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Molecular mechanisms controlling neuronal Bak expression

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More than the cherry blossoms,
Inviting a wind to blow them away,
I am wondering…what to do,
With the remaining springtime

Asano Naganotori

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LIST OF ABBREVIATIONS

A1	Bcl-2-related protein A1
AIF	apoptosis-inducing factor
APAF-1	apoptotic protease-activating factor-1
Bad	Bcl-2-associated death promoter
Bak	Bcl-2 antagonist killer
Bax	Bcl-2-associated X protein
Bcl-2	B cell lymphoma protein 2
Bcl-w	Bcl-2-homologous protein w
Bcl-x	B-cell lymphoma-extra large, Bcl-xL
BH	Bcl-2 homology
BH3	Bcl-2 homology region 3
Bid	BH3 interacting-domain death agonist
Bik	Bcl-2-interacting killer
Bim	Bcl-2-interacting mediator of cell death
Blk	Bik-like killer protein
Bmf	Bcl-2-modifying factor
Bok	Bcl-2 related ovarian killer
caspase	cysteinyl aspartic acid-specific protease
ced	cell death abnormal
cFLIP	cellular FLICE like inhibitory protein
CNS	central nervous systems
Dcp1a	decapping protein 1a
DD	death domain
DED	death effector domain
DIABLO	direct IAP binding protein with low pI
DISC	death-inducing signaling complex
DR	death receptor
eIF	eukaryotic translation initiation factor
EJC	exon junction complex
FADD	Fas-associated protein with death domain
FasL	fatty acid synthetase ligand, Fas ligand
FasR	fatty acid synthetase receptor, Fas receptor
FISH	fluorescence *in situ* hybridization
FLICE	FADD-like interleukin-converting enzyme
FMRP	fragile X mental retardation protein
G3BP	GTPase activating protein binding protein
GDNF	glial cell-derived neurotrophic factor
Hrk	Hara-kiri
HSP70	heat shock protein 70
IAP	inhibitor of apoptosis proteins
IRES	internal ribosome entry site
Luc	Luciferase
Mcl-1	myeloid leukemia cell differentiation protein
miRNA	microRNA

mRISC	micro RNA induced silencing complex
mRNP	messenger ribonucleoprotein
MOM	mitochondrial outer membrane
MOMP	mitochondrial outer membrane permeabilization
N-Bak	neuronal Bak
NGF	nerve growth factor
NMD	nonsense-mediated decay
NPC	neural progenitor cell
HTRA2/OMI	high temperature requirement A2
ORF	open reading frame
PARP	poly(ADP-ribose) polymerase
PB	processing body, P-body
PCD	programmed cell death
PEST	proline, glutamic acid, serine, threonine-enriched sequence
PNS	peripheral nervous system
PTB	polypyrimidine tract-binding protein
PTC	premature termination codon
Puma	p53 upregulated modulator of apoptosis
qPCR	quantitative real time polymerase chain reaction
RBD	RNA binding domain
RBP	RNA-binding protein
RHG	Reaper, Hid, Grim proteins
RIP	receptor-interacting protein
RNP	Ribonucleoprotein
RUST	regulated unproductive splicing and translation
SCG	superior cervical ganglion
SG	stress granule
Smac	second mitochondrial activator of caspases
Smg1	suppressor with morphogenetic effect on genitalia-1
SURF	SMG1-Upf1-eRFcomplex
tBid	truncated Bid
TM	transmembrane domain
TNFR1	tumor necrosis factor receptor 1
TNF-α	tumor necrosis factor alpha
TRADD	TNFR1 associated death domain protein
TRAIL	TNF-related apoptosis-inducing ligand
TRAIL-R	TNF-related apoptosis-inducing ligand receptor
uORF	upstream reading frame
Upf1	up frameshift 1 (gene) WT wild-type
UTR	untranslated region
XIAP	X-linked inhibitor of apoptosis protein
PIC	preinitiation complex

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications:

I **Jakobson M**, Lintulahti A and Arumäe U. (2012)
"mRNA for N-Bak, a neuron-specific BH3-only splice isoform of Bak, escapes nonsense mediated decay and is translationally repressed in the neurons." *Cell Death and Disease*, 3, e269

II **Jakobson M**, Jakobson M, Llano O, Palgi J and Arumäe U. (2013)
"Multiple mechanisms regulate N-Bak translation in neurons." *Cell Death and Disease* 4, e777

Both publications are referred to in the text by their roman numerals. The articles have been reprinted with permission of the copyright holders.

ABSTRACT

Studies in the past three decades have provided a detailed understanding of the key mechanisms mediating apoptosis (a type of programmed cell death) in eukaryotic cells. Our knowledge of the molecular regulation of apoptotic factors is, however, incomplete. Recent evidence from genetic and molecular studies demonstrate that possibilities to activate cell death machinery in post-mitotic cells is more restricted than in most dividing cell types. The aim of our studies has been to better understand the unique features regulating apoptotic factors in neurons.

Apoptosis in mammalian cells is controlled by Bcl-2 family proteins. Of those, the BH3-only subgroup and pro-apoptotic effectors Bak and Bax are particularly important in initiating apoptosis.

In most types of neurons *Bak* expression is affected by an alternative splicing mechanism. Neuronal *bak* variant, N-Bak, encodes a putative BH3-only protein, but its existence has remained controversial. N-Bak mRNA is abundantly expressed, suggesting that post-transcriptional mechanisms may control N-Bak protein expression in neurons.

This thesis addresses the molecular mechanisms, which regulate neuronal Bak expression in more detail. Using immunoblot analysis, we show that endogenous N-Bak protein is not constitutively expressed in healthy neurons. N-Bak protein expression remained undetectable upon nerve growth factor deprivation induced apoptosis in cultured sympathetic neurons. Similar results were obtained using primary cortical neurons in etoposide-induced intrinsic apoptosis model. Challenging neurons with cytotoxic drug, thapsigargin, did not reveal N-Bak protein expression, suggesting that its mRNA is translationally silent also in stressed primary neurons. The absence of N-Bak protein was further confirmed using quantitative mass spectrometry analysis, which did not reveal any peptide form samples representing healthy, stressed or apoptotic neurons. We also show that accelerated proteosomal degradation is not responsible for the absence of N-Bak protein in neurons.

The open reading frame of N-Bak mRNA is prematurely terminated, being potential target for the nonsense mediated mRNA decay (NMD) pathway. We found that N-Bak mRNA is not a constitutively targeted by NMD, but instead, is relatively stable in neurons. Luciferase reporter assays showed that N-Bak mRNA is translationally repressed via cis-acting contextual elements in its untranslated regions. Finally, we demonstrate that N-Bak mRNA localizes into the uncharacterized granular structures in sympathetic neurons, and this distribution remains unchanged in apoptotic neurons.

Our findings collectively demonstrate that multiple post-transcriptional mechanisms control N-Bak expression in neurons. These mechanisms may be part of the program that governs tight control over apoptosis in neurons. Our results also highlight the importance to further characterize diverse, still neglected post-transcriptional regulation mechanisms that can modulate apoptotic pathways in a various post-mitotic cells.

1 INTRODUCTION

Media vita in morte sumus- the expression translating to "in the midst of life we are in death" has probably many meanings, but can be used here as a reminder that life is an impermanent phenomenon and death is an inevitable part of it. This conundrum has also been a focus of modern biosciences as cell death in multicellular organisms might be programmed or accidental, beneficial or pathological and interconnected with which signaling pathways, which ultimately determine the cellular responses to death-inducing signals. The precise transitions from reversible homeostatic disturbances to the loss of cellular activities are challenging to define (Galluzzi et al. 2015).

Naturally occurring programmed cell death (PCD) plays a fundamental role in animal development and tissue homeostasis (Jacobson et al. 1997). One of the best-characterized forms of PCD in higher eukaryotes is apoptosis. It is an active, precisely controlled cell death process that progresses through series of biochemical and morphological changes culminating in the removal of abnormal, misplaced, nonfunctional, or harmful cells. Apoptosis plays a central role in the mammalian nervous system development. Roughly 50 % of the dividing neural progenitors, differentiated neurons and glial cells are removed by apoptosis (Hamburger 1975; Buss et al. 2006b). Perturbed apoptosis is often a contributing factor in cancer as well as in major degenerative diseases, and its regulation is therefore continuously exciting topic to study.

Cultured sympathetic neurons from neonatal rodent superior cervical ganglia (SCG) are a widely used model to study neuronal apoptosis *in vitro* (Deshmukh and Johnson 1997; Easton et al. 1997). Studies on this model have revealed many features that appear to specifically refine apoptotic program in neurons (Martinou et al. 1999; Deshmukh et al. 2000; Kole et al. 2013). Research indicates that other post-mitotic cell types, such as cardiomyocytes and pancreatic β-cells, but also various cancer cells may similarly control their apoptotic death program for better survival (Wright and Deshmukh 2006; Gama et al. 2014). Dissection of regulatory layers underlying increased apoptosis resistance in different cell types or cellular settings requires further investigation.

Apoptosis in mammalian cells proceeds through two major pathways, representing cell extrinsic or death receptor-initiated and intrinsic or mitochondrial apoptosis pathways. The latter is controlled by the Bcl-2 family proteins. Particularly the pro-apoptotic BH3-only subfamily and effector proteins Bax and Bak are important for apoptosis to proceed (Li et al. 1997). Analyses of evolutionary relations in animal apoptosis network have shown that the key molecular components of apoptosis machinery are highly conserved.

Previous studies have shown that various types of neurons do not express mRNA for Bak. Instead, *bak* pre-mRNA is alternatively spliced, resulting in exclusively neuronal isoform, N-Bak that can encode a BH3-only protein (Sun et al. 2001). Detection of endogenously expressed N-Bak protein has remained controversial. This thesis investigates whether N-Bak protein is expressed in neurons and which molecular mechanisms control its expression.

Eukaryotic gene expression is a complex process that is tightly regulated by multiple control mechanisms, which coordinate the synthesis and turnover of gene products in the cell. Posttranscriptional control manifests itself at all stages from mRNA biogenesis to degradation.

Widespread mRNA alternative splicing may expand the transcriptome and proteome diversity beyond the estimated coding capacity of the genome (Pan et al. 2008; Wang et al. 2008; Nilsen and Graveley 2010; Chen 2015).

The translational control provides another essential layer for regulating gene expression in various cellular settings (Schwanhausser et al. 2011; Baltz et al. 2012). Global translational reprogramming has fundamental importance in establishing protective responses to cellular stress, as well as in initiating apoptosis when these mechanisms fail (Galluzzi et al. 2016). The transcript-specific translational control allows cells to express proteins with spatiotemporal precision and in response to various signalling cues (Tiruchinapalli et al. 2003; Mili et al. 2008). Certain regulatory elements, including upstream open reading frames (uORFs) and microRNA binding sites can significantly modulate mRNA translatability in the cell. However, their relative contribution and cooperative effects in gene expression have remained largely unclear.

The accuracy of gene expression is monitored by cellular surveillance machineries. The mRNA quality control pathway- nonsense-mediated mRNA decay (NMD) detects and degrades transcripts that harbor a premature termination codon (PTC), thus limiting the expression of aberrant transcripts (Maquat 2004; Rehwinkel et al. 2006; Muhlemann et al. 2008; Karam et al. 2013; Popp and Maquat 2014; Mockenhaupt and Makeyev 2015). NMD also regulates the fate of cellular mRNAs, affecting approximately 5-20% of mammalian protein-coding transcriptomes (Mendell et al. 2004; Wittmann et al. 2006; Chan et al. 2007; Yepiskoposyan et al. 2011; Karam et al. 2013). Some transcripts may conditionally evade NMD depending on intracellular context (Stockklausner et al. 2006; Colak et al. 2013). Altogether, NMD is a widespread and multifaceted mechanism to regulate gene expression.

Defective RNA homeostasis has been implicated in a continuum of abnormalities that contribute to the development of major neurodegenerative diseases and cancer (Wethmar et al. 2010b; Liu-Yesucevitz et al. 2011; Wolozin 2012; Bai et al. 2013).

The role of post-transcriptional gene expression regulation in apoptosis has gained little attention. It is surprising, as genes, encoding key apoptosis regulators have alternative splice variants (Schwerk and Schulze-Osthoff 2005), may be substrates for NMD (Solier et al. 2005) or are translationally controlled (Cimmino et al. 2005; Mott et al. 2007; Kole et al. 2011b; Lima et al. 2011; Lerner et al. 2012). Therefore, elucidating the post-transcriptional gene expression mechanisms that target main contributing factors in the cell life and death decisions may be essential for understanding how to overcome apoptosis resistance in cancer cells or protect various postmitotic cells, including neurons, from dying.

2 REVIEW OF THE LITERATURE

2.1 Conceptual advancements of cell death research

Cell death research started soon after the formulation of the cell theory in 1839. From there, the field developed through series of discoveries and re-discoveries, but mainly stayed in dormancy for about 150 years until late 1980s with the finding that cell death in multicellular organisms is subject to genetic control (Horvitz and Sternberg 1982; Ellis and Horvitz 1986). However, it is well acknowledged that most influential concepts in cell death research, developed in 20th century, have their roots in 19th century (reviewed in Clarke and Clarke 1996).

One of the first descriptions of cell death came from German naturalist Carl Vogt in 1842 who observed dying cells in the neuronal system of developing toad embryos (Vaux and Korsmeyer 1999). Subsequent studies in different developmental systems described the chondrocyte death during endochondral ossification and loss of an entire population of neurons in fish embryos, but never captured any wide attention in the scientific community. Also, French anatomist F. Collin, German neuroanatomists M. Ernst and A. Glücksmann described the widespread cell death in the developing nervous system, but their findings were not recognized by other researchers at the time. Still, these studies collectively provided a basis for eventual understandings that naturally occurring cell death is a part of normal development.

In 1951 Alfred Glücksmann summarized observations about cell death in normal vertebrate development that he had compiled from previously published literature (Glucksmann 1951). In this review, he declared the wide existence of cell death in various embryological processes like organ morphogenesis, tissue histogenesis, and phylogenetic degeneration. Importantly, he outlined the major morphological changes observed in dying cells during normal vertebrate development.

The broader acceptance that physiological cell death is a critical process in the development emerged from the studies of Italian neuroscientist Rita Levi-Montalcini (Hamburger 1992). She provided conceptually novel perspective to explain the phenomenon, originally described by Victor Hamburger in 1934, that wing bud removal induced decrease of certain nerve centers in developing chick embryo. She found that the hypoplasia of those nerve centers in this model is a result of death of young differentiated neurons, but not due to the inability to recruit neurons from the pool of undifferentiated precursors (Levi-Montalcini 1987). This finding let her to conclude that the peripheral target tissue defines the survival of innervating neuron populations (Levi-Montalcini 1987). In collaboration with Victor Hamburger, they demonstrated that neuronal cell survival during development is under the direct influence of their target field size (Hamburger and Levi-Montalcini 1949). They proposed the theory that target field secretes limited amount of essential nutrients or trophic factors that regulate the innervating cell number during the establishment of neuron-target interactions. As in its original context, developing neurons compete for trophic support, only neurons receiving

it sufficiently survive, while neurons that fail to receive trophic support, die. The concept got convincing evidence when the nerve growth factor (NGF), a first neurotrophic factor was isolated and purified from mouse sarcoma tissue, snake venom and subsequently from the mouse submandibular salivary gland extract .

The established neurotrophic factor theory provided a mechanism how neuronal populations are sculpted to match the requirements of the target. Significantly, the neurotrophic theory became one of the most-influential concepts of developmental neurobiology and strengthened the understanding that cell death is a significant process in organism development (reviewed in Levi-Montalcini 1987).

The advancement of techniques in biochemistry and genetics in the 1960s and 1970s laid foundation to many fascinating discoveries in cell death biology.

The existence of stereotypical cell death patterns in various model organisms emerged. Conclusively, the term "*programmed cell death*" (PCD) was introduced by Richard Lockshin by series of articles where he studied regression of intersegmental muscles during moth metamorphosis (Lockshin and Williams 1965). The term PCD denotes that, during multicellular organism development, a subset of cells die in predictable places, at certain times, according to the developmental program. For example, during embryogenesis, PCD ensures the regression of phylogenetically vestigial structures, the sculpting of organs and establishment of appropriate cell numbers in tissue (Roth and D'Sa 2001).

On 1966, Jamshed Tata demonstrated that cell death during Xenopus tadpole tail metamorphosis could be blocked by translation inhibitor cycloheximide, indicating that developmentally occurring cell death process requires new protein synthesis (Tata 1966). The requirement of active RNA and protein synthesis for cell death process was also demonstrated later in postnatal developmental systems, including developing neurons (Bowen and Lockshin 1981; Martin et al. 1988). These studies collectively suggested that PCD is an active process in which cell's own intrinsic programs are activated to cause its destruction and all cells might share a common death program. However, the molecular mechanisms by which the newly synthesized proteins contribute to death program remained unknown at that time.

In parallel, Australian pathologist John F. R. Kerr started to define the morphological features of cells undergoing a type of non-necrotic death, observed in several tumor regression and organ atrophy models. The phenomena became well described by analysis of the electron micrographs showing ultrastructural changes in dying cells, exhibiting shrinkage, condensed nuclei and break up into vesicles without compromising their membrane, but injured cells swelled and lost their membrane integrity (Kerr et al. 1972). Kerr´s collaboration with Andrew H. Wyllie and Sir Alastair R. Currie showed that cells can die during normal tissue turnover with surprisingly similar morphology as those that die naturally during development or some pathophysiological conditions. A process of naturally occurring cell death was termed "*apoptosis"* (Kerr et al. 1972) and described an active, inherently regulated cell death mechanism that proceeds through distinct morphological changes (Kerr et al. 1972). Their findings coalesced with

earlier knowledge, summarized by Glücksmann and also emphasized apoptosis as a process which balance cell proliferation in a given tissue. The significance of these findings was recognized almost 20 years later, when the apoptotic cell death mechanisms were starting to uncover.

In 1975, a promising factor, with tumour-necrotizing activity in mice, termed tumour necrosis factor (TNF), was identified, but a decade later it turned into disappointment, when it was shown to exert a lethal inflammatory shock syndrome under systemic treatment (reviewed in Walczak (2013)). Initially, discouraging results for cell death researchers, however, advanced the development of anti-inflammatory therapies for chronic inflammatory diseases, such as Crohn´s disease and psoriasis. The concept to induce cell death in cancer cells by extracellular stimulus turned into success in 1990s when many research groups identified the so-called cell death receptors and their ligands, which could mediate cell death. The expanded understanding of death receptor-mediated signalling pathways has been in the forefront in many areas of biomedical research today (Lau et al. 2013; Walczak 2013).

In 1980´s the term "*autophagic cell death"* (ACD) was coined to refer a type of cell death, showing autophagic features, such as vacuolization of the cytoplasm and lacking the characteristics of other types of cell death (Schweichel and Merker 1973; Clarke 1990). ACD appeared to be relatively common alternative route for PCD during animal development, tissue homeostasis, and in diseased tissues, as well as in cultured mammalian cells under certain experimental conditions (Clarke 1990; Nixon and Yang 2011; Liu and Levine 2015). It is important to note that the concept of ACD has been based on purely morphological descriptions and has suffered from ambiguous interpretations (Shen et al. 2012; Klionsky 2013). Hence, the views on the existence of genetically regulated autophagic cell death program in mammalian cells have remained controversial (Kroemer and Levine 2008; Clarke and Puyal 2012; Denton et al. 2012; Shen et al. 2012; Liu and Levine 2015). However, the concept that autophagy can be mechanistically involved in cell death led to the recognition that multiple intracellular events may contribute to the process programmed cell destruction (Clarke 1990).

