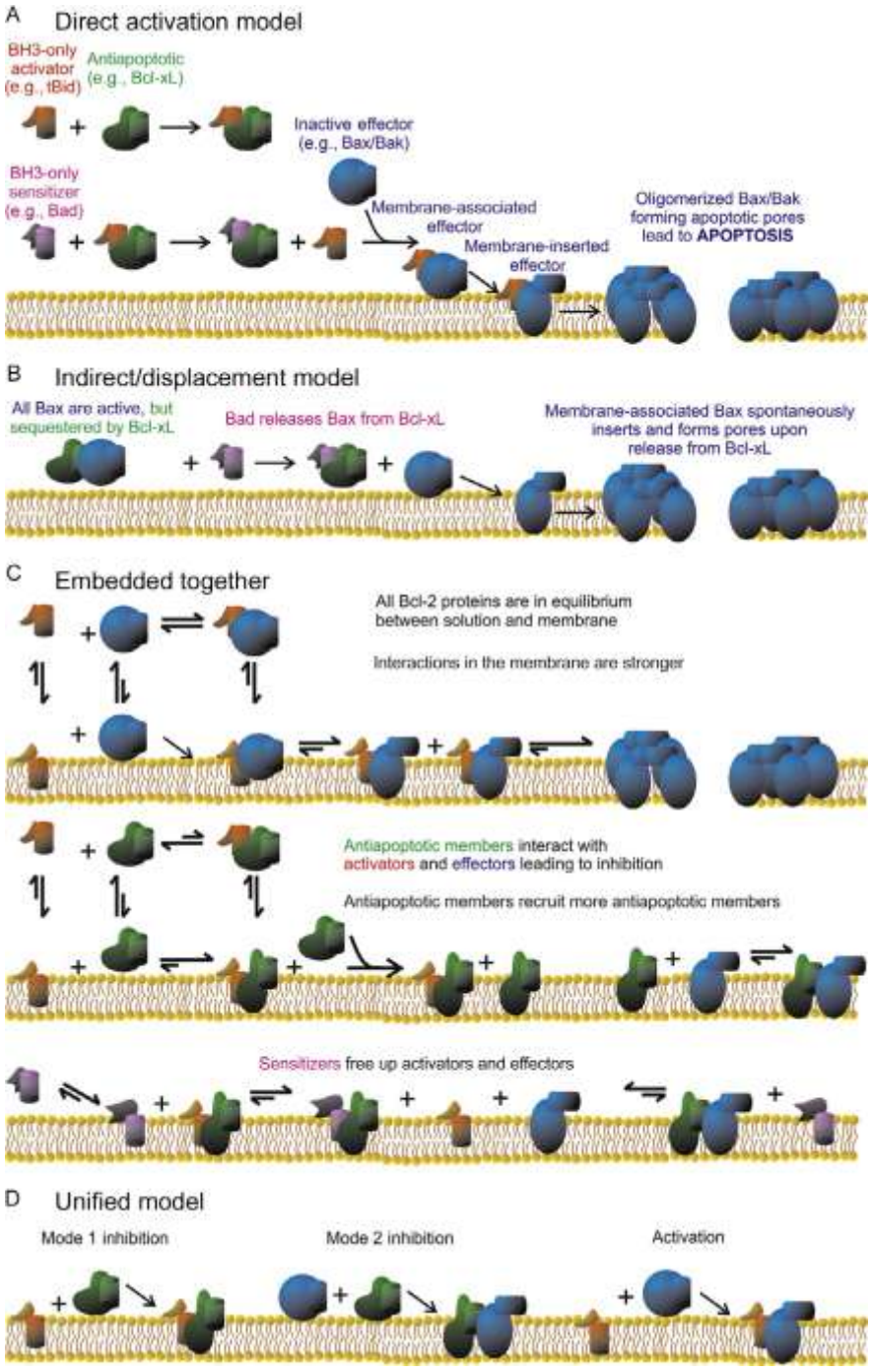


Figure 1.4 Regulation models of the Bcl-2 proteins. a) In the direct activation model Bax and Bak are inactive and have to be activated. BH3-only activators e.g. tBid bind Bax and Bak for their activation, while sensitizers inhibit the anti-apoptotic proteins such as Bcl-xL. b) In the indirect

activation model, Bax and Bak are constitutively active and suppressed by the anti-apoptotic proteins. BH3-only proteins do not activate Bax and Bak by direct contact, but rather by replacing them from the anti-apoptotic proteins.

1.1.4 Direct activation model

This model is based on the pro-apoptotic effector proteins which are inherently inactive and have to be activated through direct interaction with a BH3-only protein (Letai et al., 2002; Kuwana et al., 2005). Direct Activators Bim, tBid and Puma can bind all five anti-apoptotic proteins. Sensitizers Bad and Bmf bind to Bcl-2, Bcl-xL and Bcl-w only (Letai et al., 2002; Chen et al., 2005). Under healthy conditions, BH3-only proteins are either inactive or repressed by anti-apoptotic proteins. Upon apoptotic stimulus, activators are either activated or released from their anti-apoptotic counterparts through replacement by sensitizers (Kim et al., 2006; Shamas-Din et al., 2011).

1.1.5 Indirect activation model

In contrast to the direct activation model, the indirect activation model is based on the assumption that Bax and Bak are inherently active and their activity needs to be suppressed by anti-apoptotic proteins in order to prevent cell death. On apoptotic stimulus, BH3-only proteins engage with anti-apoptotic proteins allowing Bax-Bak mediated MOMP (Willis et al., 2007). BH3-only proteins are selective in their interaction with anti-apoptotic proteins. As such, various BH3-only proteins are requested to be present during apoptosis to bind the multitude of anti-apoptotic proteins available (Chen et al., 2005).

1.1.6 Embedded together model

The embedded together model combines the direct and indirect activation models and in addition describes the interaction between the Bcl-2 proteins as reversible, putting the membrane in the focus of action. The interactions of Bcl-2 proteins with the membrane result in conformational changes of the Bcl-2 proteins that affect their binding affinities due to their local concentrations (Leber et al., 2007; Garcia-Saez et al., 2009). In the embedded together model, BH3-only sensitizers bind exclusively to anti-apoptotic proteins and the BH3-only activators possess a dual role in directly activating the pro-apoptotic effectors Bax and Bak and sequestering the anti-apoptotic proteins by binding to them. Upon apoptotic stimulus, BH3-only activators engage with anti-apoptotic proteins and inhibit them to bind Bax and Bak, therefore function as sensitizers. Simultaneously the same BH3-only activators interact with Bax and Bak, promote their insertion in the OMM and mediate MOMP (Garcia-Saez et al., 2009; Shamas-Din et al., 2013).

1.1.7 Unified model

This model builds on the embedded together model (Llambi et al., 2011). It describes the interaction affinities of the anti-apoptotic proteins in inhibiting the BH3-only

activators (Mode 1) and the active Bax and Bak (Mode 2), whereas the inhibition of Mode 2 is more efficient in blocking MOMP. In addition, this model describes the role of Bax and Bak in the mitochondrial dynamics (Shamas-Din et al., 2013).

1.1.8 Integrated model

This model includes multiple parallel interactions between the Bcl-2 proteins in solution and in membranes in order to regulate apoptosis. Compared to the previously described models, the integrated model is based additionally on the spontaneous activation and self-amplification of Bax at the OMM, in the absence of an apoptotic stimulus and under a physiological temperature. Furthermore, it describes that membrane-bound Bax serves as an adaptor to recruit more Bax from the cytosol. The anti-apoptotic counterparts interact directly with membrane-embedded Bax and retrotranslocate it from the membrane to the cytosol, therefore inhibiting its oligomerization. Upon an apoptotic stimulus, the activated BH3-only proteins would be neutralized by the anti-apoptotic proteins, as their affinity is higher than that of anti-apoptotic proteins and pro-apoptotic effectors. In this case, the low level of Bax activation would still be counterbalanced by the excess anti-apoptotic proteins, but would prime the cells to die (Bleicken et al., 2017).

The direct interactions between the anti- and pro-apoptotic proteins are featured in all the models, based on the BH3-domain interaction with the canonical binding groove. Other interactions between the Bcl-2 proteins than the BH3 region are still missing (Kale et al., 2018). Encountered issues of studies examining the interactions between BCL-2 family proteins are:

- The use of truncated proteins or peptides of the BH3 region at physiologically irrelevant concentrations
- The absence of membranes leading to confusion in defining the core mechanisms of the BCL-2 family proteins
- The differential expression in various tissues are not considered
- The posttranslational modifications that contribute to the regulation of interactions are missing (Kale et al., 2018).

All these are under extensive investigations to understand the regulation of the apoptosis mechanism by the Bcl-2 protein family.

1.2 Overview on apoptosis assays

Several methods exist to distinguish between cell viability, proliferation and cell death, in the latter case to differentiate between different types of cell death. Here we focus on assays to monitor apoptosis. Apoptosis assays provide information on the

extent and timing of apoptotic progression by measuring key cellular events in programmed cell death, such as:

- Loss of membrane phospholipid asymmetry
- Pro-apoptotic Bcl-2 protein activation
- Mitochondrial Outer Membrane Permeabilization (MOMP)
- Caspase activation
- Cleavage of caspase substrates
- DNA fragmentation

The kinetics of the apoptosis progression is influenced by multiple factors: In cells the kinetics is much faster than in tissues, healthy tissue might react differently to apoptosis activators than cancer tissue, differences in gene expression as well as post translational modification activities of cells or tissue might lead to variances in the EC / IC 50 of apoptosis activators or inhibitors.

1.2.1 Loss of membrane phospholipid asymmetry

An initial step in apoptosis as well as in necroptosis is the translocation of phosphatidylserine (PS), a membrane phospholipid, located in healthy cells at the inner leaflet of the plasma membrane, to the outer leaflet of the plasma membrane. Annexin V, a phospholipid binding protein has a high affinity to PS and can bind to it once PS is exposed to the extracellular environment after its translocation. For detection purposes Annexin V is labeled with a fluorophore that can be measured by flow cytometry or microscopy (van Engeland et al., 1998).

1.2.2 Pro-apoptotic Bcl-2 protein activation

Bcl-2 proteins, considered to be the major regulators of apoptotic signaling pathways are divided into pro- and anti-apoptotic groups. Whereas anti-apoptotic Bcl-2 proteins protect against MOMP, the opposite is true for pro-apoptotic Bcl-2 members such as Bax. Upon exposure to various death stimuli the conformation of Bax is changed and its membrane-anchoring domain is exposed and inserted in the MOM. Studies suggest that the binding of Bid and Bim to Bax induces its activation (Zhao et al., 2014). Methods such as fluorescence correlation spectroscopy and its variants or Fluorescence Loss In Photobleaching to monitor the interactions of pro- and anti-apoptotic proteins as well as their intracellular translocations are described in the following chapters 1.3 and 3.3. Other methods to detect Bcl-2 protein activation such as cross-linking to study protein dimerization and oligomerization, the detection of active Bax by 6A7 antibody or the insertion of the proteins in the mitochondrial membrane are used as well.