The mechanisms that account for apoptotic cell death morphology were not clear, but began to emerge when the main molecular components of the apoptosis machinery were discovered. Out of 1090 somatic cells of worm *Caenorhabditis elegans*, 131 die during development in predictable and precise manner (Sulston 1976). Series of genetic experiments, in particular the selection of mutant worms with defects in the physiological death program, led Robert Horvitz and colleagues identify dozen of genes, essential for the regulation of programmed cell death (Horvitz and Sternberg 1982; Ellis and Horvitz 1986). Among these, three genes: *Ced-3*, *Ced-4*, *Ced-9* (Yuan and Horvitz 1992; Yuan et al. 1993; Hengartner and Horvitz 1994a) and later fourth gene *Egl-1* (Conradt and Horvitz 1998) were found to be indispensable for the normal progression of cell death during early development of *C. elegans*.

The first mammalian apoptosis regulator was identified when *bcl-2*, the gene, activated by chromosomal translocation in human follicular lymphoma (Tsujimoto et al. 1984), was found to rescue hematopoietic cells from cytokine deprivation induced cell

death (Vaux et al. 1988). The finding that *bcl-2* overexpression increases cell survival instead of promoting the proliferation overturned the oncogene paradigms that were accepted at that time. The demonstration that mammalian *bcl-2* gene could replace the worm survival gene *ced-9* revealed the evolutionary conservation of the apoptotic machinery (Vaux et al. 1992; Metzstein et al. 1998).

Since the identification of key apoptosis regulators in *C. elegans* and their counterparts in mammals (Hengartner and Horvitz 1994b; Horvitz et al. 1994) the apoptosis has attracted extensive research interest. It was soon realized that molecular pathways of apoptosis can be a potential target for therapeutic interventions to treat numerous human diseases. In addition, recent discoveries have led to the revelation that previously believed passive and accidental form of cell death, necrosis, can also occur in a highly regulated and genetically controlled manner (Vercammen et al. 1998; Holler et al. 2000; Vandenabeele et al. 2010b).

In 1998, P. Vandenabeele group described an alternative form of cell death that showed many hallmarks of cellular necrosis, but appeared to depend on some molecular factors, functioning in the apoptotic cell death machinery (Vercammen et al. 1998; Holler et al. 2000). This type of programmed cell death was therefore termed "necroptosis" or programmed necrosis. Necroptosis is now shown to be closely interconnected with apoptototic signaling and seems to represent a backup mechanism for apoptotic cell death. This knowledge has only recently changed the the whole concept of therapeutic targeting of pathological cell death process in mammalian cells and represents a promising field for the further development of innovative therapeutic approaches (Vaux et al. 1988; Giampietri et al. 2014).

The interconnected nature of various cell death and survival signalling pathways challenges the modulation of cell death response in many clinical settings today. It will be interesting to follow how the new knowledge obtained from biochemical analyses of cells and animal models, combined with modern techniques in proteomics and systems biology is translated into novel therapeutic approaches that enable to treat and circumvent the cell death related pathological conditions. These advancements will unfold in the future.

2.2 Apoptosis

The term *apoptosis* originates from an ancient expression in Greek meaning *"falling off"* and refers to a mechanism of naturally occurring cell death (Kerr et al. 1972). Apoptosis is an active, precisely controlled cell death process that serves the normal development and homeostatic function in multicellular organisms. It plays a crucial role for sculpting the developing organs, maintaining tissue homeostasis and eliminating damaged, aged or infected cells. Deregulated apoptosis is emphasized in numerous pathological conditions including neurodegenerative and autoimmune disorders, many types of cancer and certain viral illnesses.

2.2.1 Apoptosis and cell death classification

Historically, cell death has been classified based on morphological features, such as type I- apoptotic cell death, type II- autophagic cell death, and type III- necrosis (Kerr et al. 1972; Schweichel and Merker 1973; Clarke 1990).

Apoptosis is characterized by number of morphological changes (Table 1), including cell shrinkage, chromatin condensation (pyknosis) and nuclear fragmentation (karyorrhexis) (Kerr et al. 1972). Adherent cells lose their attachment to extracellular matrix and retract. The retraction stage is followed by plasma-membrane blebbing and formation of membrane-enclosed vesicles the so-called "*apoptotic bodies*". Hence, apoptotic cells retain their membrane integrity and are rapidly eliminated by phagocytosis. The clearance of apoptotic cells is efficient process and does not elicit an inflammatory response *in vivo*. Cells grown *in vitro*, proceed to a terminal phase through process termed "*secondary necrosis*" showing swelling and lysis of apoptotic bodies.

Majority of developmentally and physiologically occurring cell death has the classical morphological characteristics described to define apoptosis (Kerr et al. 1972). Therefore, the terms "*apoptosis*" and "*programmed cell death*" are often used as synonyms. However, PCD is irrespective of the modality by which it is executed. All PCD is not apoptotic in appearance, however, the most encountered form of PCD is apoptosis. It would be most suitable to use the term PCD to describe a temporally and spatially restricted cell death that follows a distinct biological program in the developing organism or tissue homeostasis (Kroemer et al. 2009).

Autophagy is an important catabolic process by which cells degrade and recycle their non-essential or redundant proteins, cellular aggregates and damaged organelles (Hotchkiss et al. 2009; Yang and Klionsky 2010). It serves as a vital homeostatic and survival mechanism in healthy cells and in various cellular stress conditions (Levine and Yuan 2005). Type II or autophagic cell death has been associated with enhanced autophagy and is characterized by typical morphological features, such as cell pyknosis, abundance of autophagic vacuoles (autophagosomes) and progressive disappearance of organelles (Schweichel and Merker 1973; Hornung et al. 1989; Clarke 1990). Whether autophagy is a *bona fide* cause of mammalian cell death or does its deregulation accelerate cell death indirectly has remained controversial (Long and Ryan 2012; Nixon and Yang 2012; Shen et al. 2012; Liu et al. 2013). Recently, Liu and colleagues (2013) identified a process, termed "autosis" in mammalian cells, that can be induced by autophagy-inducing peptides and blocked by pharmacological or genetic inhibition of autophagz. It suggests that cell death by autophagy can be genetically programmed (Green and Levine 2014).

Necrosis, traditionally regarded as a passive and unregulated form of cell death, (Zhou and Yuan 2014) occurs when cells are exposed to adverse conditions resulting in irreparable damage to the plasma membrane. Necrotic death can be evoked by traumatic injury, prolonged hypoxia and hypodermia, but also by viral infection. Characteristic morphological features of necrosis (Table 1) include an early onset of plasma membrane permeabilization, cellular swelling and subsequent disruption of organelle membrane

integrity. (Vandenabeele et al. 2010a). Lysosomal disintegration leading to release of hydrolases helps catalyse cellular destruction (Boya and Kroemer 2008). Unlike apoptosis, nuclei of necrotic cell nuclei remain intact while cell rupture releases its contents to the surrounding tissue, resulting in inflammation.(Vandenabeele et al. 2010b).

The discovery of necroptosis overturned the traditional belief that necrosis is merely a passive process (Zhou and Yuan 2014). Necroptosis is now the most understood form of genetically controlled necrotic cell death program. It engages the death receptor signalling and protein kinases of the receptor interacting protein (RIP) family under the conditions where apoptosis execution is prevented (Degterev and Yuan 2008). Necroptosis is negatively regulated by functional apoptotic signalling and inhibitable by specific Rip-kinase inhibitors, termed as necrostatins (Degterev et al. 2008; Kaczmarek et al. 2013). In addition, several other examples of regulated necrosis have emerged, but have remained still poorly characterized. Although the intracellular events leading to necroptosis and unregulated necrosis are distinct, these cell death modes exhibit similar morphological features (Degterev and Yuan 2008; Vandenabeele et al. 2010b), demonstrating that only morphological descriptions are not sufficient to discriminate between different necrotic cell death types (Vandenabeele et al. 2010b).

The practicality of morphological categorization of diverse types of cell death has been substantially debated (Zeiss 2003; Vandenabeele et al. 2010b). For instance, classical apoptotic cell death, although morphologically similar, can be reached through different biochemical routes (Danial and Korsmeyer 2004; Thorburn 2004), with or without the contribution of mitochondria (Yu et al. 2008). It is also appreciated that under pathological conditions the cell death process often follows mixed routes (Kroemer et al. 2009), yielding a complex morphological picture which defies the cell death classification by distinct patterns (Nixon and Yang 2013). Therefore, morphological definition of cell death subroutines are gradually being substituted by precise, measurable biochemical features, taking also into account cell type and death stimulus (Galluzzi et al. 2012b). Typical apoptosis and typical necrosis can be regarded as two extremes of apoptosis-necrosis continuum (Zeiss 2003).

Apoptotic cells classically exhibit many biochemical changes (Table1), including mitochondrial outer membrane permeabilization (MOMP), release of mitochondrial intermembrane space proteins to the cytoplasm, activation of caspase family proteases, widespread cleavage of cytoskeletal and nuclear proteins by caspases and endonucleolytic DNA nucleosomal fragmentation (Williams et al. 1974). Also plasma membrane changes, in particular exposure of internal plasma membrane lipid, phosphatidylserine to the outer leaflet of the membrane, are the biochemical hallmark of apoptosis (Savill and Fadok 2000). Due to the degree of biochemical interconnections of different programmed cell death pathways, it is suggested that the term apoptosis should be applied exclusively to cell death events that occur while manifesting several among these features (Kroemer et al. 2009).

Table 1. Typical morphological and biochemical features of apoptosis-necrosis continuum.

	Morphological features	Biochemical features
Apoptosis	Cell shrinkage	MOMP
	Chromatin condensation	Mitochondrial transmembrane poteintial dissipation
	DNA fragmentation	Release of IMS proteins
	Nuclear fragmentation (karyorrhexis)	Activation of caspase cascade
	Membrane blebbing	Phosphatidylserine exposure
	Membrane integrity maintained	Internucleosomal DNA cleavage
	Formation of apoptotic bodies	ATP depletion
	Phagocytosis	Activation of other enzymes (cathepsins, calpains)
Necrosis	Dilation of intracellular membranes	RIP1/RIP3 activation
	Disintegration of organelles	MOMP
	Irregular chromatin condensation	Sphingoside and ceramide overproduction
	No karyorrhexis	Lysosomal membrane permeabilization
	Cellular swelling	Release of cytotoxic hydrolases into the cytosol
	Plasma membrane brakedown	Cytosolic Ca2+ waves
		Activation of cathepsins and calpains
		PARP1 hyperactivation
		ATP and NAD+ depletion
		Possible phosphatidylserine exposure
		Random DNA digestion
		Postlytic DNA fragmentation

Abbreviations: IMS-mitochondrial intermembrane space; MOMP-mitochondrial outer membrane permeabilization; RIP-receptor-interacting protein kinase; PARP-poly-(ADPribose) polymerase 1; Adapted from Galluzzi et al., (2011).

2.2.2 Evolutionary conservation of animal apoptosis network

The description of characteristic morphological and biochemical criteria for apoptotic cell death (Kerr et al. 1972) led to conceptual advancement in cell death research. The studies, showing the PCD dependency on macromolecule synthesis generalized the idea that certain cell death program might be inherent in every cell of a multicellular organism (Lockshin 2016). Numerous genetic and biochemical studies using nematode, insect and vertebrate models, in combination with genome analysis of animals from lower taxa, have demonstrated that core components of the apoptotic machinery are evolutionarily conserved in the whole animal kingdom (Metzstein et al. 1998; Cheng et al. 2001; Zmasek et al. 2007; Quistad et al. 2014). However, there are several differences in the way apoptosis is regulated and how individual molecules contribute to the propagation of the death signal (Kornbluth and White 2005).

In the nematode *C. elegans*, the developmental cell death is controlled by a simple genetic pathway (Hengartner and Horvitz 1994b) involving the sequential and coordinated actions of evolutionarily conserved proteins, such as apoptosis initiator protein Egl-1, inhibitor protein Ced-9, proteolytic enzyme Ced-3 and its adaptor molecule Ced-4 (Figure1). Similar pathway operates in the fruit fly *D. melanogaster* (Richardson and Kumar 2002) (Figure 1), although no homologues for Egl-1 in fruitflies have been

described. Functional homologue of Ced-3 in the fruitfly is Dronc which recquires Ced-4 like adaptor molecule Dark for its function. Other apoptotic proteases in *Drosophila* are Drice and Dcp1. Drosophila apoptotic proteases are negatively regulated by the inhibitor of apoptosis protein (IAP) family member Diap. However, closely related proteins Reaper, Hid, Grim (RHG proteins) and Sickle, counteract the activity of Diap and are essential for the induction of apoptosis in *Drosophila* cells (White et al. 1994; Richardson and Kumar 2002). RHG proteins have been also shown to interact with mammalian IAPs and induce apoptosis in human cells (Vucic et al. 1997; McCarthy and Dixit 1998; Vucic et al. 1998), demonstrating evolutionarily conserved mechanism of apoptosis. Genetic interaction studies suggest that Drosophila Ced-9 homologs Debcl and Buffy act downstream of RHG, and upstream of Dronc proteins (Quinn et al. 2003).

The mammalian apoptotic network is more complex (Vaux and Korsmeyer 1999) (Figure 1). About a dozen homologs for the nematode Ced-3 and fruitfly Drice and Dronc proteins exist in mammalian cells (Alnemri et al. 1996; Nicholson and Thornberry 1997). These proteins are known as caspases (cysteine-specific aspartic acid residue-specific proteases) comprising a group of key proteases in apoptosis (Yuan et al. 1993).

Nematode Ced-9 gene is functionally and structurally homologous to a family of pro-apoptotic and anti-apoptotic proteins that have phylogenetic relationships with mammalian Bcl-2 protein (Hengartner and Horvitz 1994b; Rech de Laval et al. 2014). Recent findings indicate the pro- or anti-apoptotic Bcl-2 family proteins are functionally conserved from sponges to mammals (Moya et al. 2016), however, the number of apparent Bcl-2 family member genes in different genomes varies widely (Aouacheria et al. 2005; Youle and Strasser 2008; Moya et al. 2016).

Nematode *Egl-1* encodes a pro-apoptotic protein, analogous to large group of mammalian proteins which all share the amino acid sequence similarity with each other only within the short BH3 (Bcl-2 homology region 3) domain, hence termed as BH3-only proteins. Based on the presence of BH3 domain these proteins are classified as Bcl-2 family members. However, it has been suggested that classical BH3-only proteins have obtained the BH3 domain through convergent evolution and are otherwise evolutionarly unrealted (Aouacheria et al. 2013).

Nematode *Ced-4* is homologous to mammalian gene that encodes apoptosis activating factor Apaf-1, an essential adaptor molecule for caspase activation (Li et al. 1997). Intriguingly, some mitochondrial factors, have acquired additional roles in mammalian apoptosis network. Particularly mitochondrial respiratory chain component cytochrome c participates in caspase activation, whereas in nematode or insect cells Ced-3 activation does not involve the cytochrome c (Vaux 1997).

The genome analyses of several animal species representing diverse taxa in animal kingdom now indicate that complex apoptotic regulatory network, found in vertebrates, most likely existed already in the common metazoan ancestors (Zmasek and Godzik 2013). Accordingly, the relative simplicity of the apoptosis regulatory networks in the model organisms, such as nematodes and insects does not represent their ancestral state, but is the result of secondary simplifications in these model species, i.e. it has resulted from extensive gene loss in evolution of ecdysozoans (including nematodes and

insects), but also in arthropod lineage (Zmasek and Godzik 2013). Therefore, genome analysis of basal phyla has provided an important view to re-evaluate the evolution of apoptosis network in different animal species (Zmasek and Godzik 2011; Zmasek and Godzik 2013).

Caspases, Apaf-1, canonical Bcl-2 family members and IAP family proteins comprise the core apoptotic machinery in animal cells. Animal apoptosis network also contains the members of IAP antagonists, such as Smac/Diablo and tumor necrosis factor (TNF) receptor family, their ligands and various interacting adaptor proteins (Nagata 1997, Diez 2010). Bioinformatics analyses have revealed that all of the major components of the death receptor pathway are present in corals (Quistad et al. 2014). In addition to caspases, some other protease families, such as cathepsins and serine proteases, nucleases and their regulatory proteins are also involved to apoptosis. It has been estimated that whole regulatory network for apoptosis involves several hundreds of genes in mammals (Doctor et al. 2003; Diez et al. 2010). Several proteins display redundant functions in vertebrate cells and it has created many challenges for dissecting the exact role of individual homologues in apoptosis network. Information on structure, evolution and function of proteins involved in apoptosis and other forms of cell death has been collected to the database, called DeathBase (Diez et al. 2010).

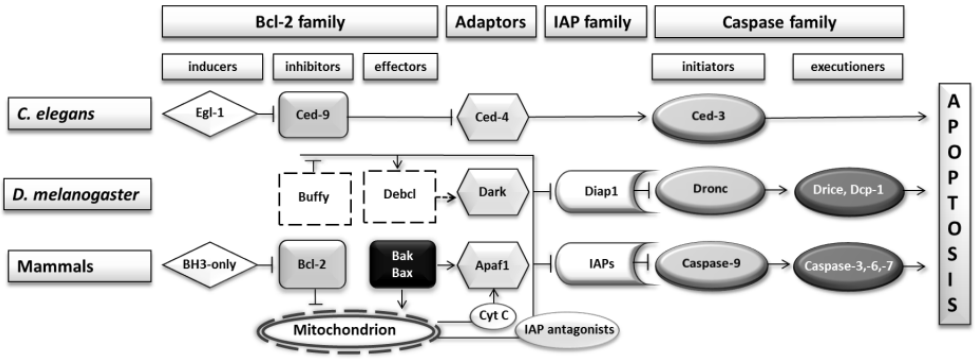

Figure 1. Evolutionary conservation of core apoptosis machinery in selected model organisms. Shown are the components of so-called intrinsic apoptosis machinery in *C.elegans, D.melanogaster* and mammals. Those include the Bcl-2, IAP and caspase protein families and apoptosome adaptor proteins. Homologues across the species are indicated by the same shape and colour. Dashed lines indicate controversial position in apoptotic cascade.

2.2.3 Mechanism of apoptosis in mammalian cells

Apoptosis can be subdivided into three intimately connected phases: an initiation, execution and clearance phase (Vaux and Strasser 1996; Kroemer and Reed 2000). Initiation phase involves signalling, related to cell life and death decisions. It is often reversible and thus, is not lethal to the cell (Harvey et al. 1998). Execution phase is essential for actual death and employs effector molecules, most notably caspases, for cell demise (Alnemri et al. 1996; Nicholson and Thornberry 1997). The engulfment of dying cell fragments by phagocytizing cells represents the clearance phase and guarantees the non-inflammatory nature of the apoptotic cell death (Savill and Fadok 2000).

Apoptosis in mammalian cells can be initiated by large range of stimuli originating from the extracellular cues or from the intracellular stress or damage sources (Berthelet and Dubrez 2013). Different stimuli induce parallel signalling cascades within the cells, which modulate the cell death and survival decisions (Figure 2). While mild or transient perturbations in cellular homeostasis trigger adaptive responses that enable extended survival or even reverse cell death initiation, severe or prolonged metabolic challenge often results in cell death. Therefore, cell fate is largely determined by its capacity to adapt (Fulda et al. 2010). Current concepts in cell commitment to apoptosis are discussed in the section 2.2.4.

The initiation of apoptotic death program as well as cell adaption to stress requires *de novo* gene expression and protein synthesis (Martin et al. 1988; Tang et al. 2012; Lu et al. 2014), where numerous gene expression control mechanisms play important role.