1.2.3 Mitochondrial Outer Membrane Permeabilization (MOMP) and cytochrome C release

The role of MOMP in promoting caspase activation is generally accepted and it is also generally believed that blocking caspase activity is sufficient to prevent cell death by apoptosis. However blocking caspase activity is not considered to be sufficient to prevent the ultimate demise of the cell (Christensen et al., 2013). Triggered by the intrinsic apoptosis pathway, MOMP is typically identified by the release of Cytochrome C (Cyt C) into the cytosol. The extent of Cyt C translocation is often determined by Western blot analysis of cytosolic and enriched mitochondrial fractions. As described in literature, these assays have several limitations that can be overcome by quantifying Cyt C release by flow cytometry (Christensen et al., 2013).

MOMP can also be detected by measuring the mitochondrial membrane potential ($\Delta\Psi m$), which is disrupted once the outer membrane is permeabilized. The loss of $\Delta\Psi m$ can be observed using cationic dyes such as Tetramethylrhodamine ethyl ester (TMRE), MitoTracker or JC-1 that accumulate in the mitochondrial matrix when $\Delta\Psi m$ is maintained. Fluorescence signals of intact cells are therefore higher than the ones of apoptotic cells.

1.2.4 Caspase activation

The conventional method to follow caspase activity is by monitoring cleavage of either model peptide substrates or physiological substrates of the enzyme. The caspases involved in specific apoptosis scenarios can be identified using substrate-based inhibitor studies. Although the available caspase inhibitors as well as model peptides show limited selectivity for individual caspases, and the substrate specificity of the caspase itself can change depending on the activation state, by performing complementary assays, the identification of activated caspases in the apoptotic cascade can be confirmed. (McStay and Green, 2014)

1.2.5 DNA fragmentation

A characteristic feature of cells undergoing late stage apoptosis is DNA fragmentation by a specific endonuclease called Caspase-Activated DNAse (CAD). CAD cleaves chromosomal DNA into 180-bp nucleosomal fragments which give an appearance of DNA laddering when run on an agarose gel. DNA ladder assays are useful for quick screening of apoptotic changes in cell populations and can also be applied for cell lysates (Rahbar Saadat et al., 2015).

Other methods for detecting DNA fragments resulting from apoptosis, particularly for intact tissues, are in situ nick translation and terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) (Apoptosis Handbook, BR_IHCGuide_011617).

1.3 Fluorescence Correlation Spectroscopy (FCS)

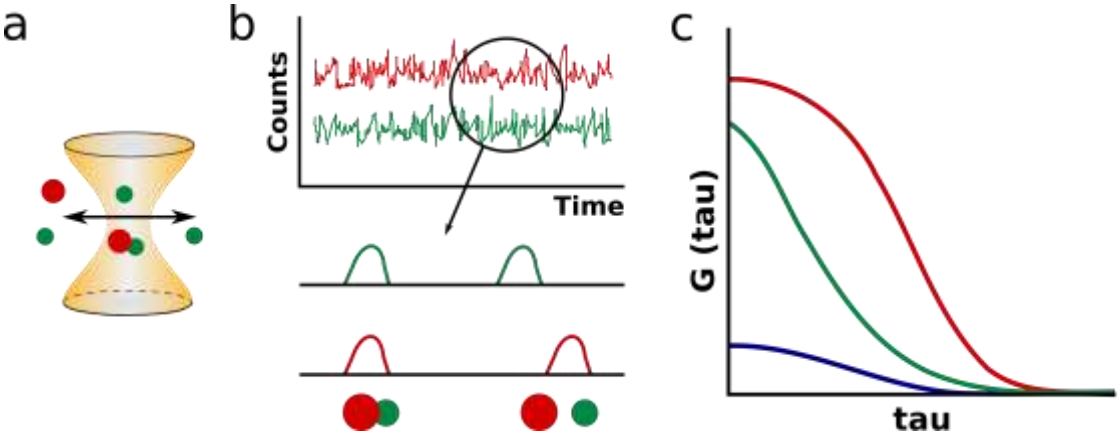
FCS allows quantitative analysis of dynamic biological processes in real-time on a single molecule level. FCS allows to determine concentrations, diffusion coefficients and association constants for nanomolar concentrations of fluorescently-labeled targets (Garcia-Saez and Schwille, 2008; Ries and Schwille, 2008; Ries et al., 2010). In contrast to other fluorescence-based methods, FCS relies on intensity fluctuations for information extraction. Intensity fluctuations are caused by the transient movement of fluorophores through a tiny detection volume. To detect these fluctuations with high precision, it is necessary that only a few molecules are excited and detected in the volume. To satisfy this requirement, pico- to nano- molar concentrations of fluorophores are typically used in FCS experiments. Signal fluctuations are measured and correlated to determine the molecular characteristics of fluorescently-labeled molecules. Bigger and slower molecules remain longer in the detection volume causing a slower decay of the correlated signal (Magde et al., 1972; Murad and Garcia-Saez, 2018). The temporal correlation describes the probability to see a molecule once detected at time t still at a later time point (t + τ). The faster the molecule, the faster the correlation decays over time. The autocorrelation is calculated by the function:

$$G\ (\tau) = \frac{\langle \delta F(t)\ *\ \delta F(t+\ \tau)\rangle}{\langle F(t)\rangle^2}$$

Where G (τ) is the autocorrelation function, *F* (t) is the fluorescence intensity as a function of time t, and τ is the correlation time or time lag.

1.3.1 Fluorescence Cross-Correlation Spectroscopy (FCCS)

FCCS is a variant of FCS that measures interactions by simultaneously detecting fluorescence fluctuations of two molecules labeled with different fluorophores. Association of the molecules leads to co-diffusion through the detection volume and, thus to highly-correlated fluctuations of the two emission channels. Non-associated molecules move independently of each other, thus leading to low cross-correlation (Figure 1.5). FCCS provides information on the total concentrations of both the molecules and the fractions participating in complex formation (Murad and Garcia-



Saez, 2018).

Figure 1.5 Graphic overview of two-color FCCS. a) Confocal volume with interacting and non-interacting green and red fluorophores. b) Intensity traces of fluorophore fluctuations in the red and green channels. c) Auto- and cross- correlation curves calculated from the intensity fluctuations.

1.3.2 Scanning Fluorescence Correlation Spectroscopy (SFCS)

FCS is preferentially applied to measure freely moving molecules in aqueous solutions. The measurement of slow-moving molecule interactions in membranous environments represents a significant challenge due to membrane motion and photo-bleaching. SFCS was developed to address these problems (Ries et al., 2009; Unsay and Garcia-Saez 2013; Hermann et al., 2015). Scanning the detection volume through a line path crossing the membrane plane shortens the residence time of the laser, reduces photo-bleaching, increases the signal-to-noise ratio and allows longer acquisition times of moving matrices such as membranes. The individual scans can be assembled to provide an image of e.g. the membrane section of interest over a certain time period. This is achieved by aligning the individual scans based on the brightest pixel, which represents the fluorescent proteins in the membrane. Similarly to FCS, the autocorrelation of each of the two potentially interacting molecules, labeled with different fluorophores, is calculated. The cross correlation is calculated

$$G\ (\tau) = \frac{\langle \delta F_r(t)\ *\ \delta F_g(t+\ \tau)\rangle}{\langle F_r(t)\rangle\langle F_g(t)\rangle}$$

1.3.3 Scanning Fluorescence Cross-Correlation Spectroscopy (SFCCS)

SFCCS expands the capabilities of SFCS to quantifying interactions on membranes. Similar to FCCS, it measures fluctuations from two fluorescently-labeled molecules by scanning two laser beams across a membrane.

1.3.3.1 SFCCS on model membranes

Artificial, simplified cellular membrane models are used to investigate the impact of the membrane composition and structure on the diffusion of proteins. GUVs are model membranes amenable to SFCCS and were extensively used in assessing the role of lipid re-organization on protein dynamics (Kahya, 2003).

To investigate the role of membranes in protein interactions and circumvent the complexity of biological membranes, Garcia-Saez et al. used GUVs to proof the role and importance of membranes to promote the interaction between Bcl-2 proteins supporting the theory of the embedded model (Garcia-Saez et al., 2009).

1.3.3.2 SFCCS on biological membranes

Protein interactions in cells are primarily studied using co-immune precipitations and proximity ligation assays. These, and other methods, can only detect stable or strong

interactions and do not provide quantitative information (Lee et al., 2013; Avila et al., 2015). Other assays can circumvent these limitations, however on surface-bound samples only (Xing et al., 2016).

SFCCS was recently used to quantify the interaction between proteins in the cytosol and on the plasma membrane of living cells (Weidemann et al., 2003; Dross et al., 2009; Ries et al., 2009). For example, Ries et al. used SFCCS to quantify the mobility of fibroblast growth factor receptors and the binding affinities to their ligands (Ries et al., 2009).

1.4 Genome Targeting and Modification

Genome Targeting is a procedure to delete, insert or edit single nucleotides or gene segments into the genome for knocking-in or knocking-out genes in order to achieve gain- or loss-of-function. Gene Targeting has been used for years to investigate the function of genes in vivo (Brinster et al., 1981; Spradling and Rubin, 1982), thus different disease models have been created. For time and space controlled gene expression, drug-inducible transgenes in mice have been developed (Lewandoski, 2001; Sun et al., 2007). Here the addition of tetracycline can activate or inhibit the expression of the targeted gene. We have used the Tetracycline (Tet) repressor-based system (Tet-off) for regulated expression of Bcl-xL by generating a stable Tet-off Mouse Embryonic Fibroblasts (MEF) cell line.

The newest and most predictable strategy for genome targeting is genome editing by restriction endonucleases. Zinc-Finger Nucleases (ZFNs), Transcription Activator-Like Effector Nucleases (TALENs) and CRISPR-Cas nucleases are able to specifically target and cleave a genomic region creating a DNA Double Strand Break (DSB) (Urnov et al., 2005; Miller et al., 2007; Christian et al.,2010; Kim and Kim, 2014), that can be repaired by the endogenous repair machinery Non-Homologous End Joining (NHEJ). The CRISPR system belongs to the RNA-based bacterial defense mechanisms to eliminate foreign DNA from invading bacteriophages. The CRISPR system consists of an endonuclease (Cas), which cleaves a targeted sequence by gRNA. The Cas endonuclease and the gRNA are encoded in the bacterial genome. By changing the sequence of the gRNA, they are able to cleave and eliminate any target sequence. The CRISPR Cas system was developed to introduce random mutations at the site of DNA double strand break by NHEJ to generate specific gene knockouts. The application of an engineered DNA construct with homology to the DNA allows the introduction of specific mutations or insertions of whole gene segments for knocking in genes or for site-specific mutations. We have applied CRISPR-Cas 9 system to generate Bcl-xL knock out MEF and HCT cell lines.

2. Objectives

Apoptosis is a complex process relying on extra- and intra- cellular communication for its regulation. In the extrinsic pathway, death ligand-receptors, including CD95 (FAS), regulate apoptosis. In the intrinsic pathway, the Bcl-2 proteins activate and regulate apoptosis. In both pathways, protein interactions on cellular membranes regulate the major steps.

Despite considerable research efforts made in the last years, the molecular mechanisms on the spatiotemporal organization and association between proteins in biological membranes leading to apoptosis are still poorly understood. This thesis is devoted to elucidating parts of this complex process. The focus of the research activities is on the investigation of the role of the protein diffusion (e.g. monomer vs. trimer) and protein translocation in healthy cells as well as in cells undergoing apoptosis, the effects of the phosphorylation of anti-apoptotic proteins on their interaction behavior and on stimulating apoptosis as well as the role of agonistic proteins on the activation of CD95. These topics are described in the following sections of the thesis:

Section 3.1: The role of agonistic proteins in the activation of CD95 receptor

- Effect of anti-CD95 agonist antibody on the aggregation, localization and mobility of CD95 receptor in the plasma membrane

Section 3.2: Cellular pathways modulating Bcl-xL stability

- The molecular mechanisms governing Bcl-xL degradation and in parallel TAT-Bcl-xL
- The role of TAT-Bcl-xL uptake in preventing apoptosis

Section 3.3: Measuring the diffusion and complex formation of Bcl-2 proteins using tubeSFCCS

- Quantitative determination of the association strength and complex formation between Bcl-xL and tBid at the OMM by SFCCS
- Measurement of diffusion and complex formation of fluorescently-labeled proteins in mitochondrial compartments and ER membranes of living cells

Section 3.4: Effect of Bcl-xL phosphorylation on its interaction with tBid and Bax

- The role of Bcl-xL phosphorylation in the inhibition of tBid and Bax
- Quantifying the strength of interaction between Bcl-xL and tBid / Bax in living cells
- The effect of Bcl-xL phosphorylation on the mobility and translocation of tBid and Bax
- The role of Bcl-xL phosphorylation on the anti-apoptotic function of Bcl-xL in the inhibition of the Bax pore formation

3. Results and discussion

3.1 The role of agonistic proteins in the activation of CD95

Attached manuscript: Sánchez et al. *Early activation of CD95 is limited and localized to the cytotoxic synapse*. FEBS J. 2018.

Contribution: 1. I developed an assay to investigate the localization and aggregation of CD95-receptor on the plasma membrane of CD95-knock-out Glioblastoma (T98G) by confocal microscopy. 2. I applied and optimized an apoptosis assay based on caspase 3/7 activity to measure apoptosis as endpoint step: I optimized cell cultivation parameters and agonistic antibody treatment conditions.

Summary: Defense against infected and tumor cells is an important role of the adaptive immune system. A cytotoxic synapse is formed between a cytotoxic T-lymphocyte or a Natural Killer cell – both expressing a CD95 ligand - and an infected cell, expressing a CD95-receptor. The synapse allows direct cell-to-cell contact and activation of the extrinsic pathway of apoptosis. Anti-CD95 antibody can bind to the extracellular domain of the CD95-receptor leading to its trimerization. Receptor trimerization activates the intracellular adaptor molecule Fas-Associated Death Domain (FADD) and recruits procaspase-8. Together they form the Death Inducing Signaling Complex (DISC). Accumulation of procaspase-8 at the intracellular domain of CD95 leads to the auto-activation of the procaspase-8 and the induction of a cascade of caspase activities including the executioner caspases 3, 6 and 7, inducing apoptosis. The cytotoxic synapse is one of the best-defined immunological synapses. Despite this, the spatiotemporal organization of CD95 during signaling remains unknown.

We developed an artificial cytotoxic synapse between HEK cells over-expressing CD95-eGFP receptor and Supported Lipid Bilayers (SLBs) incorporating anti-CD95 to study the aggregation and mobility of CD95 in the plasma membrane upon activation. We additionally activated the CD95-eGFP receptor with soluble antibodies to compare its aggregation and mobility to those without activation and those with activation at distinct positions.

We demonstrated that over-expressed and inactive CD95-eGFP receptor is uniformly distributed on the plasma membrane of human T98G cells and forms clusters upon activation with anti-CD95 antibody. Activated CD95 could also activate the downstream caspases including effector caspases 3 and 7. We identified changes in the distribution of CD95 on the plasma membrane of HEK cells after their addition to anti-CD95-incorporating SLBs. CD95 accumulated at contact sites between SLBs and HEK cells. This demonstrates the CD95 accumulation at the site of activation, confirming the results of Siegel and co-workers who showed that clustering of CD95 is essential to its activity (Siegel et al., 2004; Sánchez et al., 2018). Others have shown that stimulation of CD95 decreases membrane fluidity by increasing lipid

packaging (Grassmé et al., 2001; Carrer et al., 2009). We could further demonstrate that CD95 mobility decreases at contact sites and in the presence of an agonist antibody. These results were confirmed by z-scan FCS, two-focus SFCS and Fluorescence Recovery After Photobleaching (FRAP).

3.2 Cellular pathways modulating Bcl-xL stability

Attached manuscript: Kollek et al. *Transient apoptosis inhibition in donor stem cells improves hematopoietic stem cell transplantation*. JEM. 2017.

Contribution: I implemented and optimized methods for bacterial overexpression, purification and fluorescent labeling of recombinant full-length mouse Bcl-xL fused to a TAT-domain. The particular requirements regarding the overexpression were to over express large amounts of the protein that are soluble and correctly folded retaining function.

Summary: Mature blood cells are continuously produced by bone marrow from less-differentiated precursors derived from primitive progenitors and Hematopoietic Stem Cells (HSC). Stem cells, including HSCs, can produce daughter cells that retain the properties of their parents (Copelan, 2006). Hematopoietic Stem Cell Transplantation (HSCT) is a clinical procedure in which cells replicating bone marrow function are used for the treatment of hematologic and lymphoid cancers, non-malignant diseases and malfunction of the immune system (Marquez-Curtis et al., 2011; Snowden et al., 2012). Healthy stem cells transplanted into hematopoietic niches differentiate into multi-lineage blood cells, providing the host patient with a healthy immune system (Sullivan et al., 2010). Hematological diseases like Leukemia and Lymphoma can be cured by transplantation of allogeneic Hematopoietic Stem and Progenitor Cells (HSPCs) (Demirer et al., 1996; Copelan, 2006). Unfortunately, this treatment is associated with risk of graft failure, delayed engraftment or graft-versus-host disease. Clinical studies have shown higher HSPC numbers to correlate with faster hematopoietic regeneration; lowering the risk of graft failure (Demirer et al., 1996). A major step in the grafting process is the collection of HSCs from a donor by removal of HSCs from the bone marrow. The bone marrow controls stem cell quiescence, self-renewal, differentiation and apoptosis as well as HSPCs localization and migration (Jones and Wagers, 2008). HSPCs are retained in the bone marrow by adhesion molecules promoting cell-to-cell and cell-to-extra cellular matrix contact (Marquez-Curtis et al., 2011). Loss of the niche leads to withdrawal of signaling molecules (growth factors, cytokines, chemokines and hormones), disappearance of cellular contacts and apoptosis (Varnum-Finney et al., 2000; Greco et al., 2006; Qian et al, 2007; Butler et al., 2010; Marquez-Curtis et al., 2011).

Removal of cells from the extracellular matrix results in intrinsic apoptosis (Cory et al., 2003; Labi et al., 2006; Labi et al., 2013). Generation and maintenance of the hematopoietic system requires a regulated interplay between pro- and anti-apoptotic proteins (Kollek et al., 2016). HSPCs survival largely depends on anti-apoptotic

proteins Bcl-xL and Mcl-1 and BH3-only proteins Bim and Bmf (Labi et al., 2013). Apoptosis in HSPCs has been studied but the understanding of the molecular signaling pathways involved is still lacking. More precisely, it remains unclear whether inhibition of apoptosis is realizable and beneficial in HSCT regimens (Labi et al., 2012).

Labi and coworkers modulated Bim and Bmf levels to inhibit apoptosis in murine and human HSPCs extending their life span, which is beneficial for HSCT during engraftment and the early phases of regeneration. However, since permanent apoptosis inhibition in lymphocytes or their progenitors can trigger lymphadenopathy (i.e. increased risk of malignant transformation of these cells), such inhibition needs to be transient when used therapeutically to satisfy biomedical and bioethical safety requirements (Labi et al., 2012).

It is known that overexpression of Bcl-xL inhibits the action of BH3-only proteins. Therefore, transient overexpression of Bcl-xL should be an alternative strategy to extend the survival of HSPCs and to prevent their malignant transformation. To test this hypothesis, we transiently inhibited Bim and Bmf in mouse HSPCs by ex vivo overexpression of Bcl-xL, using adenoviral vectors or protein transduction of recombinant full-length Bcl-xL (Kollek et al., 2017).

We transiently-transfected a Bcl-xL-containing adenoviral vector (Ad5) in Lineage marker negative, Sca-1 positive, cKit positive (LSK) cells. To control transfection efficiency, expression levels and toxicity, we transfected the LSK cells with a mVenus-containing adenoviral vector. Results showed that transiently over-expressed Bcl-xL could prevent apoptosis induced by Etoposide and Cytokine deprivation.

To investigate the effectiveness of transient overexpression of Bcl-xL in vivo, we transduced donor LSK cells ex vivo with either Ad5-Bcl-xL or Ad5-mVenus and grafted them to lethally irradiated bone marrow of recipient mice. We competitively reconstituted the bone marrow by grafting a mix of Ad5-Bcl-xL / Ad5-mVenus, Ad5-mVenus / Ad5-mVenus or non-transduced / Ad5-mVenus. Cells from the bone marrow and spleen were collected 10 days after transplantation and analyzed for engraftment. Compared to mVenus and non-transduced controls, Bcl-xL-overexpressing LSK cells showed higher engraftment by displacing mVenus-overexpressing cells. We have also observed that the engraftment of mVenus-overexpressing cells was lower than that of non-transduced cells, indicating toxicity of the adenoviral transduction. Both results demonstrate the effectiveness of transient Bcl-xL overexpression in protecting cells from adenovirus- and transplantation-induced apoptosis. The recipient mice were monitored for a year and found to not have developed lympho- or myelo- proliferation, which supports our hypothesis that transient Bcl-xL expression does not lead to lymphomagenesis.

To circumvent the toxicity of adenoviral transfection and avoid the risk of genomic integration, we used protein transduction by cell-penetrating peptides. Here, TAT-

BH4, a protein consisting of the BH4 domain of Bcl-xL and reported to inhibit apoptosis, was tested (Sugioka et al., 2003). We observed that TAT-BH4 could not inhibit apoptosis induced by Etoposide or Taxol in LSK cells. Therefore we generated and purified mouse TAT-Bcl-xL (Figure 3.1).