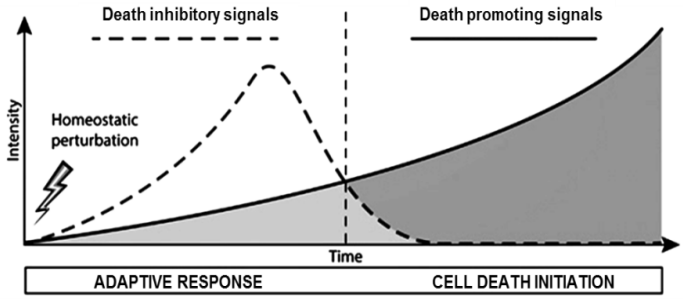

Figure 2. Cell survival and death decisions in relation to its capacity to adapt.
Cellular stress response pathways depend on the cell type and the intensity, location and duration of the stress. Activation of cellular adaption mechanisms promote survival and counteract the effect of the stress on cellular homeostasis.However, activation of death signaling pathways occurs in parallel with protective responses and becomes prevailing, if the stress remains unresolved. Adapted form Galuzzi et al (2015).

Cellular organelles, such as mitochondria, endoplasmic reticulum, Golgi apparatus, lysosomes and cell nucleus can sense stressful and pathogenic alterations in the cell and trigger local signal transduction pathways which contribute to cell life and death decisions (Ferri and Kroemer 2001). Mitochondria sequesters in its inter-

membrane space a number of apoptogenic proteins, which redistribution to cytoplasm could trigger caspase dependent or independent cell execution (Saelens et al. 2004). Thereby mitochondria are a central hub in coordinating apoptotic signalling. Bcl-2 family proteins control the permeabilization of outer mitochondrial membrane and release of apoptogenic factors from the mitochondrial intermembrane space (Danial and Korsmeyer 2004), thus being central regulators of life-death decisions within the cells.

Also, plasma membrane plays an important role in the life-death decisions of the cell (Jaattela 2004). The extracellular death ligands promote clustering of their cognate receptors and initiate apoptosis effector phase more directly than events, coordinated by mitochondria and Bcl-2 family proteins.

Translocation of apoptogenic factors from mitochondria to the cytoplasm triggers cell execution phase by promoting caspase activation or by acting as caspase-independent death effectors (Saelens et al. 2004). Mammalian caspases can be divided into three functional groups: the apoptotic initiators, executioners and the cytokine activators (McIlwain et al. 2013). Apoptotic caspases are constitutively expressed as latent zymogens, but become proteolytically activated after apoptotic stimulus. Initiator caspase activation occurs in molecular platforms that enable sufficient proximity for homodimerization and auto-activation. Executioner caspases become activated after proteolytic cleavage by initiator caspases and other executioner caspases, signifying the distinct feature of caspases - to act in self-amplifying cascades. Sequential activities of initiator and executioner caspases lead to coordinated cleavage of structural and functional components in cell, resulting in characteristic morphological indicators of apoptosis, such as nuclear condensation, cell shrinkage, and membrane blebbing (Liu et al. 1997; Enari et al. 1998; Coleman et al. 2001). The subtle destruction of dying cell into apoptotic bodies facilitates its subsequent phaocytosis by macrophages or parenchymal cells (Elmore 2007).

Focused proteomics methods have begun to reveal the natural substrates of caspases in the thousands (Poreba et al. 2013). For example, multiple kinases and transcription factors, a range of translation initiation factors and ribosomal proteins are cleaved by caspases (Luthi and Martin 2007). Caspase-3 cleavage releases also CAD (caspase-activated DNAse) from its inhibitory subunit allowing its translocation to the nucleus to initiate DNA degradation (Liu et al. 1997; Enari et al. 1998). Caspase-dependent loss of ROCK I kinase auto-inhibition has been implicated in the production of plasma membrane blebs during apoptosis (Luthi and Martin 2007). Caspase-3 cleaves nuclear lamin B and alpha-fodrin (Janicke et al. 1998), caspase-6 cleaves lamins A and C (Lazebnik et al. 1995; Slee et al. 2001) and both, caspase-3 and -7 cleave Poly (ADP-ribose) polymerase (PARP) (Distelhorst and Shore 2004), an enzyme that can otherwise deplete the ATP of a cell in an attempt to repair the damaged DNA. Loss of caspase-3 abolishes or dramatically delays the kinetics of the majority of substrate proteolysis seen during apoptosis, therefore it is considered as the major effector caspase (Janicke et al. 1998; Slee et al. 2001; Luthi and Martin 2007). The expanding list of caspase substrates has been collected to an online database, containing all of the reported mammalian caspase substrates (Luthi and Martin 2007).

The clearance phase is orchestrated by interactions of phosphatidylserine (PS) and calreticulin, exposed at dying cell plasma membrane, to multiple engulfment receptors, found in phagocytizing cells (Savill and Fadok 2000; Gardai et al. 2005). The engulfment of apoptotic corpses is accompanied with suppression of the pro-inflammatory signalling in macrophages or immune reaction in T-cells (Savill and Fadok 2000) which ensure the non-inflammatory nature of apoptotic cell death (Savill et al. 2002; Kurosaka et al. 2003).

2.2.4 Cell commitment to apoptosis

Cell commitment to die is reflected by irreversible activation of cell execution machinery (Harvey et al. 1998). In principle, the mode of cell death is determined by the cell´s physiologic milieu, its metabolic state as well as the intensity and nature of the cell death signals (Zeiss 2003). For example, autophagy can support apoptosis by fuelling it with ATP and facilitate the membrane blebbing (Qu et al. 2007), but energy depletion may result in biochemical collapse and cell commitment to necrotic death (Leist et al. 1997; Nicotera et al. 1998; Leist et al. 1999; Denecker et al. 2001; Jin and White 2007). Therefore, the selected route defines the death commitment point in every cell.

In mammalian cells apoptosis manifests in two major molecular execution programs downstream of the initiation phase, but the precise death commitment point has remained controversial (Kroemer et al. 2009). In most cell types the mitochondrial outer membrane permeabilization (MOMP) is considered as irreversible commitment point in apoptosis (Adams and Cory 1998; Martinou et al. 1999; Deshmukh et al. 2000; Keeble and Gilmore 2007). However, it may be closely linked to caspase activation (Harvey et al. 1998; Chang et al. 2002). For example, neurons and cardiomyocytes can recover from mitochondrial permeabilisation as long as caspase activation is prevented downstream (Deshmukh et al. 2000; Potts et al. 2005). In addition, in transient stress situation, the apoptotic program can be reversed, even after caspase activation (Tang et al. 2012). The controversy arises because cells contain numerous anti-apoptotic mechanisms which operate in parallel to apoptotic signalling (Griffiths et al. 1999; Bortner and Cidlowski 2002). All this ensures that the cell dies only when irrevocable damage has been reached (Sommer et al. 2001). Therefore, certain signalling thresholds appear to shape the decisions in apoptosis commitment (Sheridan and Martin 2008).

The MOMP is controlled by pro- and anti-apoptotic members of the Bcl-2 family (Kuwana et al. 2002; Chipuk et al. 2010). Also caspase activation is subject to regulation by the inhibitor of apoptosis (IAP) family proteins (Xu et al. 1999; Deveraux et al. 2001; Shi 2002). IAPs might mediate ubiquitination and degradation of mature initiator and effector caspases (Hu and Yang 2003; Morizane et al. 2005) thus restricting their availability for cell demise. The latter may have relevance in resisting transient stress (Tang et al. 2012), but could also drive the oncogenic transformation, as cells, although recovered from apoptosis, might have acquired permanent genetic changes (Sheridan and Martin 2008; Tang et al. 2012). In many instances, tumour cells counteract death

signals, by increasing the levels of survival-promoting proteins (Tamm et al. 2000; Carter et al. 2003; Juin et al. 2013).

Pro- and antiapoptotic mechanisms may operate in cell- or tissue-type or developmental stage specific manner (Bortner and Cidlowski 2002). Developing neurons have remarkably active apoptotic program (Kole et al. 2013). In mature neurons, however, the classical apoptotic pathway is uniquely refined including several inhibitory mechanisms, which operate at the posttranscriptional level (Wright and Deshmukh 2006; Kole et al. 2011b; Kole et al. 2013).

The connection of apoptotic pathways with other cell death related networks has become more and more evident (Su et al. 2013). It has been realized that alternative cell death pathways become apparent under the conditions where apoptotic mechanisms are compromised (reviewed in Nixon and Yang 2012; Jain et al. 2013). Several programmed cell-death modes may have evolved to back-up apoptosis and guarantee flexible response to environmental stresses, but also for safe and non-inflammatory removal of dying cell (Leist and Jaattela 2001).

Collectively, the precise death commitment point may vary between different cell types and conditions (Kroemer et al. 2009). Hence, more flexible concept states that cell commitment to undergo apoptosis cannot be determined by a single factor or event but relies on quenching multiple anti-apoptotic mechanisms in a particular cell type along with the activation of pro-apoptotic mechanisms that ultimately lead to caspase activation above irreversible threshold (Bortner and Cidlowski 2002; Sheridan and Martin 2008). Once a cell has committed to apoptosis, the process is remarkably efficient, and may be completed within one hour of initiation (Goldstein et al. 2000; Keeble and Gilmore 2007).

2.2.5 Molecular pathways of apoptosis in mammalian cells

In mammals, two major signalling pathways have been described that culminate in apoptotic mode of cell death (Figure 3).

The most direct apoptotic pathway, the extrinsic pathway, is initiated by extrinsic death ligands, involves the engagement of so-called cell-surface death receptors and proceeds to the activation of initiator caspases at distinct death-inducing signalling complexes (Rathmell and Thompson 1999). The mitochondria-initiated apoptotic pathway, termed intrinsic pathway, is tightly regulated by Bcl-2 family proteins, centres on the mitochondrial membrane permeabilization to control cytosolic death signalling complex formation and caspase cascade activation for apoptosis execution. Both apoptotic pathways operate rather independently (Strasser and Anderson 1995), but converge at the level of effector caspases. In some cell types, these pathways are interconnected to each other at several molecular nodes. Both apoptotic pathways can stimulate the caspase independent cell destruction machinery.

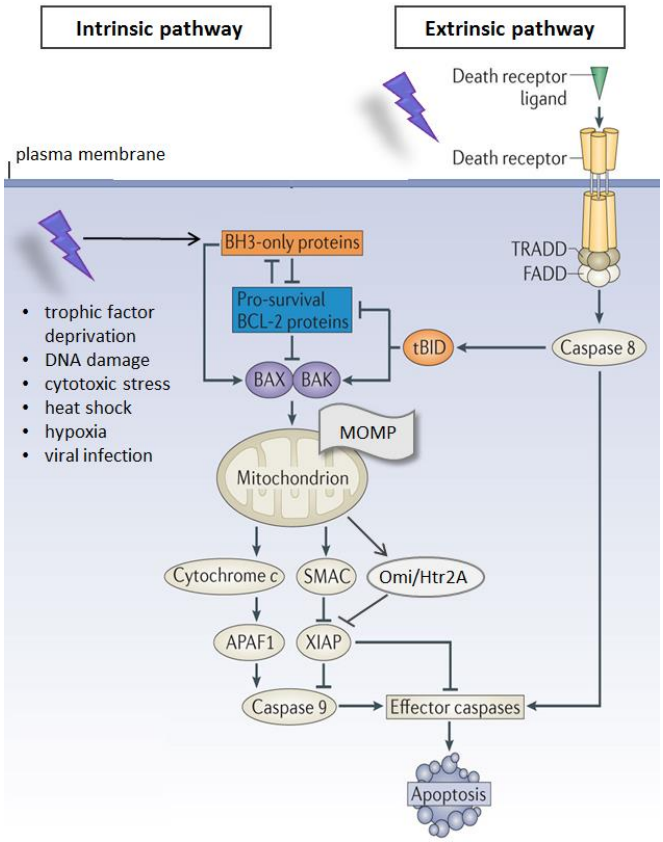

Figure 3. Intrinsic and extrinsic apoptosis pathways in mammalian cells. Apoptosis can result from the activation of two biochemical cascades, termed as the intrinsic (mitochondrial) and the extrinsic apoptosis pathway. The intrinsic pathway is triggered by intracellular stimuli, such as, trophic factor deprivation, DNA damage or cytotoxic stress that all can induce or activate BH3-only proteins. The BH3-only protein mediated death signal is either neutralized by anti-apoptotic Bcl-2 family proteins or delivered to the pro-apoptotic effector proteins Bax and Bak, which on activation trigger mitochondrial outer membrane permeabilization (MOMP). Permeabilization of MOM results in release of Smac/DIABLO (SMAC), Omi /HtrA2 and cytochrome c to the cytosol. Smac and Omi /HtrA2 neutralize the inhibitor of apoptosis family proteins (IAPs), including the X-linked IAP (XIAP), whereas cytosolic cytochrome c forms a platform with APAF-1 for the initiator caspase-9 activation. Activation of caspase-9 triggers the cleavage and activation of downstream effector caspases that then degrade cellular proteins, amplify caspase cascade, but also activate chromatin degradation via caspase-activated DNase. This leads to the coordinated fragmentation of cells into apoptotic bodies and engulfment by neighboring cells.

The extrinsic pathway is initiated by the ligation of death receptors with their cognate ligands at the plasma membrane. It leads to the recruitment of adaptor molecules such as FAS-associated death domain protein (FADD) and TNFR1 associated death domain protein TRADD, followed the dimerization and activation of caspase-8. Activated caspase-8 cleaves the executioner caspases, thereafter a caspase cascade lead to characteristic hallmarks of apoptosis. Caspase-8 may also cleave the pro-apoptotic protein Bid, which links the extrinsic and intrinsic pathway in certain cell types. Adapted from Czabotar et al., (2014).

2.2.5.1 The extrinsic pathway

Extrinsically induced apoptosis signalling pathway (Figure 3) is mediated by the cell surface death receptors, which belong to a subgroup of TNF receptor superfamily. Death receptors are transmembrane proteins, their cysteine-rich extracellular domain is required for ligand binding and an intracellular death domain (DD) is essential for recruiting specific adaptors that define their downstream interactors and signals (Itoh and Nagata 1993; Tartaglia et al. 1993). Death receptor ligands belong to TNF superfamily and are transmembrane proteins, thus they stimulate their cognate receptors through cell-cell contact.

In particular, the death receptors that mediate death signals are TNF-R1, Fas (Apo/CD95) and TRAIL-R1 and –R2 (for TNF-related apoptosis-inducing ligand receptor). Their respective ligands are the founding member tumour necrosis factor (TNF), FasL (CD95L) and TRAIL (Walczak 2013).

Prototypic activation of extrinsic apoptosis pathway can be described in Fas receptor system. The FasL binding to Fas receptor stabilizes the receptor trimers at the plasma membrane and induce conformational change, which allows the assembly of a dynamic multiprotein complex at the cytosolic tail of the receptor. Central to this is the adapter molecule FADD (Fas-associated protein with death domain) that has the ability to form oligomers when bound to receptor (Boldin et al. 1995; Chinnaiyan et al. 1995; Vincenz and Dixit 1997; Tourneur and Chiocchia 2010). The resulting supramolecular complex, known as "death-inducing signaling complex" (DISC), constitutes a platform that regulates the activation of initiator pro-caspases, such as caspase-8 or- 10 (Kischkel et al. 1995; Muzio et al. 1996). Activated caspase-8 cleaves and activates directly downstream effector caspase-3 which in turn cleaves other caspases (caspase-6 and -7) thereafter a cascade of proteolytic events lead to characteristic hallmarks of apoptosis.

TRAIL-R1 and-R2 activate pro-caspase-8 (Walczak et al. 1997; Kischkel et al. 2000; Kischkel et al. 2001) similarly to Fas, but require also a TNFR1 associated death domain protein (TRADD) to activate caspases (Micheau and Tschopp 2003).

Fas, TRAIL-R1 and -R2 primary signalling output is apoptosis induction, their secondary function is gene activation via NF-κB and MAPK pathways (Walczak 2013). TNF-R1 death receptor signalling leads to transcriptional upregulation of cyto- and chemokines for inflammatory response, is most prominent (Walczak 2013). The induction of cell death by TNF is a secondary function, triggered only when cell's response to induce inflammatory cascade remains weak (Walczak 2013). TNF-R1 mediates also necroptosis, depending on the presence of functionally active caspase-8/FADD complex (Vandenabeele et al. 2010a). The modulatory protein cFLIP (cellular FLICE like inhibitory protein) regulates the decision between TNF-R mediated apoptosis and necroptosis (Kavuri et al. 2011; van Raam and Salvesen 2012).

In various lymphoid cells (type I cells) the activation of caspase-8 is sufficient to activate the effector caspases (Budihardjo et al. 1999; Scaffidi et al. 1999). In other type of cells (type II cells), where caspase-8 activation is very slow or blocked by X-linked inhibitor of apoptosis protein (XIAP). Therefore additional apoptotic signal amplification steps are used to interconnect the death receptor and mitochondrial apoptosis pathways

(Luo et al. 1998; Budihardjo et al. 1999). Central to this is Bcl-2 family protein Bid (BH3 interacting-domain death agonist) which is N-terminally cleaved by caspase-8. Truncated Bid (tBid) translocates from the cytosol to the mitochondria (Li et al. 1998; Luo et al. 1998; Gross et al. 1999b) where it induces MOMP through interaction with pro-apoptotic Bcl-2 family members Bax and/or Bak.

Death receptor pathways are crucial for proper homeostasis and function of the immune system, although expressed in other tissues, their physiologic function have remained poorly studied (Krammer 2000; Wehrli et al. 2000).

2.2.5.2 The intrinsic pathway

The intrinsic or mitochondrial apoptotic pathway (Figure 3) can be initiated by wide array of intracellular stress conditions such as growth factor deprivation, endoplasmic reticulum stress, accumulation of DNA damage or other insults, that disrupt or alter cellular processes at various locations throughout the cell (Galluzzi et al. 2009; Tait and Green 2010).

Majority of the cell death signals are integrated to common intrinsic apoptotic pathway through induction and recruitment of one or more members of the BH3-only subset of the Bcl-2 protein family (Huang and Strasser 2000; Scorrano and Korsmeyer 2003). Once induced, they hinder the prosurvival function of anti-apoptotic Bcl-2 family proteins and enable direct and indirect activation of key Bcl-2 family effector proteins Bak and Bax.

Activation of Bak and Bax proteins involves their oligomerization at mitochondrial membranes, which triggers MOMP (Galluzzi et al. 2009). MOMP is a critical event preceding the apoptotic cell death in most of the mammalian cell types (Jurgensmeier et al. 1998; Gross et al. 1999a; Du et al. 2000; Adams and Cory 2002; Green and Evan 2002; Danial and Korsmeyer 2004; Chipuk and Green 2005). Upon MOMP a set of proteins, normally found in mitochondrial intermembrane space, are released to the cytosol (Saelens et al. 2004) where they contribute to downstream execution pathway activation (Liu et al. 1996). Several of these factors, such as cytochrome c, Smac/DIABLO (second mitochondria-derived activator of transcription/direct IAP-binding protein with low pI) (Du et al. 2000; Verhagen and Vaux 2002) and high temperature requiring enzyme A2 (Omi/HtrA2) (Suzuki et al. 2001), apoptosis initiating factor (AIF) (Susin et al. 1999) and endonuclease G (EndoG) (Li et al. 2001) have acquired functionally double identities during evolution**.**

Cytochrome c is a component of the mitochondrial electron transport chain that is required for the generation of ATP in healthy cells. Once released to the cytosol it interacts with an apoptotic protease-activating factor-1 (Apaf-1) (Zou et al. 1997; Green and Reed 1998) and dATP, and promotes the assembly of multiprotein complex called the apoptosome (Green and Reed 1998; Zou et al. 1999). Apoptosome is a cytosolic death signalling complex that recruits the initiator procaspase-9 and support it´s autoactivation by induced-proximity mode (Pop, 2006). The activated caspase-9 cleaves and activates the executioner procaspases-3, -6 and-7 (Li et al. 1997). Active executioner caspases proceed to cleave multiple downstream targets, including many vital cellular proteins

and cytoskeletal elements, which result in ordered destruction of the cell (Wright and Deshmukh 2006; Luthi and Martin 2007).