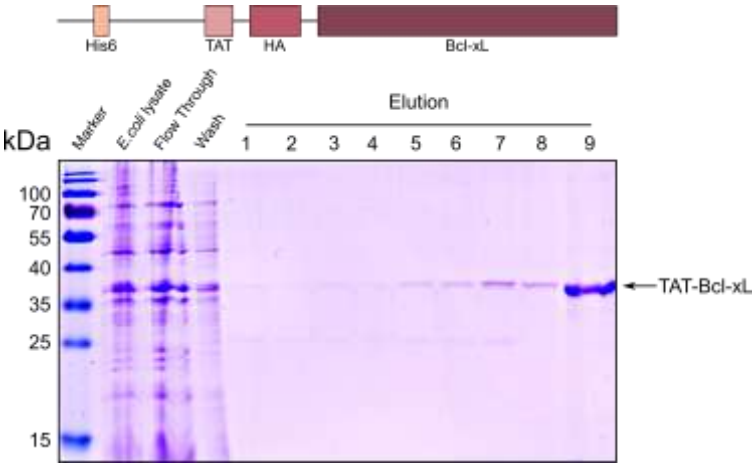


Figure 3.1 Purification of mouse TAT-Bcl-xL. a) Schematic overview of the mouse TAT-Bcl-xL with 6x-Histidine-tag and HA-tag. b) Expression of the His-HA-tagged mouse TAT-Bcl-xL was induced in *E.coli BL21* by IPTG for 4 hours. Cells were disrupted in lysis buffer complemented with protease inhibitors and DNase I using French Press. Soluble fractions were separated by centrifugation, purified using NTA-beads and eluted by increasing Imidazole concentrations. The purified proteins were desalted using PD-10 column and analyzed by SDS-page and coomassie staining.

Intracellular flow cytometry and fluorescence microscopy showed TAT-Bcl-xL internalized into the cells, localized on their mitochondria and effectively inhibited apoptosis by Etoposide or Cytokine deprivation.

To investigate the effect of TAT-Bcl-xL on HSPCs, we performed a competition assay between TAT-Bcl-xL-transduced and untransduced LSK cells. A mixture of both cell populations was transplanted in mice. TAT-Bcl-xL-transduced cells showed better survival over their untransduced counterparts under healthy conditions and on Etoposide treatment.

In addition to the above experiments performed with mouse Bcl-xL, we have also generated human TAT-Bcl-xL and optimized its overexpression and purification. Although largely identical (Fang et al., 1994), mouse and human Bcl-xL behave differently regarding the growth kinetics during overexpression and regarding interactions with the column material and other proteins during the purification. The uptake of human TAT-Bcl-xL in human CD34+ cells was insufficient and could not be enhanced. Nevertheless, the few transduced human CD34+ cells were more resistant to apoptosis compared to untreated cells.

Unpublished results: We have shown that transient inhibition of apoptosis for a few days is sufficient for the survival of lethally-irradiated bone marrow and reduces the risk of leukemogenesis compared to permanent apoptosis inhibition (Kollek et al., 2017). One strategy to improve transplantation therapy is through the inhibition of apoptosis in HSPCs by removal from stem cell niche and transplantation, thereby increasing the number of transplanted cells. It was shown that degradation of Bcl-xL in donor HSPCs leads to apoptosis and reduces the number of available HSPCs. We have observed that TAT-Bcl-xL was internalized into cells and could effectively

reduce apoptotic cell death, however Bcl-xL has a short life time in the cell (Kollek et al., 2017).

Some of the post-translational modifications of Bcl-xL described in the literature are initiated by different apoptotic signals involving TNF-alpha activation, bacterial sepsis and exposure to UVB. Upon apoptotic stimulus, Bcl-xL is phosphorylated by the c-Jun N-terminus Kinase (JNK), Protein Kinase C (PKC) and Cyclin-Dependent Kinase (CDK) (Kajihara et al., 2014). There are indications that the phosphorylation of Bcl-xL triggers its poly-ubiquitination. Several studies have shown that the proteasome regulates Bcl-xL cellular levels (Ji et al., 2008; Park and Lee 2009; Ren et al., 2011; Kajihara et al., 2014), with Keap1 and PGAM5, in particular, involved in Bcl-xL poly-ubiquitination (Niture and Jaiswal, 2011; Tian et al., 2012). Calcium-dependent Cysteine Proteases (Calpains) were also found to degrade Bcl-xL (Liu et al., 2009; Kraemer et al., 2012; Dho et al., 2013). Another regulatory post-translational modification of Bcl-xL could be deamidation. Dho and coworkers demonstrated that Bcl-xL is deamidated prior to degradation by Calpains (Dho et al., 2013). Some reports also suggest the involvement of additional degradation pathways in controlling the cellular levels of Bcl-xL such as mitophagy, lysosomal degradation (Kawaguchi et al., 2010; Shirane-Kitsuji and Nakayama, 2014) and pro-apoptotic cleavage (Arena et al., 2013). Despite so, the degradation mechanism of Bcl-xL in donor HSPCs remains unknown. Understanding of the molecular mechanism of Bcl-xL intracellular regulation is of fundamental importance to improving Bcl-xL half-life and the survival of HSPCs consequently. We reasoned that by genetically-modifying Bcl-xL at the residues involved in its post-translational modification and eventual degradation we would be able to increase its stability without affecting its anti-apoptotic activity.

Protein degradation and post-translational modification can be studied with TAT-Bcl-xL to provide information on these processes under normal and stress conditions of HSCT. Traditional protein stability assays detect a protein of interest after inhibiting the cellular protein translation system. However, this affects all endogenous proteins, perturbs the metabolic state and leads to cell death. To circumvent this problem, we developed an alternative assay that monitors the stability of Bcl-xL and TAT-Bcl-xL control by a promoter that can be turned off. To this aim, we generated a Tetracycline controlled Gene Expression (Tet)-Off stable Bcl-xL-ko cell line (Figure 3.2).

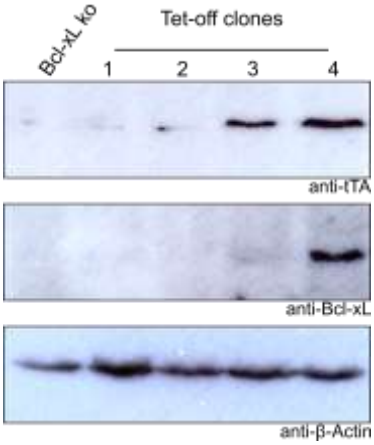


Figure 3.2 Western blot analysis of tTA and Bcl-xL. Bcl-xL knock-out MEF cell-clones stably expressing tTA were investigated for their ability to transiently overexpress Bcl-xL.

Bcl-xL-ko MEF cells were stably-transfected with pCA-tTA2 and the linear Hygromycin marker, selected under hygromycin selection medium, transfected with Bcl-xL or TAT-Bcl-xL and treated with doxycycline 24 hours after transfection to stop protein expression. Protein levels were analyzed by western blot analysis (Figure 3.3). We found that 1 ng / ml of doxycycline is sufficient to stop Bcl-xL synthesis in Tet-Off Bcl-xL-ko MEFs.

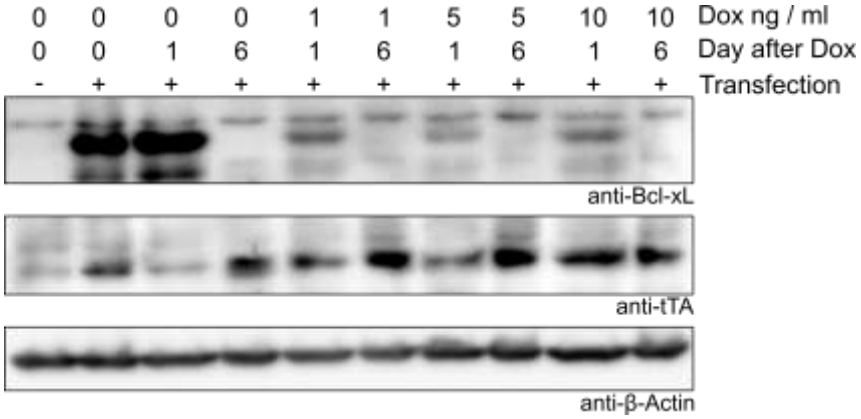
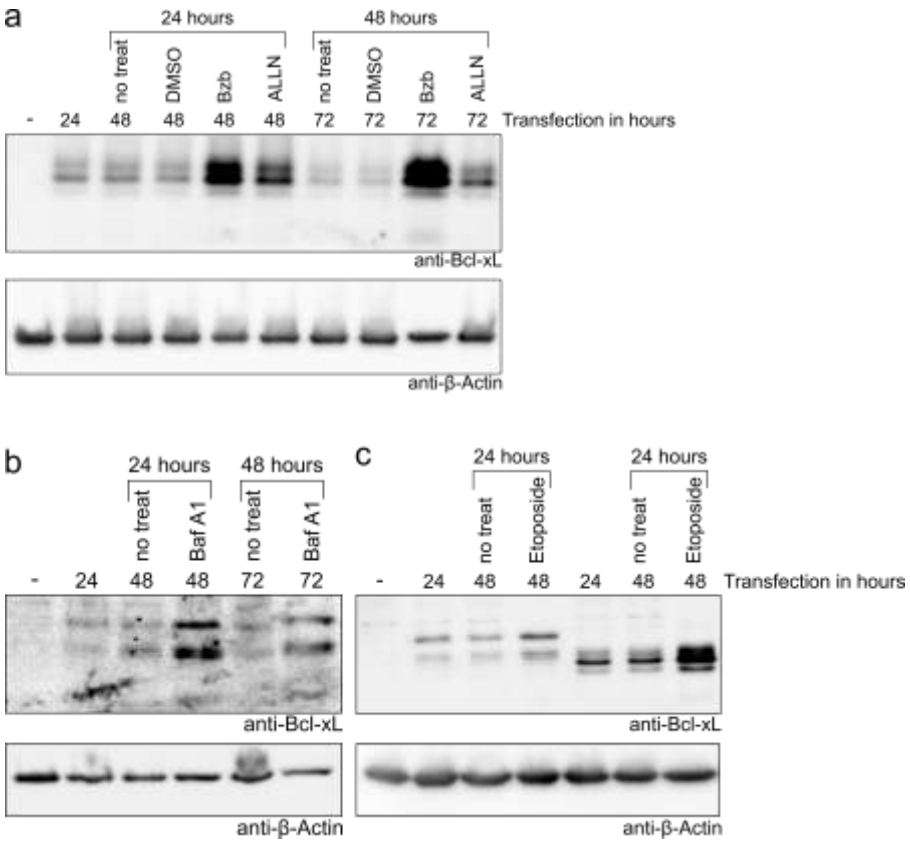


Figure 3.3 Western blot analysis of Tet-off Bcl-xL-ko MEF cells treated with Dox. The cells were transfected with Bcl-xL and treated with 1, 5 and 10 ng / ml of Doxycycline (Dox) 24 hours after protein expression. In the upper blot the dose-response effect of Dox on tTA-dependent Bcl-xL expression is shown. Bcl-xL expression is maximal in the absence of Dox whereas adding effector decreases expression with already 1 ng / ml.

To investigate the molecular mechanisms controlling Bcl-xL degradation, we applied the optimized assay described above. We transfected Tet-Off Bcl-xL-ko MEFs with Bcl-xL and TAT-Bcl-xL, terminated protein expression with doxycycline 24 hours after transfection and treated the cells with either DMSO, the proteasome inhibitors Bortezomib (Velcade®) or MG132, calpains inhibitor ALLN, autophagy inhibitor Bafilomycin A1 or Etoposide to induce pro-apoptotic cleavage of Bcl-xL. Transfected but untreated cells were used as control samples and analyzed similarly.