In parallel, Smac/DIABLO, and serine protease HtrA2/Omi ensure the full activation of mitochondrial apoptosis pathway (Danial and Korsmeyer 2004). Both proteins are able to neutralize the ubiquitously expressed IAP family proteins by antagonizing their inhibitory effect on active caspase-9 and -3 (Chai et al. 2000; Shi 2002; van Loo et al. 2002), thus allowing apoptosis to proceed.

In contrast to other mitochondrial proapoptotic factors, the released flavoprotein AIF and mitochondrial endonuclease Endo G can translocate to the nucleus and induce caspase-independent chromatin condensation and DNA fragmentation (Lorenzo et al. 1999; Susin et al. 1999; Li et al. 2001).

The functional *in vivo* significance of many apoptotic genes (e.g. some *bcl-2* family genes, *Smac/Diablo, Htr2/Omi* and *EndoG*) is masked by genetic redundancy (Lindsten et al. 2000; Okada et al. 2002; Jones et al. 2003; Irvine et al. 2005).

2.3 Bcl-2 family proteins

2.3.1 Classification

The founding member Bcl-2 (B-cell leukemia/lymphoma 2) and its relatives comprise the Bcl-2 family of proteins that are part of the core apoptotic machinery (Chipuk et al. 2010). Classical Bcl-2 family members share the amino acid sequence homology in Bcl-2 homology (BH) domains or motifs (Yin et al. 1994; Huang et al. 1998; Reed 1998). Several Bcl-2 family proteins contain more than one BH motif and are typically termed as "multi-motif" members. Others contain only the BH3 motif and are designated as "BH3-only" proteins (Figure 4).

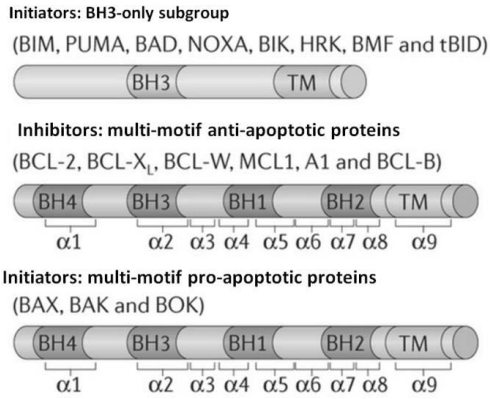

Figure 4. Scematics of Bcl-2 family protein structure and functional subgroups. Bcl-2 protein family consists of three main sub-groups. The group of anti-apoptotic proteins such as Bcl-2, Bcl-xL, Bcl-w, Mcl-1, and the other, consisting of pro-apoptotic effector proteins including Bax, Bak contain up to four conserved α-helical segments, called BH (Bcl-2 homology) domains or motifs (BH1–4) and the carboxy-terminal hydrophobic domain, termed as transmembrane domain (TM) or membrane binding region. The group of pro-apoptotic initiators, such as Bim, Puma, Bad, Noxa, Hrk, Bik, Bmf and tBid share only the third BH motif with other family members and are designated as BH3-only proteins. *Only some BH3-only proteins contain TM domain at the C-terminal end. Adapted from Czabotar et al. (2014).

Bcl-2 family members are also divided into anti-and pro-apoptotic proteins based on their functions in apoptotic process (Adams and Cory 2001).

The collection of mammalian Bcl-2 family proteins consists of phylogenetically related multi-motif anti-apoptotic members (Bcl-2, Bcl-xL, Bcl-w and Mcl-1, Bfl1/A1, and in humans Bcl-B). These proteins are able to inhibit MOMP by counteracting the functions of pro-apoptotic members (Cheng et al. 2001; Billen et al. 2008). The pro-apoptotic subset includes proteins that operate in a functionally distinct manner and can be further divided into two subgroups: the pro-apoptotic effectors (Bak, Bax and probably Bok), which on activation mediate MOMP. Structure-based alignment of Bcl-2 family proteins has revealed that multi-motif proteins share homology in three to four BH domains (BH1-4) and also in three-dimensional structure (Boise et al. 1993; Kozopas et al. 1993; Gibson et al. 1996; Willis and Adams 2005; Kvansakul et al. 2008; Czabotar et al. 2014). Additional group of pro-apoptotic Bcl-2 family members comprises classical BH3-only proteins (Bid, Bim, Puma, Bad, Bik, Bmf, Hrk, Noxa) that can activate the pro-apoptotic effectors, either directly or indirectly (Wei et al. 2001; Kuwana and Newmeyer 2003; Youle and Strasser 2008; Moldoveanu et al. 2014). The BH3-only proteins are activated by diverse intracellular stress stimuli, hence functioning as apical sentinels and initiators of the canonical apoptotic program in animal cells (Fernandez-Luna 2008), (BCL-2: database Blaineau and Aouacheria 2009; Rech de Laval et al. 2014). Classical BH3-only proteins share the sequence homology with other family members and between each other only within a signature BH3 motif (Figure 4) (Hinds et al. 2007; Day et al. 2008; Hardwick et al. 2012; Aouacheria et al. 2013; Hardwick and Soane 2013; Rech de Laval et al. 2014). The BH3-only protein Bid is exceptional, as it resembles the multi-motif Bcl-2 family members in its three-dimensional structure. It has been proposed that Bid belongs to independent subfamily with novel, less-characterized family members, such as Bpr, Mil1/Bcl-Rambo, Bcl-G, Bfk, which all share similar globular fold as Bcl-2, despite of having nonconventional BH domain organization or atypical BH domains (BCL-2: database Blaineau and Aouacheria 2009; Rech de Laval et al. 2014). In addition, some virus-encoded structural or functional Bcl-2 homologs could interfere the functions of cellular family members in animal cells (Kvansakul and Hinds 2013). Whether structural homology of these proteins allows also similar regulation and function in cells is not well-understood and remains to be studied (Blaineau and Aouacheria 2009; Aouacheria et al. 2013; Hardwick and Soane 2013; Rech de Laval et al. 2014). The functional and structural information about the apoptosis related Bcl-2 proteins has been combined and organized into the Bcl-2 family database (Blaineau and Aouacheria 2009; Rech de Laval et al. 2014)

It has been claimed that traditional classification of Bcl-2 family proteins is biased towards their established functional properties in apoptosis (Aouacheria et al. 2013). Namely, some virus-encoded Bcl-2 structural mimics and Bcl-2 sequence homologs encoded by Drosophila genome (Debcl and Buffy), have very limited or not recognizable roles in apoptosis (Lamb and Hardwick 2010; Kvansakul and Hinds 2013). There is also a growing list of BH3-only proteins, that do not exhibit clear functions in apoptosis regulation (Hardwick and Soane 2013) or potential members, whose role in apoptosis has remained experimentally unproven (Beverly 2012). These arguments have led to

initiative to re-evaluate the Bcl-2 protein family nomenclature, specifically regarding the BH3-only family members (Aouacheria et al. 2013; Hardwick and Soane 2013; Rech de Laval et al. 2014). Apparently, a subgroup for non-classical BH3-only proteins, exhibiting diverse functions in cell, is emerging, It includes proteins such as $Bnip_{1-3}$, $Bnip_3L$/Nix, Beclin1, Spike, Rad-9, Apolipoprotein L1, MULE/ARF-BP1 and many others (Blaineau and Aouacheria 2009; Zhang and Ney 2009; Rech de Laval et al. 2014). Some of these proteins have death-inducing activity and/or ability to interact with multidomain Bcl-2 family proteins (Boyd et al. 1994; Oberstein et al. 2007). However, the integration of these proteins in the apoptosis signalling network is not well-established.

Most transcripts of the *bcl-2* family genes are alternatively spliced, producing multiple mRNA variants that could encode different protein isoforms. Alternative splicing may result in functionally different or even antagonistic isoforms, as has been shown for *bcl2l1* (Boise et al. 1993), encoding both, a multidomain antiapoptotic protein Bcl-xL, but also a BH3-only protein Bcl-X_s with proapoptotic properties. Also *bak, bax, mcl-1* and *bcl-g* could encode the alternative BH-3 only isoforms, N-Bak, Bax-s, Mcl-1_s Bcl-G_s respectively (Boise et al. 1993; Bae et al. 2000; Bingle et al. 2000; Guo et al. 2001; Sun et al. 2001), although the existence or functionality of particular BH3-only proteins has been questioned in several cases. Nevertheless, regulation of the alternatively spliced mRNA transcripts, including their expression patterns and capacity to express functional protein are poorly characterized and waits for careful functional analysis.

2.3.2 General features of Bcl-2 family proteins

Simultaneously expressed pro-and anti-apoptotic Bcl-2 family proteins operate in the spatially and functionally dynamic interaction network in the cell. Prevailing homo- and heterotypic interactions between the family members allow positive or negative allosteric regulation of their activities (Antonsson et al. 2000; Grinberg et al. 2002; Youle and Strasser 2008; Rautureau et al. 2010a; Moldoveanu et al. 2014). Interactions with other cellular proteins are less characterized, but are likely involved in Bcl-2 family protein functions in normal physiology (Chipuk and Green 2004; Leu et al. 2004; Chipuk et al. 2010; Hardwick and Soane 2013; Moldoveanu et al. 2014).

Bcl-2 family proteins associate with intracellular membranes (Gross 2001; Garcia-Saez 2012), most notably with mitochondrial outer membrane (MOM), but also to smooth endoplasmic reticulum (ER), nuclear envelope and Golgi (Krajewski et al. 1993; Akao et al. 1994; Lithgow et al. 1994; Hsu et al. 1997b; Nutt et al. 2002; Scorrano et al. 2003; Echeverry et al. 2013). Several multi-domain members, such as Bak, Bok and Bcl-2 are constitutively tethered to the membranes, whereas others are partitioned between the cytosol and intracellular membrane surfaces and become integrated to target membranes after apoptotic stimulus (Schinzel et al. 2004; Shamas-Din et al. 2013b). Intracellular membranes have central importance in orienting the interactions between Bcl-2 family members (Garcia-Saez 2012; Czabotar et al. 2013).

Perhaps one of the most fascinating features of Bcl-2 family proteins is their remarkable structural mobility and plasticity (Rautureau et al. 2010a). Bcl-2 family

proteins could expose their membrane binding region and other interaction surfaces after certain conformational changes (Goping et al. 1998; Suzuki et al. 2000; Dewson et al. 2012; Czabotar et al. 2013). Pro-apoptotic effectors, Bax and Bak undergo significant conformational transitions during their activation process and it is particularly important for their pro-apoptotic function (Antonsson et al. 2000; Wei et al. 2001; Grinberg et al. 2002; Lindenboim et al. 2010; Wang et al. 2011).

The expression of individual Bcl-2 family members is controlled by heterogeneous mechanisms, involving the positive or negative regulation at transcriptional and post-translational level. Some posttranscriptional regulatory mechanisms, such as alternative splicing and mRNA translational suppression have been also described. Several of these regulatory mechanisms could operate in a context-specific manner, presumably to guarantee a proper response to various cell death and survival signals.

Considerable evidence suggests that also classical Bcl-2 family proteins regulate several other aspects of cell physiology. For instance, mammalian Bcl-2 family proteins are engaged into a network of interactions that control mitochondrial dynamics (Karbowski et al. 2006; Berman et al. 2009; Cleland et al. 2011), calcium homeostasis (Scorrano et al. 2003; Oakes et al. 2005) and autophagy (Maiuri et al. 2007a; Maiuri et al. 2007b; Oberstein et al. 2007). These functions are thought to represent their apoptosis-independent roles in healthy cell (Desagher and Martinou 2000; Galluzzi et al. 2008; Galluzzi et al. 2012a; Hardwick and Soane 2013). An open question is to what extent the non-canonical functions of Bcl-2 proteins influence the cell fate and what biochemical aspects of these functions overlap with apoptosis regulation.

2.3.3 Characteristic structural features of Bcl-2 family proteins

Most of the Bcl-2 family proteins harbour a membrane-binding region on their carboxyl-termini, which are required for their targeting into intra-cellular membranes. The activity of multidomain Bcl-2 family members in membranes is also facilitated by a hydrophobic infrastructure (Suzuki et al. 2000).

The characteristic BH domains correspond to one or two α-helical segments in Bcl-2 family protein structure (Gross et al. 1999b; Hinds and Day 2005) The BH3 domain is particularly important in mediating the pro-apoptotic activity of Bcl-2 protein family members (Boyd et al. 1995; Chittenden et al. 1995; Muchmore et al. 1996; Inohara et al. 1997; Kelekar et al. 1997; Sattler et al. 1997; Huang and Strasser 2000; Dewson et al. 2008; Czabotar et al. 2013). It is a relatively short (~13 residues), conserved sequence motif (Day et al. 2008) that folds into the amphipathic α-helix, capable of interacting to the distinct hydrophobic surfaces (Gavathiotis et al. 2008; Gavathiotis et al. 2010; Rautureau et al. 2010b). Mutations within the BH3 domain abolish the pro-apoptotic protein activity (Chittenden et al. 1995; Huang and Strasser 2000). Markedly, most of the classical BH3-only proteins are intrinsically disordered (Hinds et al. 2007; Day et al. 2008), but undergo a localized conformational change at their BH3 domain upon binding to their cognate partner protein (Petros et al. 2000; Hinds et al. 2007; Rautureau et al. 2010a). Such transition from disordered structure to the ordered α-helix allows BH3

domain to adopt the optimal helical structure, so improving the binding affinity (Hinds et al. 2007; Rautureau et al. 2010a).

The crystal and solution structure analyses have revealed that multi-domain Bcl-2 family members as well as BH3-only protein Bid, exhibit a similar α-helical bundle fold (Muchmore et al. 1996; Aritomi et al. 1997; Chou et al. 1999; McDonnell et al. 1999; Suzuki et al. 2000; Petros et al. 2001; Petros et al. 2004). Their canonical globular structure has central hydrophobic helix (α5), surrounded by 7 amphipathic α-helices (α1-α4 and α6- α8) (Petros et al. 2000; Hinds and Day 2005) (Figure5 a, c). Some members, such as Bax, Bak and Bcl-w, bear also carboxyl-terminal α9 helix that is involved in the regulation of activity and subcellular localization of these proteins (Suzuki et al. 2000; Denisov et al. 2003; Hinds et al. 2003). Several viral proteins, with anti-apoptotic properties, lack obvious BH motifs in their primary structures, but their three-dimensional helical structures are similar to cellular multidomain members (Kvansakul and Hinds 2013).

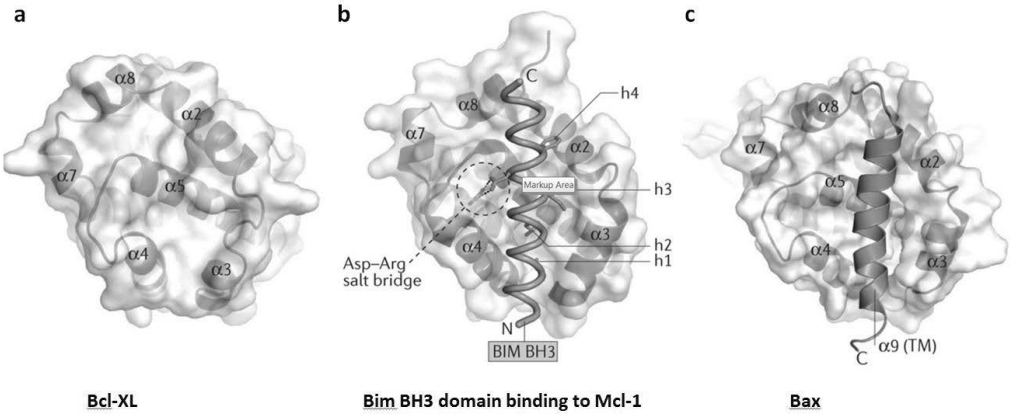

Figure 5. **Similar structural features of multidomain Bcl-2 family proteins.** Multidomain Bcl-2 family proteins have similar structural folds, displaying a characteristic hydrophobic cleft at the protein surface that serves as the binding site for the BH3 peptides or similar α-helical fold of the C-terminal part. (A) Cartoon representating a Bcl-xL surface structure. (B) Representation of anti-apoptotic family member Mcl-1 bound to Bim BH3 peptide. Shown are the four hydrophobic residues (h1–h4) of the BIM-BH3 domain that interact with the hydrophobic groove and the invariant aspartic acid residue on the BH3 domain that makes contact with the arginine residue on the Mcl-1 to form the Asp–Arg salt bridge. Proapoptotic Bcl-2 family proteins Bax and Bak bind BH3-only proteins in a similar manner. (C) Characteristic helical fold of soluble, inactive Bax. In this structure, the putative transmembrane anchor α9 (in grey) binds in the hydrophobic cleft of Bax. Modified from Czabotar et al (2014).

The canonical Bcl-2 fold is also remarkably similar to the pore-forming domains of bacterial colicin and diphtheria toxin (Muchmore et al. 1996). Indeed, Bcl-2, Bcl-xL, Bax and Bid have have capability to model artificial membranes. Other studies suggest that only effector proteins (e.g. Bax) exhibit a channel-forming activity in cells (Soriano and Scorrano 2010; Czabotar et al. 2013).

All multi-domain Bcl-2 family proteins exhibit shallow hydrophobic groove or cleft at their surface (Muchmore et al. 1996; Sattler et al. 1997; Czabotar et al. 2013). This cleft in the anti-apoptotic proteins is the canonical binding site for the BH3-only proteins and activated forms of Bak or Bax (Muchmore et al. 1996; Sattler et al. 1997; Antonsson et al. 2000; Petros et al. 2000; Grinberg et al. 2002; Rautureau et al. 2010b; Czabotar et al. 2011; Czabotar et al. 2013; Moldoveanu et al. 2013). The BH3-in-groove interactions between family members occur also during Bax or Bak activation and their self-dimerization (Letai et al. 2002; Dai et al. 2011; Dewson et al. 2012; Czabotar et al. 2013). Hence, formation of BH3-in-groove interface is a key feature that mediates interactions between Bcl-2 family proteins (Huang et al. 1998). Structural and biochemical studies have revealed that subtle variations in residues along the hydrophobic groove and BH3 domain dictate the binding specificities between different family members (Chen et al. 2005). Therefore, the BH3 domain coupling to hydrophobic groove is highly selective (Chen et al. 2005).

In addition to homologous hydrophobic groove, some other hydrophobic surfaces have been characterised in Bax molecule (Gavathiotis et al. 2010; Ma et al. 2012). The roles of these surfaces have been implicated in regulation of Bax activity, but their relevance in this process has remained controversial (Gavathiotis et al. 2008; Gavathiotis et al. 2010; Ma et al. 2012; Czabotar et al. 2013; Lamb and Hardwick 2013; Czabotar et al. 2014).

The realization that the BH3 binding pocket can be also pharmacologically targeted by peptides and small molecules that mimic the action of a BH3 domain (Kang and Reynolds 2009; Llambi et al. 2011) has led to development of whole class of new molecules (BH3 mimetics) with the aim of therapeutically intervene the functions of multi-domain Bcl-2 family members (Bajwa et al. 2012). Several BH3 mimetics that target main anti-apoptotic proteins have reached into clinical development (Bai and Wang 2014). The alternative structural states, resolved recently for proapoptotic effector Bax (Czabotar et al. 2013), have opened the avenue to develop small molecules that either activate or supress Bax for therapeutic purposes (Lamb and Hardwick 2013).

Precise features that determine the pro-or anti-apoptotic properties of cellular Bcl-2 homologs have remained obscure. Czabotar and colleagues demonstrated (2013) that binding of BH3 molecule to hydrophobic groove of pro-apoptotic Bax protein destabilizes the Bax molecule hydrophobic core and facilitates its insertion and homo-oligomerization in phospholipid membrane, whereas BH3 peptide binding to anti-apoptotic protein forms a stable complex which leads to mutual inactivation of interaction partners (Czabotar et al. 2013; Czabotar et al. 2014). Such intramolecular structural differences may account, in part, to the opposed functions in otherwise structurally similar Bcl-2 homologs in apoptosis regulation.

The BH3 domain is essential for proapoptotic function, hence its availability for interactions is strictly regulated (Chittenden et al. 1995; Antonsson 2001; Kvansakul and Hinds 2013). Also the hydrophobic binding surfaces may not be constitutively exposed for interactions, but can be masked by other structural elements, such as C-terminal α-helix (α9) (Suzuki et al. 2000; Petros et al. 2004).

Posttranslational modifications can trigger either activation or silencing of protein activity in a context-specific manner (Yang and Seto 2008). For example, phosphorylation of Bak hydrophobic cleft regulates its conformational change and multimerization (Azad et al. 2012). Intrinsically disordered regions that connect the helices in multi-domain family members (Hinds, Day 2005) are involved in post-translational regulation through phosphorylation, proteolytic cleavage or some other type of modification (Hinds and Day 2005; Rautureau et al. 2010a).

2.3.4 Bcl-2 family proteins in apoptosis regulation

2.3.4.1 The BH3 only proteins

Classical BH3-only proteins are responsible for triggering apoptosis in response to various apoptotic signals that arise from developmental cues and diverse range of stress stimuli (Adams and Cory 2002). Their expression and activity is controlled by heterogeneous mechanisms.

Several BH3-only proteins, including Bik, Bim, Hrk, Noxa and Puma are targeted to ER and/or mitochondrial membranes through their C-terminal membrane binding regions (Nakano and Vousden 2001; Seo et al. 2003; Yu et al. 2003a; Shamas-Din et al. 2013a) or distinct posttranslational modifications, as it has been described for Bad, Bid (Li et al. 1998; Luo et al. 1998; Polzien et al. 2009).

BH3-only proteins regulate the activities of the pro- and anti-apoptotic multidomain family members. Depending on their action mode, typical BH3-only members are subdivided into "direct activators" and "de-repressors/sensitizers" (Letai et al. 2002; Kuwana et al. 2005). Direct activators, such as Bim, tBid and Puma have a board interaction range within the Bcl-2 family. They are able to activate the pro-apoptotic effector proteins Bax and Bak (Wei et al. 2000; Elkholi et al. 2011; Dai et al. 2014). BH3-only activators can also neutralize the anti-apoptotic members (Figure 6) (Kuwana et al. 2002; Letai et al. 2002; Kuwana et al. 2005; Certo et al. 2006; Kim et al. 2006; Deng et al. 2007; Gavathiotis et al. 2008; Letai 2009; Dai et al. 2011; Llambi et al. 2011). The rest of the BH3-only proteins are known as de-repressors/sensitizers. De-repressing activity includes the displacement of the activator BH3-only proteins and/or Bax/Bak from the inhibitory interactions with anti-apoptotic members, but they lack the ability to directly activate Bak or Bax. Sensitizer activity involves the occupation of the binding sites in anti-apoptotic proteins, which, in result sensitizes the cells to apoptosis (Cheng et al. 1996; Letai et al. 2002; Kuwana et al. 2005; Certo et al. 2006). De-repressors could bind and inactivate only distinct set of anti-apoptotic members (Figure 6). For example, Bad interacts with Bcl-2, Bcl-xL and Bcl-w, whereas Noxa binds avidly to Mcl-1 and A1. Recent studies have identified also sequence determinants that distinguish activator and sensitizer BH3-domains (Czabotar et al. 2013).

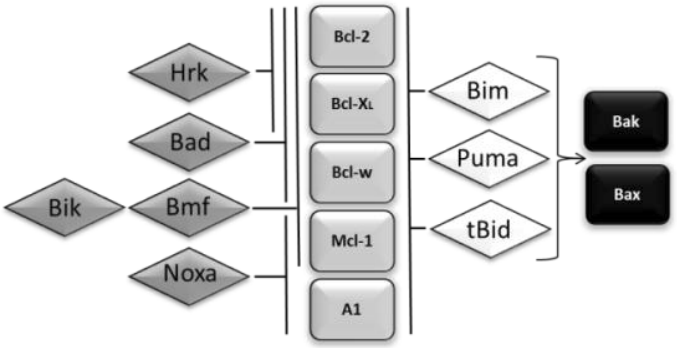

Figure 6. Different binding specificity and apoptotic potency of BH3-only proteins to the multidomain family members. The sensitizer- BH3-only proteins (Bik, Hrk, Bad, Bmf and Noxa) bind to subsets of anti-apoptotic memebers. The activators (Bim, Puma and tBid) bind to all anti-apoototic members, as well as pro-apoptotic effectors Bak and Bax. Modified from Renault and Chipuk (2013).

Genetic deletion of any one BH3-only proteins, (except Bim), do not have profound effects on animal development, but show often stimulus and/or tissue specific function in animal cells. It is consistent with their roles as sensors of intracellular stress in development and tissue homeostasis. Different studies have indicated that loss of expression of several BH3-only proteins might contribute to cancer development (Lomonosova and Chinnadurai 2008).

Bim has prominent function in lymphoid cells, but is also readily detectable in many other cell types (O'Reilly et al. 2000). In *bim* deficient mice, a significant proportion (approximately 40%) of embryos die *in utero* and survivors show lymphoid and myeloid cell hyperplasia as well as development of a fatal autoimmune disease (Bouillet et al. 1999). Resistance to growth factor deprivation in *bim* deficient cells (Bouillet et al. 1999; Putcha et al. 2001; Whitfield et al. 2001), indicates that Bim has central role in mediating developmental and homeostatic apoptotic signals. Bim also mediates most of the cell death induced by endoplasmic reticulum (ER) stress (Puthalakath et al. 2007).

Full-length Bid is expressed in most tissues, but is inactive until cleaved proteolytically (Li et al. 1998; Luo et al. 1998). *Bid* deficient mice are developmentally normal, but resistant to CD95/Fas or TNF induced cell death in certain cell types (type II cells), such as hepatocytes and pancreatic β cells (Yin et al. 1999; Kaufmann et al. 2007; Kaufmann et al. 2009). *Bid* deficient mice also develop a myeloid cell hyperplasia that can progress to a malignancy resembling chronic myelomonocytic leukemia (Zinkel et al. 2003), which have been mechanistically explained by Bid mediated response to replicative stress or cell-cycle arrest (Zinkel et al. 2003).

Puma deficiency does not cause any significant developmental defects in mice (Shibue et al. 2003; Villunger et al. 2003). *Puma* deficiency protects cells from genotoxic stress that could normally activate p53 dependent apoptotic response, especially following γ- irradiation, but these cells are also resistant to several p53-independent death stimuli, such as cytokine withdrawal or application of glucocorticoids or phorbol

ester (Villunger et al. 2003). Other studies have implicated a role for Puma in ER stress, ischemia/reperfusion as well as bacterial, viral or fungal infection (Chipuk et al. 2010).

Bim, tBid and Puma are the most potent activators for Bax and Bak (Letai et al. 2002; Kim et al. 2009). Accordingly, *bim*, *bbc3* (*puma)* and *bid* triple deficient cells exhibit near full resistance to intrinsic stress signals (Ren et al. 2010). However their genetic ablation in mice does not fully phenocopy the severe cell-death associated phenotype seen in *bax/bak* double deficient mice (Villunger et al. 2011). It indicates that proteins other than BH3-only activators Bid, Bim and Puma may be responsible for Bak or Bax activation under certain conditions (Chipuk et al. 2004; Villunger et al. 2011; Moldoveanu et al. 2014). Also the tumor suppressor p53 is able to directly bind/activate Bax and Bak *in vitro* (Moldoveanu et al. 2014). However, using a combinatorial genetic approach in human colon cancer cells, it was recently found that Bax and Bak can become active also in the conditions where tBid, Bim, and Puma and p53 were genetically deleted and both, Bcl-xL and Mcl-1 were simultaneously suppressed (Zhang et al. 2016). It indicates that direct activation is not essential for Bax/Bak activation (Zhang et al. 2016). Alternatively, other activators, such as Noxa, may still activate Bax in those conditions. Therefore, the mechanism of Bax activation remains controversial.

Mice lacking Bad, Bmf, Bik, Hrk, or Noxa are fertile and lack an obvious phenotype. Analysis of these individual BH3-only deficient mice has demonstrated functional redundancy but also a certain degree of cell type/organ specificity, especially in a stress conditions. *Bad* loss renders cells only partially insensitive to the pro-apoptotic effect of cytokine deprivation or other cytotoxic stimuli, indicating that it synergises with more potent BH3-only protein to mediate apoptotic signals to Bax and Bak (Ranger et al. 2003; Danial 2008; Kelly et al. 2010). *Bad* deficient mice exhibit spontaneously developing diffuse B cell lymphomas (Ranger et al. 2003; Kelly et al. 2010) that is probably caused by reduced inhibition of Bcl-xL in the absence of Bad (Mason et al. 2007). *Bmf* deficient mice accumulate excess B cells, demonstrating its role in B-cell homeostasis (Labi et al. 2008). Bmf has also overlapping functions with Bim in sensing detachment of adherent cells (anoikis) (Puthalakath et al. 2001). *Bik* deficient mice do not have developmental abnormalities (Coultas et al. 2004), however, male mice lacking both *bim* and *bik* show impaired apoptosis in immature testicular progenitor cells, resulting in infertility (Coultas et al. 2005). *Hrk* is expressed mainly in the central and peripheral nervous system (Imaizumi et al. 2004). *Hrk* deficiency in mice protects sensory neurons from nerve growth factor deprivation-induced apoptosis (Imaizumi et al. 2004; Coultas et al. 2007). Noxa appears to mediate UV-radiation-induced apoptosis of fibroblasts and keratinocytes (Naik et al. 2007). Noxa functions often synergize with the activities of Puma, in DNA damage induced apoptosis from etoposide and radiation (Villunger et al. 2003).

2.3.4.2 Activation of BH3-only proteins

Activation of BH3 only proteins provides a major link between intrinsic stress signals and downstream events governed by core apoptotic machinery. Their activation

allows cells to properly respond to cell death and survival signals (Huang and Strasser 2000).

In healthy cells, some BH3-only proteins, such as Bid, Bim, Bmf and Bad, may be constitutively expressed. These BH3-only proteins usually have distant location from their target membrane(s) where they often participate in functions independent of apoptosis (Cheng et al. 2006). Post-translational modifications may restrict the BH3-only proteins to one of the alternative functions (Zinkel et al. 2005). For instance, Akt kinase mediated survival signalling inactivates Bad by phosphorylation that promotes its sequestering by 14-3-3 scaffold proteins (Zha et al. 1996; Tan et al. 2000; Zhou et al. 2000). Bad has been also shown to reside in a functional holoenzyme complex involved in glucose-driven mitochondrial respiration (Danial et al. 2003). This function of Bad is specifically dependent upon the phosphorylation of its BH3 domain (Datta et al. 2000). Bim is expressed as several alternatively spliced isoforms (Marani et al. 2002). Most abundant Bim isoforms and Bmf are attached to the cytoskeletal components via dynein and myosin motor complex, respectively (Puthalakath et al. 1999; Puthalakath et al. 2001). Bid phosphorylation at different sites modulates its pro-apoptotic activity, particularly in sensing the replicative stress and DNA damage (Desagher et al. 2001; Zinkel et al. 2005). Bid has been also associated the transport and recycling of mitochondrial phospholipids (Esposti et al. 2001).

In response to apoptotic signals, cytoplasmic BH3-only proteins are released from sequestering complexes (Puthalakath et al. 1999; Puthalakath et al. 2001), enabling their translocation to mitochondria, to induce apoptosis. For example, growth factor deprivation triggers Bad dephosphorylation and release from sequeresting protein complex (Zha et al. 1996; Datta et al. 1997; Harada et al. 1999) The specific proapoptotic signals are also responsible for the release of Bim and Bmf, mostly by inducing their phosphorylation (Lei and Davis 2003). For example, loss of substrate adhesion triggers Bmf phosphorylation and disruption of its interaction with dynein, allowing Bmf to interact with other Bcl-2 family members in the mitochondrial membrane (Puthalakath et al. 1999; Puthalakath et al. 2001). Phosphorylation of human Bik appears to increase its pro-apoptotic activity through enhanced interaction with anti-apoptotic proteins (Verma et al. 2001).

Proteasomal degradation pathway may down-regulate the BH3-only protein levels. For example, Bim protein levels are downregulated in osteoclasts by constitutive ubiquitylation and rapid proteasomal degradation. This process is significantly reduced in growth factor removal, resulting in rapid and sustained increase in Bim protein levels (Akiyama et al. 2003). Bim phosphorylation at different residues modifes its sensitivity to proteasomal degradation as well as its interactions with other family members., (Hubner et al. 2008; Lomonosova and Chinnadurai 2008). Cell culture treatment with various proteasome inhibitors lead to extensive upregulation of Bim, Bik and Noxa (Marshansky et al. 2001; Hur et al. 2004; Fernandez et al. 2005; Nikrad et al. 2005). It indicaties that proteasmal targeting might control the levels of several constitutively expressed BH3-only proteins in cells.

Bid is activated by proteolytic cleavage by caspase-8 or other proteolytic enzymes (Muchmore et al. 1996; Li et al. 1998; Luo et al. 1998; Eskes et al. 2000), resulting in an N-terminally truncated form (tBid) (Li et al. 1998; Luo et al. 1998). Truncated Bid becomes fully activated by myristoylation of its N-terminus (Zha et al. 2000). These modifications facilitate tBid targeting to mitochondria (Garcia-Saez et al. 2004; Rautureau et al. 2010a).

Several BH3-only proteins are transcriptionally up-regulated in response to intracellular stress, such as DNA damage, growth factor deprivation, and endoplasmic reticulum stress, to initiate apoptosis. For instance, transcription factor p53 induces Noxa and Puma transcription, particularly following DNA damage (Oda et al. 2000; Yu et al. 2001). Noxa activation has been also reported by HIF-1α in response to hypoxia (Kim et al. 2004). JNK-mediated signalling increases the mRNA expression of Bim and Hrk upon survival factor withdrawal in neurons (Imaizumi et al. 1997; Harris and Johnson 2001; Putcha et al. 2001; Whitfield et al. 2001; Ma et al. 2007). Bim expression is also up-regulated by the Forkhead transcription factor, Foxo3a in growth factor deprived neurons or by a member of the C/EBP transcription factor family CHOP, during endoplasmic reticulum (ER) stress (Gilley et al. 2003; Puthalakath et al. 2007). The transcription factor E2F-1 could directly up-regulate expression of *Puma*, *Noxa*, *Bim*, *Hrk* and *Bik* in response to unscheduled DNA replication and cell cycle progression (Hershko and Ginsberg 2004; Real et al. 2006; Subramanian et al. 2007).

2.3.4.3 The multidomain pro-apoptotic proteins

Pro-apoptotic effectors, Bax and Bak have a key role in mitochondrial outer membrane permeabilization during apoptosis, although the mechanism by which Bak and Bax compromise organelle integrity is not fully resolved. It has been demonstrated that Bak and Bax physically constitute the release channel in model membranes (Korsmeyer et al. 2000; Nechushtan et al. 2001; Lovell et al. 2008), but also other models, including those that question the role of Bak and Bax oligomerization in this process have been proposed (Volkmann et al. 2014).

Gene targeted loss of function studies in mice have shown, that combined loss of *Bak* and *Bax* result in perinatal lethality, due to an inability to eliminate excess cells during development (Lindsten et al. 2000). The few long-term survivors (<10%) display significant developmental defects that correlate with the deficiency in cell death (Lindsten et al. 2000; Mason et al. 2013). Deletion of *Bax* and *Bak* render various types of cells profoundly resistant to almost all intrinsic death stimuli, including the enforced expression of the BH3-only proteins (Zong et al. 2001). The loss of either *Bak* or *Bax* shows virtually no decreased susceptibility to apoptosis (Cheng et al. 2001; Wei et al. 2001; Zong et al. 2001).It indicates that both proteins are functionally redundant and the presence of either Bak or Bax is sufficient to mediate apoptotic signal in the mitochondrial pathway. Yet, Bak appears to have a principal role in apoptosis of platelets and certain cell populations in thymus (Mason et al. 2007; Dunkle et al. 2010). Analysis of Bax-deficient mice has revealed its critical role in regulating neuronal death in several neuronal populations of the brain and retina, but also demonstrated its non-redundant

requirement for germ cell death in spermatogenesis (Deckwerth et al. 1996). Bax deficiency decreases also cardiac myocyte apoptosis after myocardial infarction (Hochhauser et al. 2007). Bax/Bak-deficient cells have also alterations in mitochondrial morphology, indicating their role in modulating mitochondrial dynamics (Karbowski et al. 2006).

Bcl-2-related ovarian killer (Bok) (Hsu et al. 1997a) is also widely expressed potential effector molecule, however the mechanism of Bok pro-apoptotic activity is not understood (Ke et al. 2012; Echeverry et al. 2013). Mice lacking Bok or its combined loss with either Bax or Bak are largely normal (Ke et al. 2012; Ke et al. 2013). Some impact on primordial follicle oocyte survival in aged female mice has been observed in mice deficient for both Bok and Bax, indicating that Bok may have an overlapping pro-apoptotic role with Bax in this tissue (Ke et al. 2013). Biochemical evidence suggests that Bok induces apoptosis in a Bax and Bak dependent manner (Chipuk et al. 2010; Echeverry et al. 2013).

In healthy cells, Bax either exists as a monomer in a cytosol, or loosely attached to the outer mitochondrial membrane (Goping et al. 1998). Upon induction of apoptosis, cytosolic Bax translocates into the outer mitochondrial membrane (Hsu et al. 1997b; Annis et al. 2005; Lovell et al. 2008; Oh et al. 2010). Bak is constitutively localized at the outer membrane of mitochondria and does not change its localization in apoptotic cells (Breckenridge et al. 2003; Scorrano et al. 2003; Zong et al. 2003). Bax and Bak have been also detected at the endoplasmic reticulum/nuclear outer membrane where these proteins are thought to exert some specific physiological functions (Scorrano et al. 2003; Zong et al. 2003; Hetz et al. 2006; Lindenboim et al. 2010; Wang et al. 2011). The Bok localizes predominantly to Golgi and ER in healthy and apoptotic cells (Echeverry et al. 2013).

2.3.4.4 The Bak and Bax activation and activity in membranes

The activation of Bak and Bax proteins requires notable conformational changes within their monomeric species, which by currently preferred model, enables their assembly into large oligomeric complexes that permeabilize the intracellular membranes, including MOM to kill the cell (Korsmeyer et al. 2000) (Figure 7). The key conformational changes that occur in Bax and Bak during activation are the exposure of an N-terminal segment, transient dislodgement of the BH3 domain (in the α2 helix), and in the case of Bax initial exposure of the C-terminal α9 helix for membrane localization (Hsu and Youle 1997; Griffiths et al. 1999; Griffiths et al. 2001; Dewson et al. 2008; Dewson et al. 2009; Czabotar et al. 2013). Further changes in membranes include the N terminal bundle separation from the C-terminal hydrophobic core (core/latch structure) resulting in formation of symmetric dimers by BH3 interaction of one monomer to the reminiscent canonical hydrophobic cleft of another (Dewson et al. 2008; Bleicken et al. 2010; Oh et al. 2010; Dewson et al. 2012; Czabotar et al. 2013).