Bcl-xL and TAT-Bcl-xL demonstrated a similar behavior with no influence of the TAT-domain on the stability of the protein. Both untreated and DMSO-treated cells did not affect the stability of the protein. Inhibition of the proteasome by MG132 or Bortezomib for 24 and 48 hours prevents the degradation of Bcl-xL and TAT-Bcl-xL and leads to their accumulation. In contrast to the proteasome inhibition, blocking the Calpains by ALLN for 24 and 48 hours leads to a slight stabilization of Bcl-xL and TAT-Bcl-xL in the first 24 hours followed by degradation at a rate similar to that in untreated cells (Figure 3.4 a). Inhibition of autophagy / mitophagy by Bafilomycin A1

stabilizes Bcl-xL (Figure 3.4 b). To avoid pro-apoptotic cleavage of Bcl-xL, we generated a Bcl-xL D61A mutant lacking the caspase cleavage site and treated the cells with Etoposide. We showed that elimination of the caspase cleavage site



inhibits the cleavage of Bcl-xL (Figure 3.4 c).

Figure 3.4 Inhibitory effects of Bortezomib (Bzb), ALLN and Bafilomycin A1 (A1) on Bcl-xL degradation. a) Tet-off Bcl-xL-ko MEF cells were transfected with Bcl-xL, treated with 10 nM Bzb, 10 µM ALLN or DMSO for the indicated times. Protein lysates were immunoblotted for Bcl-xL and β-Actin. b) Tet-off Bcl-xL-ko MEF cells were processed according to (a) and treated with 10 nM Baf A1 for the indicated times. c) Tet-off Bcl-xL-ko MEF cells were transfected with Bcl-xL wildtype or Bcl-xL D61A, treated with Etoposide for the indicated time and analyzed according to (a).

After gathering sufficient evidence suggesting that the degradation of Bcl-xL in MEFs is primarily controlled by proteasome degradation, mitophagy and pro-apoptotic cleavage, we will use the same assay to study the degradation mechanisms of Bcl-xL in LSK cells under healthy and nutrients-deprived conditions. To stabilize the protein, we mutated the residues that could be implicated in its degradation in LSK cells. Furthermore, to inhibit Bcl-xL ubiquitination and, therefore, its degradation, we exchanged the Lysins at positions 16 and 20 with Alanines. The mutant Bcl-xL K20A was found to be more stable than the wildtype and the K16A mutant Bcl-xL upon the treatment with Staurosporine (Figure 3.5).

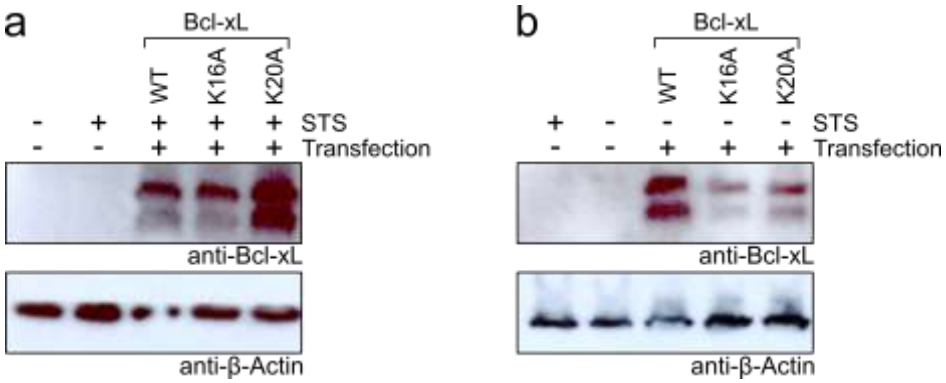


Figure 3.5 Inhibition of ubiquitination affects Bcl-xL protein levels after Staurosporine (STS) treatment. Tet-off Bcl-xL-ko MEF cells were transfected with Bcl-xL wildtype, Bcl-xL K16A or Bcl-xL K20A and treated with 1 nM STS (a) or untreated (b). Protein lysates were immunoblotted for Bcl-xL and β-Actin.

3.3 Measuring the diffusion and complex formation of Bcl-2 proteins using tubeSFCCS

Attached manuscript: Unsay et al. *Scanning Fluorescence Correlation Spectroscopy for Quantification of the Dynamics and Interactions in Tube Organelles of Living Cells*. ChemPhysChem. 2018.

Contribution: I generated Bcl-xL / Bid Double Knock-Out (DKO Mouse Embryonic Fibroblasts (MEFs) from Bid knockout MEFs by CRISPR / Cas9. I optimized transient transfection of mCherry-Bcl-xL and tBid-GFP to allow the expression of low protein levels above the detection limit of the microscope but low enough for single molecule detection, and I performed SFCCS measurements.

Summary: The members of the Bcl-2 family share sequence and structure homologies and form a protein interacting network that regulates apoptosis. The protein binding preferences have a significant impact on the activation of the apoptosis signaling pathway. The activation of apoptosis next to the inhibition of proliferation is one of the hallmarks of cancer treatment. To be able to influence this pathway, it is of importance to understand the underlying mechanisms, in which protein-protein and competitive interactions play a major role. Methods to detect protein-protein interactions, including a wide range of advanced fluorescence methods, already exist (Hashemi et al., 2017; Meyer-Almes, 2017; Sandord and Palmer, 2017), however they cannot quantify the strength of their association.

Interactions amongst Bcl-2 proteins occur on the membranes of organelles, namely, OMMs and ER membranes. Quantification of protein-protein association strengths in complex membrane environments is a considerable analytical challenge. Although this can be overcome by biochemical assays and mass spectroscopy on simplified membrane models (Bleicken et al., 2011; Siligardi et al., 2014; Khadria and Senes, 2015), these poorly mimic, important biological properties do not have the right lipid

composition, structure and the rich content of cellular membranes. Various methods are available to investigate protein-protein interactions in vivo in cellular systems such as co-immunoprecipitation assays, but these are limited to qualitative analysis (Qian et al., 2012; Fricke et al., 2014). Two-focus SFCS was developed to record fluorescence fluctuations in membranes allowing the quantification of concentrations, diffusion constants and association of fluorescently labeled proteins. Two-focus SFCS was previously applied to measure interactions between proteins in the cytosol and in the nucleus and on flat membranes like the plasma membrane but to our knowledge never used for more complex tubular membranes such as the OMM or ER.

We developed tubeSFCCS to quantify protein associations on the mitochondrial inner and outer membranes. Our method is further applicable to other cellular organelles of tubular form such as the ER. Expansion of SFCCS to tubeSFCCS required the following major adaptations.

- **Measurement protocols:** Molecules diffuse slower on complex cellular membranes compared to artificial ones. To ameliorate this problem and any adverse effect(s) it might have on our measurements, we performed repetitive 500s scans to capture a sufficient number of fluorescence fluctuation events and to avoid photobleaching.

- **Data handling and mathematical modelling:** 2D free diffusion models are often used to mathematically-describe diffusion on flat membranes. However, the geometry of tubular membranes compelled us to evaluate several models described in literature by comparing the diffusion coefficients obtained by tubeSFCCS measurements with the ones obtained by theoretically calculated diffusion coefficients. The 1D Free diffusion model was identified to be optimum for the tubeSFCCS measurements on complex membranes.

The novel protocol and the mathematical data handling method were validated by comparing the tubeSFCCS data obtained

- for the mobility to related Fluorescence Recovery After Photobleaching (FRAP) data

- for the diffusion coefficients to related super resolution single molecule tracking data

- for the concentration values to western blot analysis data.

The results obtained by tubeSFCCS and reference methods correlated very well, a prerequisite to use the novel method for the investigation of a relevant biological system in living cells.

Quantification of molecular interactions on membranes of cellular organelles is an important step to elucidate and understand intracellular signaling mechanisms. We used tubeSFCCS to quantify the interaction between the anti-apoptotic protein Bcl-xL and the pro-apoptotic tBid on OMMs of living cells. This interaction has a key function in the regulation of mitochondrial apoptosis and therefore is of major interest as a target for apoptosis inducing drugs.

Point FCCS was used to measure complex formation in the cytosol and tubeSFCCS on the OMM of living cells. We calculated the total number of proteins and fraction forming complexes in the cytosol and on OMMs. As negative control, we used mito-GFP and mCherry-Bcl-xL and as positive control a self-generated mito-GFP-mCherry construct that represented the highest interplay of the fluorescent proteins at the OMM. We showed that the % cross-correlation, proportional to complex formation, between Bcl-xL and tBid was higher at the OMM compared to the cytosol. Furthermore, we estimated that all Bcl-xL and tBid molecules on the OMM were associated with each other.

To further confirm the applicability of the tubeSFCCS on other tubular organelles, we applied it on Succinate Dehydrogenase (SDH) located on the Inner Mitochondrial Membrane (IMM). SDH is an enzyme complex and part of the respiratory chain. I tagged GFP and mCherry to the subunits SDH-A, SDH-C and SDH-D to measure their fluctuations on the IMM. This and other investigations confirmed the applicability of tubeSFCCS to calculate concentrations, diffusion coefficients and protein/protein interactions inside cellular organelles with tubular structure.

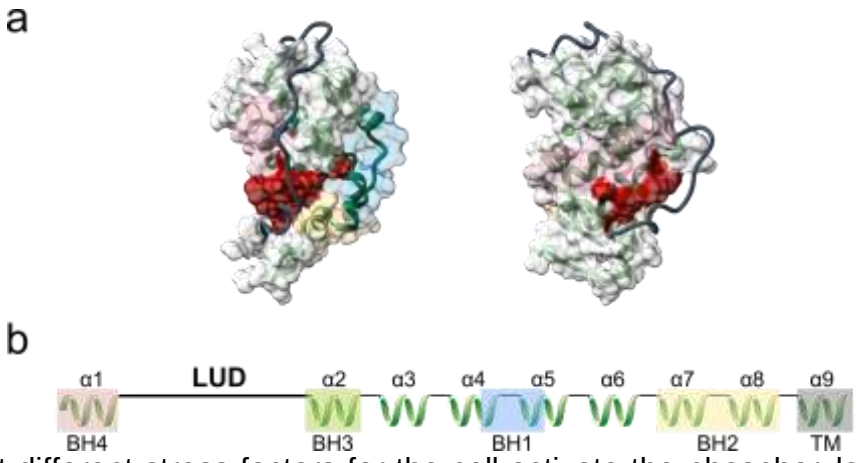
3.4 Effect of Bcl-xL phosphorylation on its interaction with tBid and Bax

Attached manuscript: Murad and Garcia-Saez. *Impact of Bcl-xL phosphorylation on the regulation of the pro-apoptotic activity of Bax and tBid* (In preparation).

Contribution: I developed the research strategy to investigate the effect of Bcl-xL phosphorylation on the activation of apoptosis and on the association with tBid and Bax. I designed and performed all experiments, statistical analysis and data interpretation. I authored the manuscript.