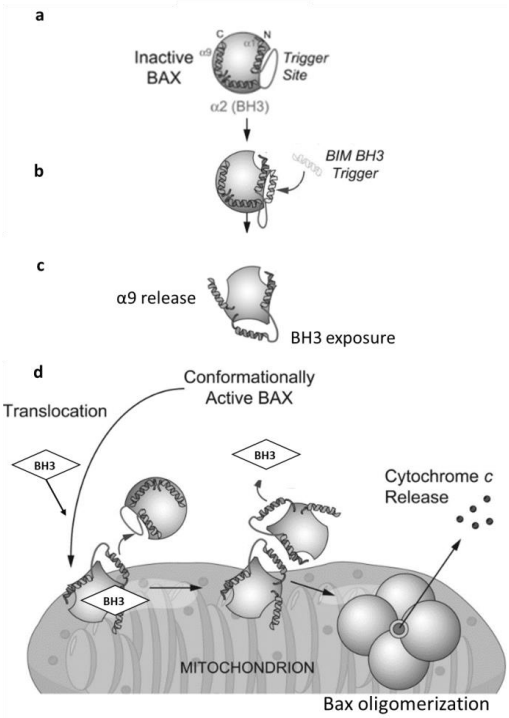

Figure 7. Proposed model of BAX activation, mitochondrial translocation, oligomerization and focal clustering on the mitochondrial membrane. a) Inactive, soluble Bak. b) Bax activation is initiated by "activator" BH3-only protein that binds directly to the soluble Bax protein "rear pocket" or "trigger site". c) Conformational changes in Bax result in dislodgement of the helix α9, which directs it to the outer mitochondrial membrane. Also a BH3 domain (α2) becomes exposed. d) Sequential conformational changes in Bax are mediated by BH3-only protein binding to the canonical hydrophobic groove of Bax. The core bundle separates from the C-terminal part and the BH3-only protein is released. Activated and membrane-bound Bax is competent to oligomerize and trigger MOMP. Modified from Gavatioitis (Gavathiotis et al. 2010).

Precise dynamics of Bak and Bax activation in living cells is not completely understood. Several studies have provided support for the so-called "hit-and run" activation model where transient interactions with BH3-only proteins induce the effector protein activation and oligomerization (Wei et al. 2000; Kuwana et al. 2005; Walensky et al. 2006; Gavathiotis et al. 2008). It is likely that also non-canonical activation occurs in some biological condition (Chipuk et al. 2010; Moldoveanu et al. 2014).

Structural and biochemical analyses have revealed a non-canonical hydrophobic site, termed as "rear pocket" in the Bax molecule (Gavathiotis et al. 2008). Stabilized α-helical peptide, corresponding to Bim BH3 domain, binds to Bax protein rear pocket and causes the conformational changes that favour Bax mitochondrial translocation, oligomerization and focal clustering on the mitochondrial membrane (Gavathiotis et al. 2008; Gavathiotis et al. 2010). These results suggest that direct binding of an "activator" BH3-only protein to Bax molecule "rear pocket" triggers its activation (Roy et al. 2014), although stochastic activation of Bak and Bax has been also suggested (Edlich et al. 2011; Schellenberg et al. 2013).

In the membrane vicinity Bax nestles the Bim-derived stabilized BH3 peptide to its canonical hydrophobic groove (Czabotar et al. 2013). Hence, BH3 domain interactions with two different sites in Bax may play hierarchical roles in its activation (Kim et al. 2009). Biochemical data have shown that constitutively membrane bound Bak accommodates the interacting BH3-helices only within its canonical hydrophobic groove (Leshchiner et al. 2013; Moldoveanu et al. 2013), indicating that Bax and Bak are activated at distinct trigger sites (Leshchiner et al. 2013). Some studies have revealed

that, dephosphorylation of certain amino acid residues in Bak canonical binding cleft enable interactions with activating BH3-only proteins , thus constituting the initial step in Bak activation process (Fox et al. 2010; Azad et al. 2012).

Once translocated to the membrane, Bax undergoes further structural transitions, which allow its membrane integration and oligomerization (Annis et al. 2005; Oh et al. 2010). For example, Bax inserts at least three amphipatic helices (α5, 6 and 9) into the MOM before oligomerization (Annis et al. 2005) Also constitutively membrane-bound Bak inserts at least its α6 helix into the MOM after activation (Oh et al. 2010).

Amphipathic helix (α2), which corresponds to BH3 domain in Bax and Bak molecule, is transiently exposed during an activation process (Dewson et al. 2012; Czabotar et al. 2013). Reciprocal binding of Bak or Bax α2 to the hydrophobic cleft of another molecule generates symmetric homo-dimers (Dewson et al. 2012; Czabotar et al. 2013).

It is currently unclear how important is membrane-binding region in Bax and Bak oligomerization process. Some studies have reported that it is dispensable for activated Bak oligomerization (Oh et al. 2010; Landeta et al. 2011) and similar results have been also provided for Bax (Garcia-Saez et al. 2006; Czabotar et al. 2013).

Although BH3-in groove interaction is crucial for Bax or Bak homotypic oligomerization (Dewson et al. 2008; Czabotar et al. 2013; Shamas-Din et al. 2013b), it has been suggested that the membrane association and conformational changes could stabilize Bax or Bak homodimers through new interaction surfaces (Dewson et al. 2009; Garcia-Saez 2012). Similar features may be involved in forming the high order oligomers that affect the MOM (Dewson et al. 2012). At present, the exact rearrangements in effector protein structures as well as their interactions that are responsible for MOMP are incompletely understood (Moldoveanu et al. 2014; Volkmann et al. 2014).

Aggregation of Bak and Bax onto intracellular membranes affects the integrity of these target membranes (Antonsson et al. 2000; Wei et al. 2001; Grinberg et al. 2002; Lindenboim et al. 2010; Wang et al. 2011). Experiments using synthetic model membranes and isolated mitochondria suggest that Bax and/or Bak are able to destabilize membranes by generating either proteinacious or lipidic pores (Dejean et al. 2005; Qian et al. 2008; Shamas-Din et al. 2013b) (Basanez et al. 1999; Saito et al. 2000). These pores have been demonstrated to be large enough to release intravesicular macromolecules (Saito et al. 2000), but precise mechanism for pore formation is not fully understood. Mitochondrial lipids, in particular cardiolipin, may be involved in Bax mediated pore formation (Epand et al. 2002; Kuwana et al. 2002; Ott et al. 2002), but their contribution in this process is not clear (Garcia-Saez 2012). Recent super-resolution microscopy approach revealed that Bax assembles into ring-like structures of varying sizes in the outer mitochondrial membrane of apoptotic mitochondria (Grosse et al. 2016). It suggests that Bax delineates a pore required for MOMP. The role of auxiliary mitochondrial proteins in mediating Bax or Bak induced MOMP remains poorly characterized.

Biochemical studies indicate that Bak activation is prevented primarily by Mcl-1 and Bcl-xL (Willis et al. 2005), whereas Bax is held in check by all pro-survival Bcl-2

homologues (Uren et al. 2007; Czabotar et al. 2014; Vandenberg et al. 2014). It is still a matter of considerable controversy whether the antiapoptotic proteins engage the constitutive inhibitory interactions with Bax and Bak or interact with these proteins during their activation process.

The homeostatic suppression of Bak involves hindering of its canonical binding groove (Leshchiner et al. 2013). In the absence of apoptosis signalling, the accumulation of Bax to the mitochondria is prevented by its constant retrotranslocation from the OMM into the cytoplasm in a Bcl-xL dependent manner (Edlich et al. 2011). Bax shuttling between the cytosol and mitochondria constitutes an equilibrium that maintains the predominant cytosolic localization of Bax and minimizes its proapoptotic activity in mitochondria (Edlich 2015). Apoptotic signals promote Bax accumulation on the mitochondria (Suzuki et al. 2000) which may reflect a shift in the Bax shuttling equilibrium (Czabotar et al. 2014). Beside the antiapoptotic Bcl-2 family members, several other proteins, such as 14–3-3 (Nomura et al. 2003)(Nomura 2003), humanin (Guo et al. 2003), and Ku70 (Cohen et al. 2004; Mazumder et al. 2007; Amsel et al. 2008), have been shown to negatively regulate Bax by sequestering it from the mitochondria.

2.3.4.5 The multidomain anti-apoptotic members

The multidomain anti-apoptotic members such as Bcl-2, Bcl-xL, and Mcl-1 operate at multiple locations in the cell to co-ordinately control cellular functions (Hardwick and Soane 2013). These proteins localize predominantly to mitochondrial membrane, ER and nuclear envelope (Lithgow et al. 1994), but, for example Bcl-xL shows facultative targeting to membranes and localizes from cytosol to mitochondrial and ER membranes in apoptotic cells (Youle and Strasser 2008; Susnow et al. 2009).

Antiapoptotic Bcl-2 family proteins are able to counteract the Bax and Bak pore-forming activity, but also intercept upstream proapoptotic signals which are mediated by BH3-only proteins (Michels et al. 2013).

Genetic ablation of *mcl-1* in mice results in failure of the early embryo to implant (Friberg et al. 2013). Deficiency of Bcl-2 and Bcl-xL causes embryonic lethality in mice, because of the defective development (Veis et al. 1993; Motoyama et al. 1995). Bcl-2 and Bcl-xL overexpression in mammalian cell cultures and in transgenic mice increases resistance to several type of intrinsic stresses, such as nutrient and serum deprivation, heat shock or irradiation (Vaux 1993; Arden and Betenbaugh 2004). High levels of anti-apoptotic Bcl-2 family proteins are often associated with aggressive malignancies and with resistance to chemotherapy in human cells. However, studies in mice suggest that overexpression of these proteins alone is not sufficient to drive tumorigenesis, but requires other oncogenic transformations (Strasser et al. 1990). Increased expression of Bcl-2 has been correlated also with development of asthma and certain autoimmune diseases (Dewson 2010). The upregulation of Bcl-2 expression, however, contributes to the protection of terminally differentiated cells, such as postmitotic neurons, pancreatic β-cells or cardiomyocytes, from numerous apoptotic insults.(Martinou et al. 1994; Kirshenbaum and de Moissac 1997; Tran et al. 2003; Imahashi et al. 2004). These studies

show that anti-apoptotic proteins are important for maintaining cell survival during development and following acute and chronic injury.

The primary function of anti-apoptotic Bcl-2 family members is to inhibit the multi-domain pro-apoptotic Bax and Bak proteins from multimerizing and compromising organelle integrity (Krajewski et al. 1994; Lithgow et al. 1994; O'Reilly et al. 2001). However, the mechanism of their action is not fully elucidated. The biochemical coupling of pro-apoptotic BH3 domain to the hydrophobic cleft of anti-apoptotic member, leads to mutual inactivation of binding partners. It probably accounts for a mechanism how anti-apoptotic proteins neutralize functionally opposite factions of Bcl-2 family proteins. Anti-apoptotic members bind only unique set of the BH3-only molecules (Letai et al. 2002; Chen et al. 2005; Certo et al. 2006). For example, Bcl-2, Bcl-xL, and Bcl-w bind Bad avidly, but do not bind Noxa. Mcl-1 and A1 bind Noxa, but do not bind Bad. However, all anti-apoptotic proteins bind Bim and Puma with high affinity. The interactions with pro-apoptotic members Bak and Bax are also relatively selective. Mcl-I1, Bcl-xL and A1 interact with Bak, whereas all anti-apoptotic proteins interact with Bax (Czabotar et al. 2014).

One important feature, distinguishing the anti-apoptotic Bcl-2 proteins, is their inability to oligomerize into high molecular weight structures (Billen et al. 2008). Anti-apoptotic members, however, may insert their hydrophobic core helices into membranes (Losonczi et al. 2000; Aisenbrey et al. 2007) and may act as a dominant-negative regulators for Bax (Dlugosz et al. 2006; Leber et al. 2007; Leber et al. 2010).

Intriguingly, several anti-apoptotic members are cleaved during apoptosis thereafter they acquire proapoptotic properties (Basanez et al. 1999). The molecular basis for this reversal of function is unclear, although conformational changes, like exposure of BH3 domain (α2 helix) and other structural rearrangements in membrane have been suggested as a potential mechanism.

2.3.5 Models for controlling Bax and Bak mediated MOMP in mammalian cells

A single mechanism by which members of the Bcl-2 family regulate apoptosis has not been completely resolved (Bortner and Cidlowski 2002; Czabotar et al. 2014). It is commonly accepted that MOMP in stress-induced mitochondrial pathway is mediated by Bax and Bak. It has been extensively debated whether anti-apoptotic Bcl-2 proteins block MOMP predominantly through binding to BH3-only proteins, to Bax/Bak, or to both. This ambiguity has been in the centre of different models which have been proposed over the years to explain the function of distinct Bcl-2 family protein classes in apoptosis regulation (Llambi et al. 2011; Shamas-Din et al. 2013b) (Figure 8).

In 1993, Stanley Korsmeyer provided the core concept that susceptibility of a cell death is governed by the balance between pro- and antiapoptotic Bcl-2 family members, which he called as the “rheostat model” (Korsmeyer et al. 1993; Oltvai et al. 1993; Chao and Korsmeyer 1998). This theory has remained remarkably unmodified since its original formulation although, its details have been progressively refined (Michels et al. 2013).

The displacement model states that pro-apoptotic Bax and Bak are constitutively "primed" in cells and anti-apoptotic proteins, such as Bcl-2 and Bcl-xL bind and inhibit their oligomerization and cell death (Chen et al. 2005; Uren et al. 2007; Willis et al. 2007)
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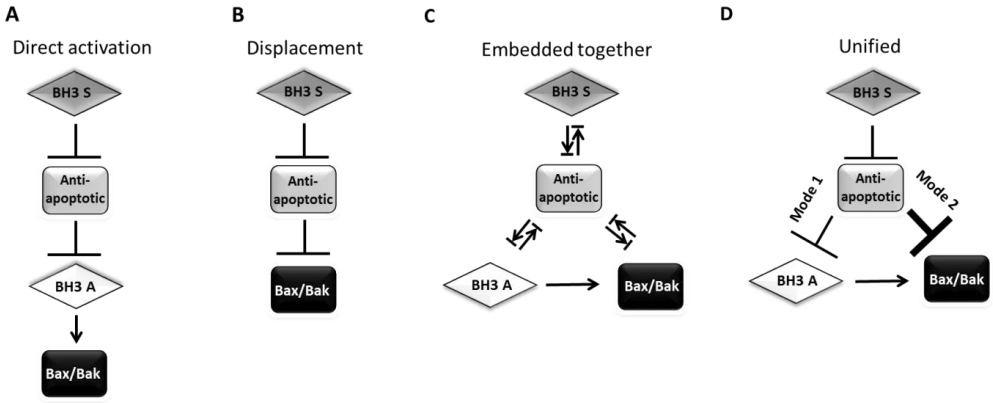


Figure 8. Schematic representation of the proposed models for Bcl-2 protein hierarchical interaction in regulating MOMP. Symbols denote: (↑) activation; (⊥) inhibition; (⊥↑) mutual recruitment/sequestration. The BH3 proteins with high affinity for binding and activating Bax and Bak are termed as "activators," (BH3 A) whereas those that only bind the anti-apoptotic proteins are termed "sensitizers" (BH3 S). (*A*) The direct activation model. (*B*) The displacement model. (*C*) The embedded together model. (*D*) The unified model. Adapted from Shamas-Din et al., (2013b)

This model proposes that activated BH3-only proteins displace pro-apoptotic Bax and Bak proteins from the inhibiting interactions with anti-apoptotic Bcl-2 members to promote MOMP (Oltvai et al. 1993; Willis et al. 2005; Willis et al. 2007). Therefore, neutralization of multi-domain anti-apoptotic proteins is likely sufficient for Bax and Bak dependent MOMP to occur (Chen et al. 2005; Willis et al. 2005; Willis et al. 2007). This assumption has been however challenged by several studies showing that only a minor fraction of Bak and Bax are bound to prosurvival proteins in healthy cells (Leber et al. 2007; Dewson et al. 2009). In addition, select BH3-only proteins and BH3 peptides have a capacity to induce Bax activation through direct, although transient binding (Kuwana et al. 2005; Walensky et al. 2006; Gavathiotis et al. 2008; Czabotar et al. 2013). The studies on the BH3 peptide binding affinity profilings have led to the development of the concept of "activating" and "de-repressing/sensitizing" BH3-only proteins (Kuwana et al. 2002; Letai et al. 2002; Kuwana et al. 2005). Together with the Bcl-2 family protein structural analyses and *in vitro* recapitulated effector-protein activation assays (Lovell et al. 2008), a few additional models have been proposed (reviewed in Shamas-Din et al. 2013b).

The direct activation model suggests that direct activators, tBid, Bim and Puma are kept in check by anti-apoptotic proteins, but are released upon displacement by derepressor BH3-only proteins, such as Bad or Noxa. It leads to activation of inactive pro-apoptotic members Bak and Bax to trigger MOMP.(Wei et al. 2000; Kuwana et al. 2002;

Letai et al. 2002; Kuwana et al. 2005; Certo et al. 2006; Walensky et al. 2006; Deng et al. 2007; Leshchiner et al. 2013).

The examination of the interaction dynamics of the Bcl-2 family proteins have revealed that Bcl-2 family proteins can enhance or delay MOMP depending on the relative stoichiometry of available binding partners in the membrane. The “embedded together” model (Leber et al. 2007; Billen et al. 2008; Lovell et al. 2008; Bogner et al. 2010) proposes that anti-apoptotic proteins are able to sequester both, the BH3-only activators and Bak/Bax in the phospholipid membrane environment or prevent their binding to membranes, hence acting as a dominant-negative regulators to inhibit apoptosis (Leber et al. 2007; Leber et al. 2010; Edlich et al. 2011). The BH3-only de-repressors, however, displace membrane bound Bax and BH3-only activators from the anti-apoptotic proteins (Billen et al. 2008; Lovell et al. 2008; Shamas-Din et al. 2013b). Displaced effector, such as Bax, is competent to oligomerize at mitochondrial membrane which results in pore formation and MOMP (Leber et al. 2007; Billen et al. 2008; Lovell et al. 2008).

Multidomain Bcl-2 homologs bind the BH3-death domains with varying efficiencies. Therefore, a “unified model” has been proposed (Llambi et al. 2011). This model differentiates two kinetically different modes by which antiapoptotic Bcl-2 proteins can inhibit MOMP. Importantly, both inhibition modes could occur simultaneously in cell. One pathway may be dominant depending on the cell death stimulus or varying biological context (Llambi et al. 2011; Czabotar et al. 2014). In this model, antiapoptotic Bcl-2 family proteins can sequester direct-activator BH3-only proteins ("mode 1") or bind to activated Bax and Bak ("mode 2"). Inhibition of apoptosis through mode 1 is less efficient and is easier to overcome by BH3 sensitizers to promote MOMP than inhibition through mode 2 (Llambi et al. 2011).

Unified model also combines the Bcl-2 family functions in apoptosis and in mitochondrial dynamics. Bax and Bak regulate mitochondrial fusion in healthy cells, presumably through interaction with mitofusin-2 (Karbowski et al. 2006; Hoppins et al. 2011). Efficient sequestration of activated Bak and Bax by antiapoptotic proteins (mode2 inhibition) appears to inhibit this process and promotes mitochondrial fragmentation, whereas in mode1 inhibition, their function in mitochondrial fusion remains unaffected (Llambi et al. 2011). Thus, two modes of inhibition have distinct physiological consequences to mitochondrial dynamics (Llambi et al. 2011). Still, integration of the existing knowledge of Bcl-2 protein family functions in the regulation of autophagy, calcium homeostasis, and metabolism to their central roles in the apoptosis requires additional studies.