Summary: Bcl-xL is a key player in the intrinsic apoptotic pathway and many cancer cells modulate the Bcl-xL activity to escape death signals. It was shown that different types of cancer cells have elevated levels and post-translationally modified forms of Bcl-xL and therefore fail to undergo apoptosis (Azmi et al., 2011). Even the aggressiveness of the tumor is defined by a possible substitution of Bcl-2 by Bcl-xL. Bcl-xL has a globular structure with an ordered domain consisting of nine α-helices with α-5 in the center, leading to a hydrophobic groove. The hydrophobic groove serves as a binding pocket for the hydrophobic tail of the protein in an aqueous

milieu (Figure 3.6 a). On approaching the OMM, the tail dislodges from the groove to anchor onto the membrane, thus freeing the pocket for interaction with the BH3-domain of pro-apoptotic proteins. Unlike other anti-apoptotic proteins, Bcl-xL but also Bcl-2 itself have a Long Unordered Domain (LUD) (Figure 3.6 b). The majority of the post-translational modifications of Bcl-xL occur in its LUD. The most extensively described post-translational modification of Bcl-xL is its phosphorylation. It was



shown that different stress factors for the cell activate the phosphorylation of Bcl-xL, thereby initiating apoptosis (Zhou et al., 2015; Nakamura et al., 2016; Seng et al., 2016; Zhang et al., **Figure 3.6 Structure of Bcl-xL.** a) Surface representation of Bcl-xL with its Large Unstructured Domain (LUD) (grey) interacting with the surface of the structured part (red, green, blue and yellow) (adapted from Priya et al., 2017). b) Secondary structure of Bcl-xL showing the helices 1 – 9, the LUD, the transmembrane (TM) domain and the Bcl-2 Homology (BH) domains BH1, BH2, BH3 and BH4.

2017). Different kinases were reported to phosphorylate Bcl-xL at different positions. Inhibition of the kinases e.g. with the cell cycle kinase inhibitor oxindol 1, inhibits the phosphorylation of Bcl-xL and therefore suppresses apoptosis. Still unknown is how the different phosphorylation positions in the LUD of Bcl-xL modulate apoptosis and which molecular mechanisms govern Bcl-xL regulation. Our goal was to elucidate this open question as follows:

Strategy: To generate phosphomimetic Bcl-xL variants for the most prominent phosphorylation sites to investigate the impact of Bcl-xL phosphorylation on the regulation of Bax and tBid and therefore on apoptosis. To overexpress, isolate and purify the full length Bcl-xL wildtype and full length Bcl-xL variants for use in *in vitro* experiments on artificial membranes. Subsequently to overexpress the wildtype and variants in the HCT 116 cell line, which lacks all of the Bcl-2 proteins (HCT116 all Bcl-2 ko) for measurements on intact mitochondria.

To create the site specifically fluorescent labeled proteins Bcl-xL, Bax and cBid for SFCCS measurements.

To measure and confirm the activity of the purified proteins by a permeability assay, based on the release of Calcein (a Fluorescein complex), from LUVs.

To investigate the interaction strength between Bcl-xL and cBid, and Bcl-xL and Bax by SFCCS, initially on a simplified model system - the membrane of GUVs - mimicking mitochondrial membranes. Furthermore, to expand and optimize the method of SFCCS to measure the interaction strength between Bcl-xL and tBid, and Bcl-xL and Bax on the OMM of living cells.

To investigate the kinetics of the interaction and the ability of Bcl-xL to retro-translocate tBid and Bax from the mitochondria to the cytoplasm by Fluorescence Loss In Photobleaching (FLIP).

To investigate the role of phosphorylation in modulating Bcl-xL stability, as phosphorylation often leads to ubiquitination and proteasomal degradation, determination of the amounts of expressed proteins by western blot analysis.

Results: Selection of seven phosphorylation sites in the LUD of Bcl-xL and one in its ordered domain. Generation of the corresponding phosphomimetic variants:

To examine the role of Bcl-xL post-translational modifications in regulating apoptosis, we generated eight phosphomimetic Bcl-xL variants by introducing Aspartic or Glutamic acid via site-directed mutagenesis Polymerase Chain Reaction (PCR) at positions reported to be relevant for phosphorylation. The phosphorylation sites are T41, S43, T47, S49, S56, S62, S73 and T115. With the exception of T115, which is located in the loop between α3 and α4, all other sites are located in the LUD between α1 and α2.

Overexpression, purification and labeling of wildtype proteins / phosphomimetic variants:

To study the interaction between Bcl-xL, Bax and cBid *in vitro*, we overexpressed the proteins in *E.coli* and purified them by affinity and anion exchange chromatography (Figure 3.7). To obtain cBid from Bid, the latter was cleaved *in situ* by caspase-8.

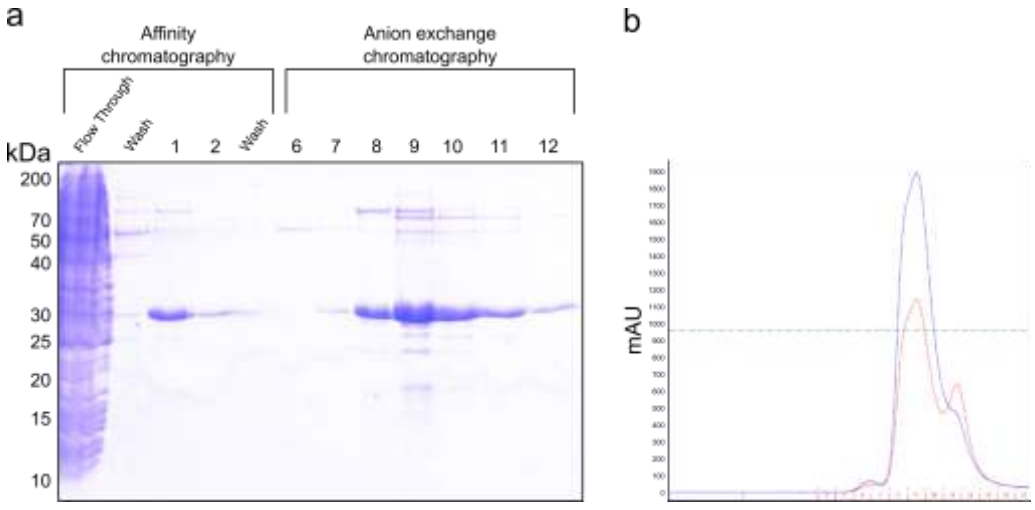


Figure 3.7 Purification of recombinant Intein-tagged Bcl-xL. a) Chromatogram of the purification of recombinant Bcl-xL. Blue: UV280 absorbance. Red: UV260 absorbance. b) Analysis of total protein by SDS-page and coomassie staining at various stages of purification using chitin-column for affinity chromatography and the Äkta for anion exchange chromatography.

Examination of recombinant proteins activity:

Using a permeability assay based on the release of Calcein (a Fluorescein complex) from LUVs, we confirmed that cBid could activate Bax and induce its oligomerization and membrane-permeabilization. Furthermore, we confirmed the ability of the purified phosphomimetic variants to inhibit Bax pore formation. From the relative calcein release we calculated the IC50 of each variant compared to Bcl-xL wildtype. Our data show variants T47E, S62E and T115E having lower inhibition potential with IC50 of 49 nM, 35 nM and 52 nM respectively compared to S56E and S73E with IC50 of 17 nM and 16 nM respectively on the Bax pore formation (Figure 3.8).

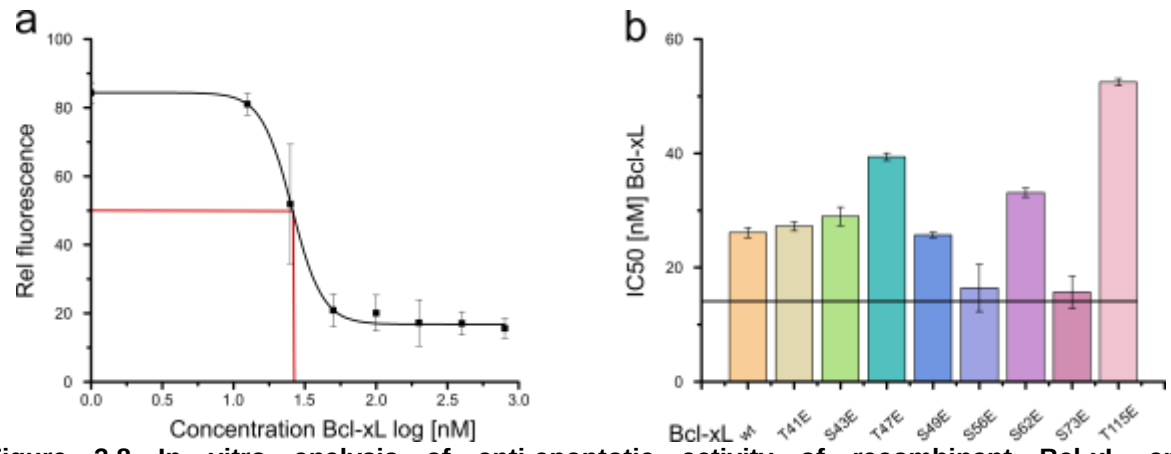


Figure 3.8 In vitro analysis of anti-apoptotic activity of recombinant Bcl-xL and phosphomimetic variants. a) Dose response and Inhibitory Concentration for half-maximal (IC50) analysis of Bcl-xL in the inhibition of the Bax pore formation in the calcein release assay. b) IC50 values of Bcl-xL wildtype and its phosphomimetic variants in the calcein release assay.

Investigation of protein interactions on GUV membranes by SFCCS:

To understand whether the differences in the inhibition of the Bax pore formation are due to changes in the association of Bcl-xL variants with cBid and Bax or not, we investigated the corresponding association interactions on GUVs by SFCCS. Comparing the association of Bcl-xL and cBid with that of Bcl-xL and Bax, we could demonstrate that the association of Bcl-xL with cBid is twice as high as with Bax (Figure 3.9 a).

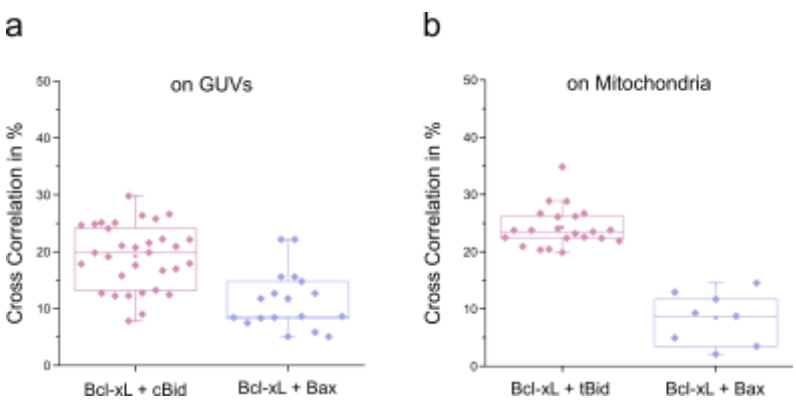


Figure 3.9 Analysis of interactions between cBid / tBid, Bax, and Bcl-xL on GUVs and on OMM. a) Comparison of the association expressed in Cross Correlation in % (CC %) between Bcl-xL and cBid and Bcl-xL and Bax on the membrane of

GUVs. b) Comparison of the association in CC % between Bcl-xL and tBid and Bcl-xL and Bax on the OMM of intact mitochondria.