2.4 General considerations of neuronal cell death and apoptosis

2.4.1 Programmed cell death in the vertebrate nervous system development

Programmed cell death is an integral process in development of the animal nervous system, particularly in the higher vertebrate species. Normal development of mammalian nervous system includes substantial overproduction of cells, followed by a programmed demise of roughly half of the originally produced cells (Oppenheim 1991). PCD occurs in almost all regions of the nervous system and involves virtually all subtypes of neuronal and glial cells (Rubin 1997; Buss et al. 2006b).

Programmed cell death in neurodevelopment occurs in several distinct waves, eliminating subsets of proliferating neuronal precursors and immature postmitotic neuroblasts prior to the establishment of connections, and young postmitotic neurons during their differentiation and synaptogenesis (Oppenheim 1991; Jacobson 1997; Kuan et al. 2000a; Roth et al. 2000b; Roth and D'Sa 2001; Buss et al. 2006b; Kim and Sun 2011). In most animal species new neurons are produced also in adulthood but considerable numbers of these cells are eliminated by PCD (Biebl et al. 2000; Kim and Sun 2011). Aging nervous tissue experiences several alterations in cell metabolism that may cause progressive, and even pathological loss of neurons (Frazzini et al. 2006).

While the mechanism of selective death of postmitotic neurons in the peripheral nervous system (PNS) could be well explained by the neurotrophic factor theory (Levi-Montalcini and Booker 1960b; Hamburger et al. 1981; Levi-Montalcini 1987; Oppenheim 1991; Buss et al. 2006b), the regulation of the early wave of naturally occurring neuronal death is not well understood (Oppenheim 1991; Buss et al. 2006b; Okouchi et al. 2007). The extent of PCD in different regions of central nervous system (CNS) is also not well characterized and the mechanism by which the number of mature neurons is finally determined is not completely understood (Song et al. 2012; Dekkers et al. 2013).

Survival and death of neuronal cell is regulated by concerted actions of various molecules, acting cell-extrinsically or intrinsically to prevent or promote cell death. Classic neurotrophic factor theory, which was mainly developed based on the studies of nerve growth factor (NGF) in the PNS, emphasizes that developing neurons are programmed to die unless they are rescued by extrinsic growth factors (e.g. neurotrophic factors) that are secreted by their targets (Oppenheim 1991; Korsching 1993; Benn and Woolf 2004; Buss et al. 2006b). Accordingly, developing neurons must compete for the limiting amounts of neurotrophic factors. Only the neurons that reach their targets and receive sufficient trophic support survive and establish a synaptic contact with their target. Those neurons that do not obtain sufficient amounts of neurotrophic factors are removed by PCD (Barde 1989; Oppenheim 1991; Davies 1996).

The concept of neurotrophism has broadened, and now includes the trophic dependence of different types of neurons at various developmental stages (de la Rosa and de Pablo 2000). For example, growth factors belonging to several molecular families and

other signals, such as neuronal activity and neurotransmitter input may profoundly regulate neuronal survival in the developing CNS (Barde 1989; de la Rosa and de Pablo 2000; Dekkers et al. 2013). It thus appears that in the developing CNS the death of most neurons may not be regulated by simple competition for growth factors (Song et al. 2012; Dekkers and Barde 2013; Dekkers et al. 2013). Recently, Southwell et al (2012) demonstrated that GABA-ergic interneuron cell death in developing cerebral cortex appears to be intrinsically determined and thus do not follow the prediction of the extended neurotrophic factor theory (Southwell et al. 2012; Dekkers et al. 2013). It has been shown that neighbouring microglia cells could actively participate in the PCD of Purkinje cells in the cerebellum (Marin-Teva et al. 2004).

The mechanism of PCD in the newly produced neurons of the adult brain could be explained by extended neurotrophic factor theory. For instance, learning and physical exercise has been shown to increase the extracellular secretory factors, such as neurotransmitters, hormones and growth factors, which in turn enhance the survival of adult-produced neurons (Kim and Sun 2011). However, newly produced immature neurons, must become incorporated into the pre-established mature circuits and these cells likely compete with mature neurons for their synaptic connections (Kim and Sun 2011).

Neural precursor cells (NPCs) include both multipotent neural stem cells and lineage-restricted neural progenitors (Rao 1999; Akhtar et al. 2006). The molecular regulation of PCD in NPC population is poorly studied, but both, trophic factor deprivation and DNA damage has been shown to serve as significant death stimulus for NPC death in the developing brain (Gilmore et al. 2000; Roth and D'Sa 2001; Akhtar et al. 2004; Akhtar et al. 2006).

There are several explanations for the role of naturally occurring cell death in the nervous system. It is well-established that PCD regulates the size of neuronal progenitor populations and eliminates the neurons that contact with inappropriate target cells, are ectopically located, form transient structures, or do not fit to the adult neuronal network(Jung et al. 2008; Kim and Sun 2011). PCD in the developing PNS is also considered as an adaptive process that serves the purpose to match the number of innervating neurons with the size of the target field therefore optimizing the connectivity between neurons and their targets (Purves 1990; Oppenheim 1991; Kim and Sun 2011). Pre-synaptogenic death may serve variety of functions including matching of the NPC population to that necessary for proper brain morphogenesis and neuronal histogenesis, but also for elimination of cells with genetic abnormalities (Haydar et al. 1999; Roth and D'Sa 2001). It thus appears that PCD in the embryonic and early postnatal development is necessary for sharpening the borders of brain compartments and ensuring that the size of the nervous system is minimal, but sufficient. The utility of PCD in maintaining the optimal function of the nervous system is more recognized in recent years. Namely, it aids to sculpt the adult neural circuits and is critical for refining of synaptic network in the adult brain (Kim and Sun 2011).

2.4.2 Neuronal apoptosis

Most neurons undergoing PCD in brain development exhibit characteristic morphological and biochemical hallmarks of apoptosis (Arends and Wyllie 1991; Edwards et al. 1991).

The molecular nature of apoptotic pathways in different neuronal subtypes may vary and seems to depend on the apoptotic stimulus and neuronal differentiation state (Zaidi et al. 2001). For example, GDNF-deprived sympathetic neurons activate caspases via mitochondria-independent death pathway (Yu et al. 2003b) but NGF-deprived sympathetic neurons utilize the classic mitochondrial pathway for dying (Putcha et al. 2002). DNA-damage induced apoptotic pathway in NPCs involves caspase-9 and p53, but neither Bax nor caspase-3 is required (D'Sa-Eipper et al. 2001).

The importance of apoptotic machinery in mammalian nervous system development has been assessed using the genetically modified mice, lacking the critical components of core apoptosis machinery. Mice, deficient for caspase-3, caspase-9 or Apaf-1, share a similar phenotype, exhibiting gross abnormalities in the brain development and embryonic lethality (Kuida et al. 1996; Cecconi et al. 1998; Kuida et al. 1998; Yoshida et al. 1998). These defects are primarily caused by the reduced apoptosis and expansion of specific NPC populations, which all result in neural overgrowth and brain exencephaly during the embryonic development (Kuida et al. 1996; Cecconi et al. 1998; Kuida et al. 1998; Roth et al. 2000a). Mice expressing apoptotically inactive cytochrome c show similar brain abnormalities (Hao et al. 2005). Abovementioned results indicate that mitochondrial apoptosis is a critical regulator of NPC apoptosis in the developing brain (Kuida et al. 1996; Kuida et al. 1998).

Bcl-2 family proteins control the entry to mitochondrial apoptosis pathway. The presence of multiple Bcl-2 family members, their asynchronous expression and incomplete exploration of all combinations of Bcl-2 family members in genetic studies has challenged understanding of their precise role in neuronal apoptosis (Lamb and Hardwick 2010; Hyman and Yuan 2012). In addition, many Bcl-2 family members are expressed as multiple isoforms, some of them are species-specific and some exhibiting even opposite functions (Sun et al. 2001; Lucian Soane et al. 2011). However, based on the expression patterns of Bcl-2 family proteins and analyses of their corresponding knockout mice, the important roles in for several family members in regulating neuronal cell death have revealed (Lamb and Hardwick 2010).

Combined deletion of pro-apoptotic Bcl-2 family members, *bax* and *bak* in mice has revealed their redundant and synergistic role in regulating the size of the neural stem cell pool (Lindsten et al. 2000; Lindsten et al. 2003; Kim et al. 2007). The redundancy, however, appears to be eliminated in differentiated neurons as Bax deletion alone is sufficient to block apoptosis in most populations of peripheral neurons and many populations of CNS neurons (Deckwerth et al. 1996; White et al. 1998; Sun and Oppenheim 2003; Sun et al. 2004; Wright and Deshmukh 2006; Kim et al. 2007). Evidence suggests that full length Bak is not expressed in post-mitotic neurons (Sun et al. 2001; Uo et al. 2005; Wright and Deshmukh 2006). Currently there is no evidence that another pro-

apoptotic Bcl-2 family member Bok is expressed in neurons (Putcha et al. 2002).The role of Bak and Bax in neuronal apoptosis is discussed further in section 2.4.5.

The expression of Bcl-2 in mice is high during the nervous system development. After birth, its expression is downregulated in CNS, but is retained in certain neuronal populations in the PNS throughout life. Overexpression of Bcl-2 in the mouse brain results in no overt changes in morphology, although the brains were larger and contained more neurons (Li and Yuan 2011). Targeted deletion of *bcl-2* causes only subtle neurodevelopmental abnormalities but results in a progressive degeneration of motoneurons, sympathetic neurons and sensory neurons at early postnatal ages (Michaelidis et al. 1996). These results indicate that Bcl-2 is necessary for the maintenance of specific neuronal subpopulations (Michaelidis et al. 1996), while its role in other regions in developing nervous system might be compensated by other anti-apoptotic Bcl-2 family members.

The anti-apoptotic Bcl-2 family member Bcl-xL is abundantly expressed in postmitotic neurons and its expression is maintained in the adult CNS. Bcl-xL deficiency causes embryonic lethality around gestational day 13.5 (E13.5) with markedly increased apoptosis in immature neurons throughout the developing nervous system (Motoyama et al. 1995; Roth et al. 1996). This neuronal phenotype can be rescued by concomitant deficiency in Bax, caspase-9, caspase-3 or Apaf-1 (Shindler et al. 1997; Roth et al. 2000a). It suggests that these molecules act in a linear death pathway, analogous to that controlling PCD in *C. elegans,* at least in regulating neuronal apoptosis (Zaidi et al. 2001). Concomitant deficiency in Bcl-xL, however, did not rescue from NPC overgrowth in caspase-9, caspase-3 or Apaf-1 deficient embryos (D'Sa-Eipper and Roth 2000; Roth et al. 2000a). Thus, Bcl-xL is a critical anti-apoptotic regulator for post-mitotic neurons (Motoyama et al. 1995).

Mcl-1 is highly expressed in both proliferating NPCs and postmitotic neurons during brain development (Li and Yuan 2011). Mcl-1 has been implicated in protecting neurons during the transition from the progenitor to the postmitotic state and in DNA damage-induced neuronal death (Arbour et al. 2008; Hyman and Yuan 2012). Bcl-w appears to protect the axons of sensory neurons from degeneration after establishing synaptic connections (Hyman and Yuan 2012).

Deficiency in individual BH-3 only genes has been shown to only reduce or delay neuronal apoptosis, suggesting their redundant role in regulating neuronal apoptosis. For example, DP5/Hrk can promote neuronal apoptosis in a Bax dependent manner, but its contribution varies from one type of neuron to another (Imaizumi et al. 1999; Imaizumi et al. 2004; Ham et al. 2005). This variability may reflect the cell ability to functionally substitute DP5 to another BH3-only member in neuronal apoptosis. Indeed, several different BH3-only proteins can be induced upon neuronal injury (Engel et al. 2011). Gene targeting studies have revealed that only simultaneous deficiency in most potent BH3-only proteins, Bid, Bim and Puma, can block apoptosis in several neuronal populations as efficiently as Bak and Bax double-deficiency, suggesting that these proteins play a central role in mediating intrinsic stress signals to core apoptosis machinery in neuronal apoptosis (Ren et al. 2010).

The death receptor mediated extrinsic apoptotic pathway has been implicated mostly in neuronal cell death during pathological conditions, although various components of this pathway may regulate normal physiology of the cell. For example, Fas-FasL system has a dual function within the CNS, specifically participating in neurite branching during development and in neuronal apoptosis during various disease states (Zuliani et al. 2006; Okouchi et al. 2007). Yu et al. (2008) demonstrated that the death pathways in GDNF and BDNF-deprived cultured midbrain dopaminergic neurons occur via a nonconventional apoptotic pathway in which death receptors and caspases, but not mitochondrial events are activated (Yu et al. 2008).

Altogether, apoptosis appears to be the central cell death mechanism in nervous system development, although not all components of the core apoptosis machinery operate in all cell populations (Kuida et al. 1996; Cecconi et al. 1998; Kuida et al. 1998; Lindsten et al. 2000; Sun et al. 2001; Zaidi et al. 2001).

Extensive research over the past decades has revealed that along with their post-mitotic maturation, neurons adopt stringent control over the apoptotic pathway (Benn and Woolf 2004; Wright and Deshmukh 2006; Leveille et al. 2010; Kole et al. 2013) (Figure 9). For example, maturing post-mitotic neurons progressively restrict the expression of critical BH3-only proteins to control the Bax activation (Kole et al. 2011b) whereas Apaf-1 levels become decreased and XIAP levels increased to control apoptosome formation and caspase activation (Yakovlev et al. 2001; Wright et al. 2004; Potts et al. 2005; Wright et al. 2007). As a consequence, mature neurons are less sensitive to apoptotic signals and diverse cellular insults (Putcha et al. 2000). Various neuronal subtypes have differential age-dependent response to insults like viral infection, axotomy, traumatic brain injury or hypoxia (Snider et al. 1992; Kole et al. 2013). Mature neurons also lose their acute dependence on trophic factors for survival, although growth factor signalling remains important in stabilizing cellular metabolism (Easton et al. 1997; Pettmann and Henderson 1998; Vogelbaum et al. 1998; Mattson 2000; Putcha et al. 2000; Benn and Woolf 2004; Leveille et al. 2010). Stringent regulation of the apoptotic program in mature post-mitotic neurons appears to serve the demand for their long-term survival, which is ultimately required for vital functioning of the organism (Wright et al. 2004; Wright and Deshmukh 2006). It has been suggested that the mechanisms promoting long-term survival of post-mitotic neurons are also used in other post-mitotic cell types, such as myoblasts, cardiomyocytes or pancreatic β-cells (Wright and Deshmukh 2006). Moreover, cancer cell evasion from apoptosis might employ similar mechanisms that are naturally used in terminally differentiated post-mitotic cells (Wright and Deshmukh 2006; Vaughn and Deshmukh 2008; Kole et al. 2013; Gama et al. 2014). For instance, a p53-associated Parkin-like cytoplasmic protein (PARC) mediated ubiquitination and degradation of cytochrome c is a strategy engaged by both neurons and cancer cells to prevent apoptosis during conditions of mitochondrial stress (Gama et al. 2014). It highlights the requirement for in depth analysis of apoptosis regulation in post-mitotic cells. Some changes in apoptotic machinery are, however clearly neuronal cell type-specific and may depend on the stage of their developmental maturation (Kole et al. 2013). It is also important to note that despite their increased capacity to survive

numerous apoptotic insults, mature neurons can undergo cell death in situations of acute injury or neurodegenerative disease (Kole et al. 2013).

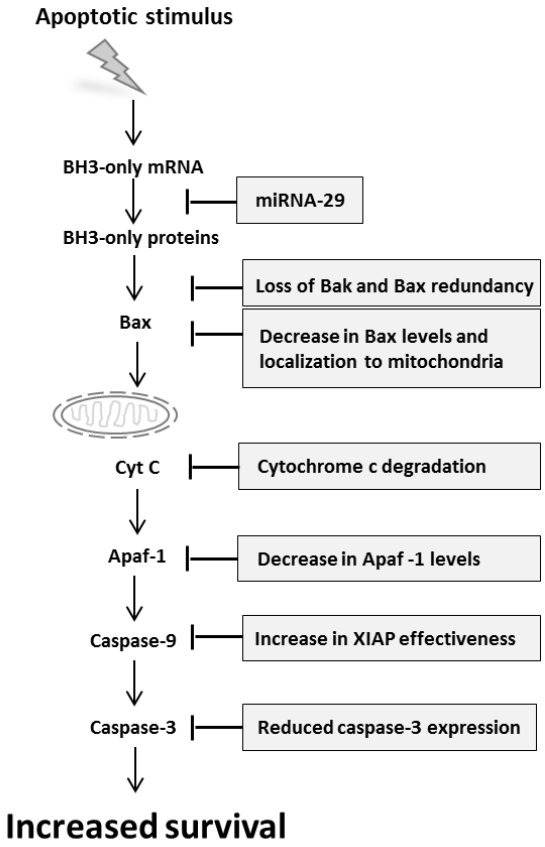


Figure 9. Schematics summarizing the mechanisms that may restrict apoptosis in various sub-types of mature neurons. Various neuronal types express a microRNA (miR-29), which can target and repress the expression of BH3-only proteins. As well, Bax and Bak redundancy could be lost. Bax expression levels and localization can change in mature neurons. PARC-mediated ubiquitinylation may trarget cytochrome c to degradation. Apaf-1 expression can be repressed via chromatin restriction. XIAP can more efficiently inhibit both caspase-9 and caspases-3. Mature neurons of the CNS have markedly decreased levels of caspases-3. Adapted from Kole et al., (2013).

The emerging data suggests that various components of the apoptotic machinery aid to refine neuronal connectivity, by regulating synaptic plasticity and axon pruning and guidance in adult neurons (Chan and Mattson 1999; Mattson 2000; Raff et al. 2002; Luo and O'Leary 2005; Hyman and Yuan 2012; Cusack et al. 2013; Dekkers et al. 2013; Hardwick and Soane 2013). For example, the pro-apoptotic proteins Bax, caspase-9, and caspase-3 promote weakening of synapses (long-term depression [LTD] (Li et al. 2010; Jiao and Li 2011; Jo et al. 2011), while the anti-apoptotic proteins Bcl-xL and the IAP-family member survivin promote synaptic strengthening (long-term potentiation [LTP] (Li et al. 2008; Iscru et al. 2013). It has been also shown that locally activated caspase-3 cleaves AMPA receptor subunits (Li et al. 2010) which may protect neuron from excitotoxic injury (Schulz and Nicotera 2000). Several non-canonical functions of caspases and Bcl-2 family proteins are increasingly implicated in normal cell physiology (Danial et al. 2010; Jiao and Li 2011; Hardwick and Soane 2013). An exciting suggestion has been put forward declaring that apoptotic cascades may function in a continuum in which low levels of activation play roles in adaptive responses to subtoxic levels of stress

or changed physiological activity, whereas higher levels of activation mediate cell death (Mattson 2000; Long and Ryan 2012; Cusack et al. 2013). The biochemical basis for such regulation is, however poorly studied. It has been demonstrated that the diffusion of activated caspases, for instance, may be prevented by the localized proteasome degradation and XIAP (Hyman and Yuan 2012; Cusack et al. 2013). Some apoptosis and cell survival related factors can be induced at the translational level directly in synaptic terminals (Duan et al. 1999; Schratt et al. 2004).