Cross-correlation analysis revealed that the T41E, S43E, S62E, S73E and T115E variants have a higher degree of association on GUVs with cBid (CC in the range of 22 % to 34 %) than the wild type (CC of 19 %). However phosphomimetic Bcl-xL variants T47E and S56E have a lower association to cBid (CC in the range of 12 % to 16 %). The phosphomimetic variant S49E behaves similar to the Bcl-xL wildtype with a cross-correlation of 20 % to cBid (Figure 3.10 a). Despite clear variations in the association of cBid with the different Bcl-xL variants, Bax associated equally to all Bcl-xL variants (Figure 3.10 b).

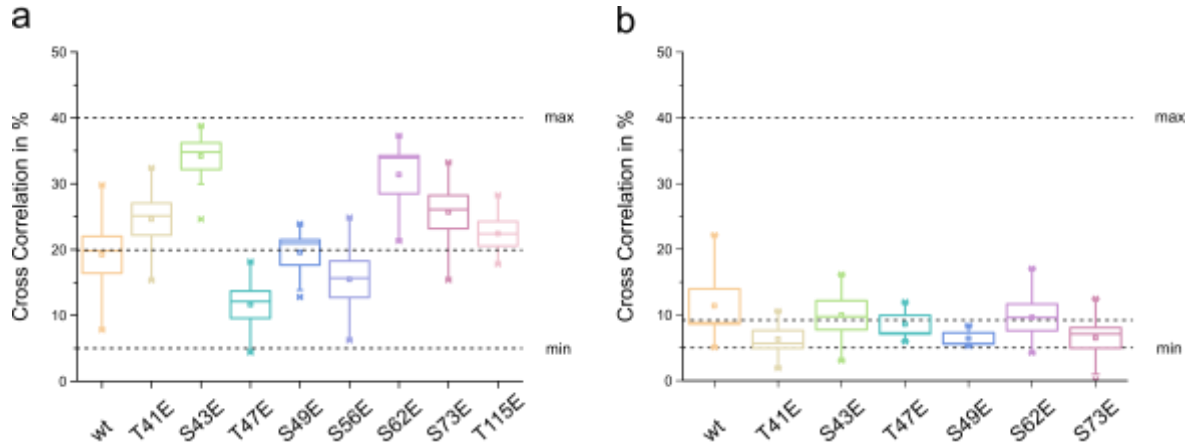


Figure 3.10 Comparison of the association strength between Bcl-xL phosphomimetic variants with cBid and Bax on the membrane of individual GUVs using SFCCS. a) The association strength of the phosphomimetic variants with cBid was compared to that between Bcl-xL wildtype and cBid. b) The association strength of the phosphomimetic variants with Bax was compared to that between Bcl-xL wildtype and Bax. The dotted line highlighted with max represents the maximal possible CC % of dimer formation considering the degree of labeling and the dotted line highlighted with min represents the minimal possible CC % of dimer formation using the fluorescence dyes (the dyes used to label Bcl-xL, cBid and Bax) incorporated in the GUV membranes. All the experiments were performed from the same batch of purified and labeled cBid and Bax.

Investigation of protein interactions on membranes of intact mitochondria:

To confirm the findings obtained on GUV membranes, we expanded the measurements to the biologically more relevant OMMs. We optimized the SFCCS to measure protein interactions on OMMs of living cells (Figure 3.11).

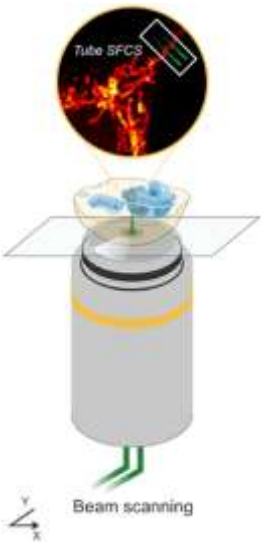
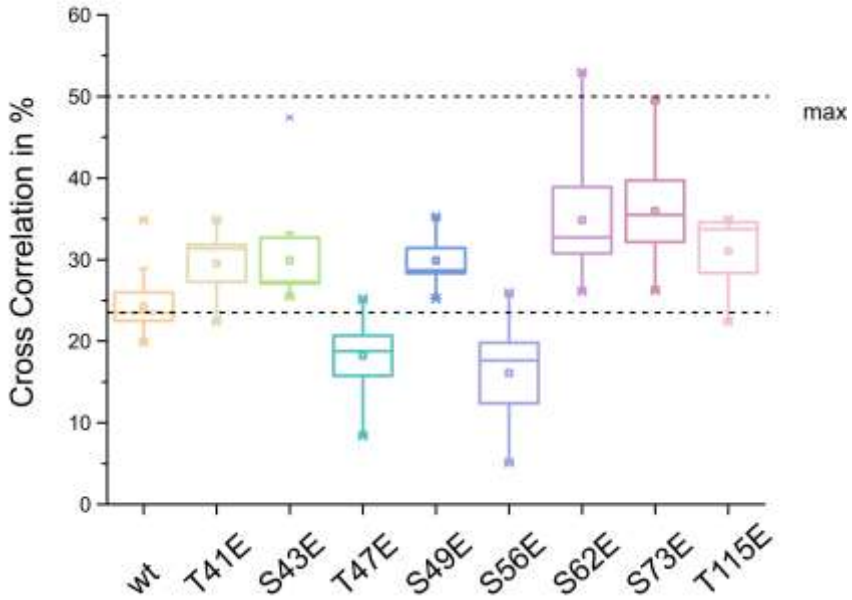


Figure 3.11 Scheme of SFCCS acquisition strategy using two-focus linear scanning across a mitochondrial tube in living cells (from Unsay at al., 2018).

The HCT 116 all Bcl-2 ko cells were transiently-transfected with mCherry-Bcl-xL and tBid-GFP or mcherry-Bcl-xL and GFP-Bax. The expressed proteins were imaged by fluorescence and SFCCS at the level of single cells. First we investigated the association differences between mCherry-Bcl-xL wildtype / tBid-GFP and mCherry-Bcl-xL wildtype / GFP-Bax on the OMM (Figure 3.9 b). Cross-correlations measurements were in good agreement with those obtained on GUVs (Figure 3.9 a). Also the cross-correlations of the phosphomimetic variants with tBid-GFP measured



on the OMM are in agreement with those obtained in GUVs (Figure 3.12).

Figure 3.12 Comparison of the association strength in CC % between Bcl-xL phosphomimetic variants and tBid on the membrane of intact mitochondria of living cells using tubeSFCCS. The HCT 116 Bcl-xL ko cells were cultivated and transfected over night with mCherry-Bcl-xL and tBid-GFP. The expressed proteins were imaged by fluorescence and SFCCS at the level of single cells. To

calculate the maximal possible CC % of dimer formation, dotted line highlighted with max, the cells were transfected with mito-GFP-mCherry and imaged by fluorescence and SFCCS.

Investigation of the Bcl-xL wildtype and phosphomimetic variants ability in retro-translocating Bax and tBid:

FLIP was chosen as the appropriate method to investigate how the Bcl-xL variants affect the retro-translocation of Bax and tBid from the OMM to the cytoplasm. The HCT 116 all Bcl-2 ko cells were transiently-transfected with mCherry-Bcl-xL and tBid-GFP or mcherry-Bcl-xL and GFP-Bax. The expressed proteins were imaged by fluorescence and FLIP at the level of single cells. We analyzed the decay in fluorescence intensity and calculated the immobile fractions of Bax as well as tBid on the OMM in presence of Bcl-xL wildtype and variants one at a time and found that:

- In the absence of mCherry-Bcl-xL, tBid-GFP retro-translocates faster between the OMM and cytosol and shows a low immobilized fraction at the OMM (Figure 3.13 a, c).
- mCherry-Bcl-xL overexpression induces 15 fold slower retro-translocation and an approximately 4 fold higher immobile fraction of tBid-GFP (Figure 3.13 a, c).
- Bcl-xL stabilizes tBid on the OMM, an effect of Bcl-xL to modulate apoptosis.
- Only the variant T115E is less able to stabilize tBid at the OMM (rel. fluorescence intensity 34 units) compared to the wildtype (rel. fluorescence intensity 54 units) (Figure 3.13 d).
- The immobile fraction of tBid with all other Bcl-xL variants was higher (rel. fluorescence intensities in the range of 56 to 80 units) than with the wild type (rel. fluorescence intensity 54 units) (Figure 3.13 d).
- mCherry-Bcl-xL overexpression causes small changes in the retro-translocation of GFP-Bax (rel. fluorescence intensity in the absence of Bcl-xL 21 units and in the present of Bcl-xL 23 units) (Figure 3.13 b, c).
- Bax retro-translocation is not significantly affected by overexpression of Bcl-xL variants (rel. fluorescence intensities in the range of 20 to 40 units) (Figure 3.13 e).

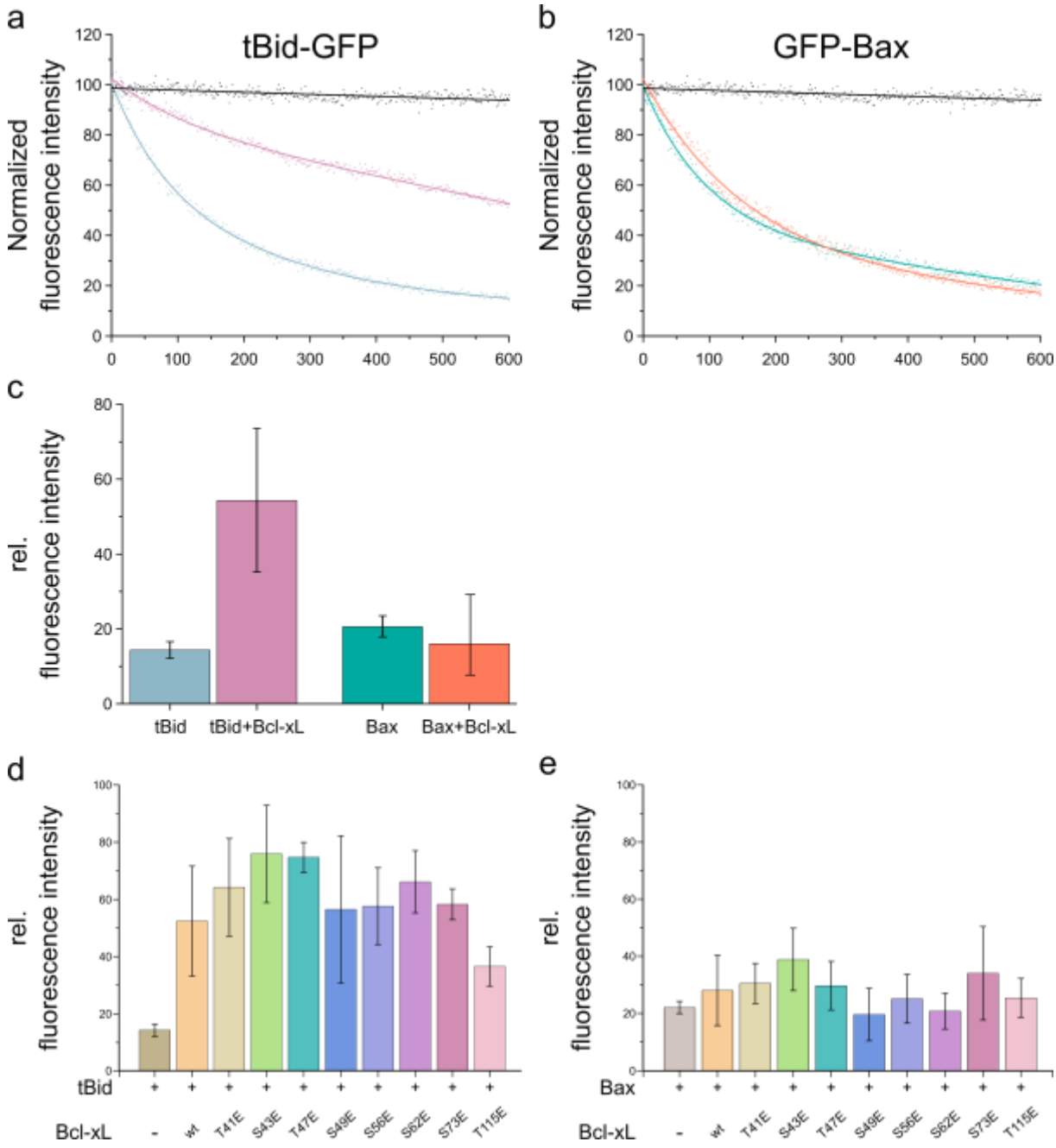
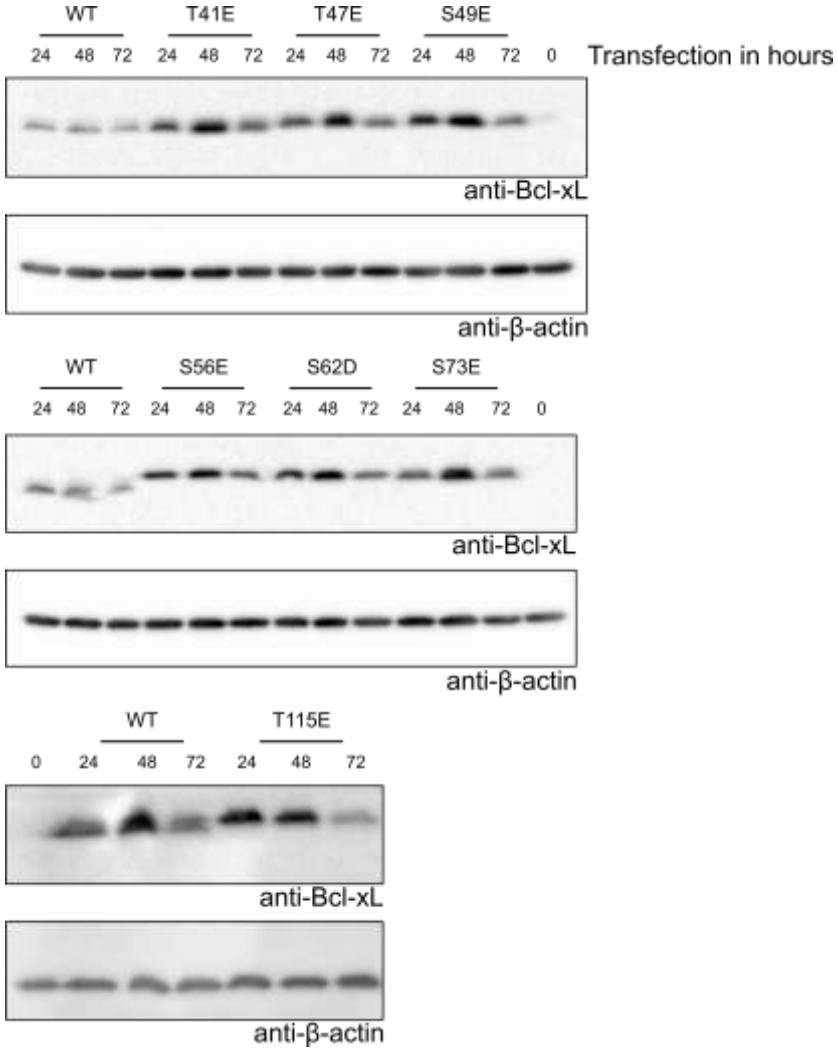


Figure 3.13 Comparison of Bcl-xL wildtype and phosphomimetic variants ability in retro-translocating tBid and Bax from the OMM to the cytoplasm. The overexpressed proteins were imaged by fluorescence and FLIP at the level of single cells, and the fluorescence of tBid-GFP and GFP-Bax were analyzed. a) The normalized fluorescence intensities represent tBid-GFP at the mitochondria in the absence (blue) and presence (purple) of overexpressed mCherry-Bcl-xL wildtype. Fluorescence of the neighboring cell is shown as control (black). b) The normalized fluorescence intensities represent GFP-Bax at the mitochondria in the absence (green) and presence (orange) of overexpressed mCherry-Bcl-xL wildtype. Fluorescence of the neighboring cell is shown as control (black). c) The rel. fluorescence intensities represent the immobile fractions of tBid-GFP and GFP-Bax in the absence and presence of overexpressed GFP-Bcl-xL wildtype. d / e) The rel. fluorescence intensities represent the immobile fractions of tBid-GFP (d) and GFP-Bax (e) in the absence and presence of overexpressed GFP-Bcl-xL wildtype and phosphomimetic variants.

These results confirm that Bcl-xL modulates apoptosis by directly associating with tBid rather than Bax and that modifications at the phosphorylation sites alter the association of Bcl-xL to tBid.

Investigation of intrinsic degradation of Bxl-xL wildtype and phosphomimetic variants:

To confirm that the differences in the interaction strength between Bcl-xL wildtype and its phosphomimetic variants to tBid and in the ability to stabilize tBid at the mitochondria are not due to the proteins´ intrinsic instability, we performed protein expression and western blotting analysis. The HCT 116 all Bcl-2 ko were transfected transiently with Bcl-xL wildtype or the phosphomimetic Bc-xL variants and the proteins were expressed for 24, 48 and 72 hours. The expression levels of the proteins were analyzed by western blot analysis (Fig. 3.14) and flow cytometry (data not shown). The results obtained by both methods confirm that the protein levels of



all the proteins decrease with a similar rate.

Figure 3.14 Protein levels of Bcl-xL wildtype and phosphomimetic variants. HCT 116 all Bcl-2 ko were transfected transiently with Bcl-xL wildtype or the phosphomimetic Bc-xL variants and the

proteins were expressed for 24, 48 and 72 hours and analyzed by western blot analysis. Protein lysates were immunoblotted for Bcl-xL and β-Actin.

3.5 Quantification of the interaction between Bcl-2 proteins. Methods and Protocols in Molecular Biology

Attached manuscript: Murad and Garcia-Saez. *Quantification of the Interactions Between BCL-2 Proteins by Fluorescence Correlation Spectroscopy*.

Contribution: I optimized the described protocols in terms of robustness and precision for the measurement of Bcl-2 proteins. I performed all the experiments including the data handling procedures. I wrote the manuscript.

Summary: This book chapter shall introduce the reader to the working principles of Fluorescence Correlation Spectroscopy (FCS), Fluorescence Cross-Correlation Spectroscopy (FCCS) and Scanning FCS and provide experimental procedures and protocols to determine the interaction strength of Bcl-2 proteins in solution and on GUV membranes. The benefits and measuring differences of FCS based methods vs. other fluorescence methods are summarized. A particular emphasis is on the comprehensive description of materials and protocols spanning from the preparation of lipid mixtures to data analysis and providing important hints to avoid experimental failures.

For the investigation of Bcl-2 protein interactions, GUVs composed of phosphatidylcholine and cardiolipin are considered to be a simplified but valid and proven model to mimic the outer mitochondrial membrane (Murad and Garcia-Saez, paper in preparation). The protocol to prepare GUVs from a mixture of the above mentioned phospholipids is provided, emphasizing that the oxidation of the lipids should be avoided by keeping them in an inert atmosphere. The GUVs are prepared in an electro formation system by distributing the lipid mixture on the electrodes in a sucrose solution and applying an alternating current voltage. Special attention should be given to the cleanliness of the electrodes and the chamber as is described in step 1 of the protocol.

The microscope settings such as laser power, emission filters and pinhole size are provided to achieve optimum results for the FCS measurements. Despite the fact that the parameters are given for a Zeiss model 710 confocor 3 system, the filter ranges and power settings are applicable to other confocal fluorescence microscopes as well. In this part, special emphasis was given on the careful calibration to be performed before each measurement series and on the aspects of photobleaching and laser instabilities due to a too high and too low power setting respectively.

For the data analysis of the calibration runs, the generally accepted and used mathematical models for fitting FCS data and the corresponding formula are provided. A step by step description including the diffusion coefficients of an often used and suitable fluorescence dye pair Alexa 488 and Alexa 633 is given.

In the two subsequent chapters, we provided the experimental procedures for the determinations of the interactions of different anti apoptotic Bcl-2 proteins with a selection of pro apoptotic proteins. Selected and suitable dye combinations as well as concentration ranges of the proteins for the investigation of their interactions are given. Particular emphasis is on the relatively complex data analysis using a 3D Brownian diffusion model and on providing the formulas to calculate the concentrations and the cross correlations of the green and the red labeled molecules.

Similarly to measurement in solutions, the experimental procedures are given for the measurements on GUVs. In this part special emphasis was given on the composition of the phospholipids to achieve optimum interaction of the different proteins with the membrane and on the preparation of the proteins regarding their membrane interactions and their activation.

Based on the Bcl-2 proteins, the relevant apoptosis regulators, we could demonstrate that FCS and its expansions are suitable methods to quantify concentrations of individual proteins and aggregates, diffusion coefficients and interaction strength in solution and on membranes and provided comprehensive and detailed protocols for successful experiments and to avoid experimental failures.

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5. List of publications

5.1 Accepted publications

Sánchez et al. Early activation of CD95 is limited and localized to the cytotoxic synapse. FEBS J. 2018

Kollek et al. Transient apoptosis inhibition in donor stem cells improves hematopoietic stem cell transplantation. JEM. 2017

Unsay et al. Scanning Fluorescence Correlation Spectroscopy for Quantification of the Dynamics and Interactions in Tube Organelles of Living Cells. ChemPhysChem. 2018

Murad and Garcia-Saez. Quantification of the Interactions Between BCL-2 Proteins by Fluorescence Correlation Spectroscopy.

5.2 Manuscript in preparation

Murad and Garcia-Saez. Impact of Bcl-xL phosphorylation on the regulation of the pro-apoptotic activity of Bax and tBid