Importantly, the major defects in the CNS development, found in the caspase-3, caspase-9, and Apaf-1 deficient mice, depend on the mouse strain background (Kuan et al. 2000a; Roth et al. 2000a; Leonard et al. 2002; Yuan et al. 2003; Green 2005). Also, the histological analysis of Bax deficient and Bax and Bak double deficient mice has revealed that supernumerary cells can be eliminated in several brain regions (Roth et al. 2000a; Fan et al. 2001; Lindsten et al. 2003; Whitmore et al. 2003; Yuan et al. 2003). These results imply that cells can be eliminated by other cell death mechanisms (Oppenheim 1991; Green 2005). Histological analysis of Bax deficient mice have revealed other strategies compensating failed PCD in certain neuronal populations (Sun and Oppenheim 2003; Buss et al. 2006a; Hotchkiss et al. 2009).

The exact molecular mechanisms that compensate defective apoptosis in developing and injured neurons are not well understood (Kim and Sun 2011; Kole et al. 2013). It challenges the development of therapies aiming to prevent or modulate neuronal cell death in diverse disease states, such as neurodegenerative diseases, brain tumours or neuropsychiatric disorders, where apoptosis intersects with other cell death pathways at the molecular level (Martin 2001; Christian et al. 2010; Engel et al. 2011; Glerup et al. 2014).

2.4.3 The model of NGF-deprived sympathetic neurons to study neuronal apoptosis

Classical trophic factor deprivation-induced neuronal apoptosis occurs in developing sympathetic nervous system, where survival and death of neonatal postmitotic neurons are almost exclusively regulated by NGF (Levi-Montalcini 1987; Cowan 2001). The requirement of NGF signalling in sympathetic neuron survival and development is evidenced by virtually complete loss of sympathetic neurons in mice, deficient for NGF or its receptor TrkA (Crowley et al. 1994; Fagan et al. 1996) and destruction of sympathetic nervous system upon treatment of early postnatal mice and rats with anti- NGF antibodies (Levi-Montalcini and Booker 1960a; Angeletti and Levi-Montalcini 1971). The injection of purified NGF to young rodents accordingly increased the number of sympathetic neurons (Levi-Montalcini and Booker 1960b). Along with their postnatal maturation, sympathetic neurons gradually lose their dependency on NGF for survival (Angeletti et al. 1971). For example, up to 34% of sympathetic neurons remained viable, though reduced in size, when adult mice were treated with NGF antiserum (Levi-Montalcini and Booker 1960a; Kole et al. 2013).

Sympathetic neurons derive from neural crest and innervate various endo- and exocrine glands, cardiac muscle and smooth muscle throughout the body (Glebova and Ginty 2005). The superior cervical ganglion (SCG) contains a homogenous population of sympathetic neurons whose neuronal complement peaks at E18 and decreases thereafter as a consequence of naturally occurring neuronal death (Levi-Montalcini 1987). The PCD of rodent sympathetic neurons occurs during the first two weeks of their postnatal development coinciding with the time of their target tissue innervation (Wright et al. 1983; Kristiansen and Ham 2014). SCG neurons mature and maintain their dependency on NGF for survival *in vitro*, equivalently to their post-natal development *in vivo*. This feature allows the experimental manipulation of these cells in varying developmental stages, addressing also the regulation of neuronal apoptosis in response to various developmental signals and pathological insults (Martin et al. 1988; Deckwerth and Johnson 1993a; Edwards and Tolkovsky 1994). Thus, cultured SCG neurons provide a good *in vitro* model for studying unique features of neuronal apoptosis.

NGF deprivation induces the intrinsic (mitochondrial) pathway of apoptosis in cultured SCG neurons (Deckwerth et al. 1996; Deshmukh and Johnson 1997; Harris and Johnson 2001; Whitfield et al. 2001; Wright et al. 2007). The NGF deprived sympathetic neurons show apoptotic morphological changes, including neurite fragmentation, cell atrophy, membrane blebbing, chromatin condensation and loss of the structural integrity prior to their death by secondary necrosis (Deckwerth and Johnson 1993b; Pittman et al. 1993; Edwards and Tolkovsky 1994; Martin et al. 1998). Neuronal death in this model system requires *de novo* gene expression and can be delayed by applying RNA or protein synthesis inhibitors to culture medium (Deckwerth and Johnson 1993b; Martin et al. 1998). NGF- deprived rat sympathetic neurons show significant changes in overall gene transcription rates and signalling pathways (Kristiansen et al. 2011). Death of NGF-deprived neurons can be efficiently blocked by broad-range caspase inhibitors, indicating that NGF withdrawal-induced death of sympathetic neurons requires caspase activity (Deshmukh et al. 1996; McCarthy et al. 1997; Martinou 1999). Nearly 100% of early postnatal neurons (equivalent to P5-P7 *in vivo*) die over a period of 24-48h when NGF is removed from culture medium (Deckwerth and Johnson 1993b; Edwards and Tolkovsky 1994; Martin et al. 1998; Kristiansen and Ham 2014). Macromolecule synthesis inhibitors cannot rescue more than 50 % of neurons by ~16 h after NGF withdrawal (Martin et al. 1998). This time point is denoted as transcriptional commitment point in this cell death model. Most of the neurons can be rescued form death by resupplying NGF at this time point (Deckwerth and Johnson 1993a; Deshmukh et al. 1996; Martinou et al. 1999). Only 50% of the neurons can be rescued by ~22h after NGF withdrawal (Deckwerth and Johnson 1993b). This time-point is referred as a death commitment point for NGF-deprived sympathetic neurons (Deckwerth and Johnson 1993b; Kristiansen and Ham 2014). These properties demonstrate the dynamics of key events in NGF-deprivation induced sympathetic neuron death model.

The molecular events that occur following removal of NGF from cultured sympathetic neurons include the activation of protein kinase cascade, involving the mixed lineage kinase (MLK) and c-Jun N-terminal kinase (JNK) proteins (Whitfield et al.

2001; Towers et al. 2009; Kristiansen and Ham 2014). JNK is an important stress inducible kinase, which mediates the induction and/or activation of a number of pro-apoptotic proteins, including the leucine-zipper transcription factor c-Jun (Putcha et al. 2003; Ham et al. 2005). Activation of JNK/c-Jun pathway results in the increased expression and activation of the BH3 only proteins, such as Bim, DP5/Hrk, Puma and Bmf (Estus et al. 1994; Ham et al. 1995; Eilers et al. 1998; Harris and Johnson 2001; Putcha et al. 2001; Whitfield et al. 2001; Putcha et al. 2003). NGF withdrawal induced death response in cultured sympathetic neurons involves also the activation of cyclin-dependent kinases (Liu et al. 2004b) which lead to an E2F1-dependent upregulation of transcription factors B-myb and C-myb, resulting in increased Bim expression (Liu et al. 2004b; Biswas et al. 2005) and Bim can be induced also by FOXO3a (Gilley et al. 2003).

The BH3-only proteins Bim, Puma and Dp5 are particularly important in promoting MOMP after NGF withdrawal (Imaizumi et al. 1999; Putcha et al. 2001; Putcha et al. 2002). Other BH3 only members, such as Noxa, Bid and Bad are also expressed in sympathetic neurons, but they do not contribute to the NGF-deprivation induced cell death (Putcha et al. 2002).

Bax deletion in mice protects sympathetic neurons from NGF-deprivation induced apoptosis (Deckwerth et al. 1996; Miller et al. 1997; Deshmukh and Johnson 1998; Putcha et al. 2002), whereas *bak* deletion does not provide any protection form this death cue (Putcha et al. 2002). Bax suppression by small interfering RNAs protects cultured sympathetic neurons from apoptosis (Aalto et al. 2007). NGF deprivation in young neurons activates Bax translocation to mitochondria, Bax-dependent MOMP and cytochrome c release (Martin et al. 1988; Deckwerth et al. 1996; Eilers et al. 1998; Martinou et al. 1999; Putcha et al. 1999; Bruckner et al. 2001; Harding et al. 2001). In mature sympathetic neurons Bax activation is efficiently blocked, providing an effective survival mechanism to these neurons (Putcha et al. 2000; Kole et al. 2013).

Bax mediated cytochrome c release is necessary step for apoptosome formation and caspase-9 activation in mitochondrial apoptosis pathway (Meier and Vousden 2007). Apoptosis in sympathetic neurons, however, requires additional step, referred as "competence-to-die", to fully initiate caspase cascade (Deshmukh and Johnson 1998). This phenomena was discovered by cytoplasmic microinjection of functional cytochrome c to the NGF-maintained sympathetic neurons, which was, however insufficient to induce cell death in these cells (Deshmukh and Johnson 1998). In contrast, microinjection of cytochrome c to the NGF-deprived, *Bax*-deficient or NGF-deprived, cycloheximide-treated neurons induced a rapid caspase-dependent death (Deshmukh and Johnson 1998). It was later revealed that NGF deprivation induces competence by releasing Smac/Diablo from mitochondria, consequently relieving XIAP-mediated inhibition on caspases (Deshmukh et al. 2002; Potts et al. 2003). This mechanism likely protects neurons from accidental leakage of cytochrome c and caspase activation (Deshmukh and Johnson 1998; Wright et al. 2004; Potts et al. 2005).

Most of the cultured sympathetic neurons can be recovered from NGF deprivation by restoring NGF supply prior to loss of membrane potential (Deshmukh et al. 1996; Martinou et al. 1999), but also by microinjecting with a cytochrome c blocking antibody

(Neame et al. 1998). It therefore concludes, that the release of cytochrome c from mitochondria during apoptosis of NGF-deprived sympathetic neurons is a reversible event (Martinou et al. 1999). Conclusively, both, cytochrome c release and development of competence-to-die can be considered as parallel post-mitochondrial pathways that are cooperatively required for apoptosis in sympathetic neurons.

Once cytochrome c is released and XIAP is neutralized, apoptosis proceeds with the activation of initiator caspase-9. It leads to the cleavage and activation of caspase -3, which in turn cleaves a diverse group of cellular targets leading to controlled destruction of the cell. Caspase-3 activity is critical for sympathetic neuron apoptosis, as these cells do not express executioner caspase-7 (Wright et al. 2007). It has been shown that caspase-2 is also activated in dying sympathetic neurons and it may stimulate the expression of Bim through c-Jun (Jean et al. 2013), indicating that caspase-2 may function upstream of the mitochondrial pathway in sympathetic neurons. However, the role of caspse-2 in neuronal apoptosis is not well understood.

Analysis of sympathetic neurons with inactivating mutations in their *FasL* or *Fas* genes suggested that the Fas pathway does not contribute to NGF withdrawal–induced death (Putcha et al. 2002).

2.4.4 Apoptosis in pathological neuronal death

Pathological neuronal death in the adult nervous system occurs through more diversified pathways than during fetal and postnatal development. For example, apoptosis is only one of the mechanisms of neuronal death following stroke, traumatic brain injury or genotoxin exposure (Raghupathi et al. 2000; Engel et al. 2011; Hyman and Yuan 2012). Several pro-apoptotic proteins have been implicated during seizures, but their contribution to regulation of cell death might be contrary to expected (Fannjiang et al. 2003; Engel et al. 2010; Murphy et al. 2010; Engel et al. 2011; Moran et al. 2013).

The general feature of nervous system injury is that cell death pattern is spatially heterogeneous and depends on the severity of damage. The magnitude of neuronal damage also evolves in time, being first evident in the acute period of injury, but may take place over a period of days and weeks after the injury. The secondary lesions likely develop due to the toxic effect of accumulating excitatory amino acids, in particular glutamate which causes excessive Ca^{2+}-ion influx and cell death, followed by the release of endogenous glutamate that propagates the excitotoxic effect on neurons (Pettmann and Henderson 1998).

Cell death in the damage core is predominantly necrotic, but it may involve morphological and biochemical features of apoptosis, particularly in the early phase of neuronal injury (Raghupathi et al. 2000; Benchoua et al. 2001). For example, in early stages of cerebral infarction may involve cytoplasmic and nuclear condensations and caspase-8 and the caspase-1 activation (Benchoua et al. 2001). The areas that are distant from the site of damage, also referred as penumbra, are usually less affected. Cell death in penumbra often displays apoptotic morphologies and involves mitochondrial apoptosis pathway (Conti et al. 1998; Raghupathi et al. 2000; Benchoua et al. 2001).

Pharmacological inhibition of caspases in mice prior to stroke or traumatic brain injury show reduced cell death and decreased pathological symptoms (Hara et al. 1997; Yakovlev et al. 1997). Whether these effects result in functional recovery of neurons is still matter of great debate. These observations, however, imply that apoptosis is a distinct component of neuronal cell death following nervous system injuries.

The tendency to undergo apoptotic cell death may decrease as neurons mature (Liu et al. 2004a; Wei et al. 2004). Caspase-dependent and independent mechanisms of neuronal cell death may depend on neuronal cell type or brain region involved. This is not surprising, since apoptotic program in mature neurons requires re-activation and may fail in several steps (Benn and Woolf 2004; Wright and Deshmukh 2006; Kole et al. 2013). The intensity of the insult and its effect on the status of cell energy levels likely determines the efficiency of apoptotic program re-initiation (Frazzini et al. 2006). For example, apoptosis pathway can be initiated in acute phase of damage, but the final morphological features likely result from aborted apoptotic process. In cerebral ischemia, apoptosis can fully develop in penumbra due to the residual blood supply, whereas neuronal cell death in the damage core suffers from energy depletion, resulting in alternative cell death mechanisms that can be characterized by necrotic morphology (Rink et al. 1995; Raghupathi et al. 2000; Benchoua et al. 2001; Yuan 2009). The regional and temporal patterns of apoptotic and necrotic cell death have been also observed following traumatic brain injury (Rink et al. 1995; Raghupathi et al. 2000).

Apoptosis contributes to pathogenesis of neurodegenerative diseases, such as, Alzheimer's and Parkinson's disease, amylotrophic lateral sclerosis and many polyglutamine diseases, including Huntington's disease (Yuan and Yankner 2000; Vila and Przedborski 2003). The molecular basis of these diseases likely involves environmental and genetic components, but is still poorly characterized (Culmsee and Landshamer 2006). One of the common pathological characteristics, shared in neurodegenerative disorders, includes an accumulation of misfolded proteins, aggregated peptides or aberrant ribonucleoprotein complexes within or around the neurons (Matus et al. 2011; Heneka et al. 2014), eventually causing death of the cells (Mattson 2000; Bredesen 2008). Misfolded proteins could also trigger neuroinflammation and thereby contribute to the disease progression (Heneka et al. 2014).

While considerable progress has been made in understanding the neuronal cell death mechanisms in various cell culture models, it is less clear to what extent apoptosis drives neurodegenerative pathologies *in vivo* (Jellinger 2006). Targeting apoptosis upstream of the execution phase results in marked attenuation of neurodegeneration, whereas interfering, for example, with caspase activation produce variable results.

Some authors suggest that loss or reversal of the mechanisms that restrict apoptosis in mature neurons contribute significantly to the initiation and acceleration of the chronic neurodegenerative diseases (Benn and Woolf 2004; Kole et al. 2013). Indeed, some apoptotic brakes that are acquired during neuronal maturation are found to be decreased or lost in several neuropathological conditions, including Alzheimer's and

Parkinson's disease, amylotrophic lateral sclerosis (Kole et al. 2013). At present these are only correlative observations and require more detailed studies to understand whether and how the loss of these mechanisms increases the vulnerability of mature neuron degeneration (Kole et al. 2013). Nevertheless, existing knowledge highlights that targeting apoptosis related pathways at multiple levels and in early phase of the disease, would likely provide beneficial outcomes.

2.4.5 Bax and Bak in neuronal apoptosis

Neither Bak nor Bax deficiency cause no overt neurodevelopmental defect in mice (Lindsten et al. 2000), but several studies have investigated neuronal cell death properties in Bax or Bak deficient mice in more detail (Deckwerth et al. 1996; Miller et al. 1997; Fannjiang et al. 2003; Lindsten et al. 2003; Sun and Oppenheim 2003). These studies have revealed that *bax* deletion has profound effect on the survival of many kinds of neurons whereas deletion of *bak* does not confer any protection to most of these stimuli (White et al. 1998; Lindsten et al. 2000; Fan et al. 2001; Putcha et al. 2002). Bak, however, co-operates with Bax, to limit the size of NPC pool in the brain. It is evident in the Bak and Bax double-deficient mice that die perinatally with severe defects in craniofacial and neuronal development (Lindsten et al. 2000). Few surviving mice show accumulations of NPCs and immature neuroblasts in the periventricular, hippocampal, cerebellar and olfactory bulb regions of the brain (Lindsten et al. 2000; Lindsten et al. 2003; Kim et al. 2007; Kim et al. 2011a). This phenotype is mild or undetectable in Bak or Bax single deficient mice (Lindsten et al. 2000), indicating that Bax and Bak function redundantly to affect NPC survival *in vivo* (Lindsten et al. 2003; Akhtar et al. 2004). Both NPCs and mature neurons derived from Bak and Bax double-deficient mice are resistant to various apoptotic stimuli (Lindsten et al. 2003), but remain sensitive to excitotoxic death signals, indicating that Bak and Bax may not be required for this type of cell death (Lindsten et al. 2003). Bak and Bax double deficient mice are also deaf and exhibit more sensitivity to spontaneous and stress-induced seizures (Lindsten et al. 2000; Lindsten et al. 2003).

Accumulating evidence suggests that Bax and Bak redundancy might be eliminated in post-mitotic neurons (Wright and Deshmukh 2006; Kole et al. 2013).
Sole Bax deficiency appears to be sufficient to block developmental PCD in several neuronal populations *in vivo* and protect neurons from various insults (White et al. 1998). In Bax deficient mice the developmental cell death is virtually eliminated in most peripheral ganglia, including in the dorsal root ganglia (DRG), sympathetic and cochleovestibular ganglia during mid-embryonic and early postnatal development (Deckwerth et al. 1996; White et al. 1998). These ganglia contain neurons that require different neurotrophin family members for survival, indicating that Bax activation is critical for neuronal apoptosis induced by neurotrophic factor deprivation during critical period of PCD (Deckwerth et al. 1996; Miller et al. 1997; Deshmukh and Johnson 1998). Consistent with that, cultured motoneurons, young sympathetic and DRG neurons from Bax mutant mice can live indefinitely in the absence of exogenous neurotrophic factors *in*

vitro (Deckwerth et al. 1996; Gillardon et al. 1996; Bar-Peled et al. 1999), whereas Bax overexpression induces apoptosis in the NGF-maintained cultured sympathetic neurons and accelerates the rate of apoptosis after NGF withdrawal (Vekrellis et al. 1997; Martinou et al. 1998).

Genetic deletion of *bax* in mice alters overall neuron number in certain sexually dimorphic nuclei of the forebrain, thus abolishing sex differences in the brain (Forger et al. 2004). Apoptosis is also markedly reduced or eliminated in the spinal cord and brainstem motoneurons, the retina, and some areas of the cerebellum and hippocampus in the Bax deficient mice (Deckwerth et al. 1996; White et al. 1998). Bax deletion in mice rescues the excessive apoptosis that occurs in developing nervous system of Bcl-xL-deficient mice (Shindler et al. 1997).

In addition to developmental death cues, Bax deficiency is shown to protect neurons from apoptosis that is induced by injuries or noxious signals (Li and Yuan 2011). In Bax deficient mice, facial motor neurons survive dissociation from their targets by axotomy *in vivo*, although survived neurons are markedly atrophic compared with contralateral motoneurons (Deckwerth et al. 1996). *Bax* null mutants show also resistance to ischemia-induced neuronal loss in the hippocampus (Gibson et al. 2001) and reduced cell death upon sciatic nerve injury (Martin and Liu 2002). Bax deficiency is sufficient to reduce striatal cell death after excitotoxic injury (Perez-Navarro et al. 2005), prevent dopaminergic neuron death in the MPTP (1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine) mouse model of Parkinson disease (Vila et al. 2001) and protect neurons from ethanol-induced apoptosis (Young et al. 2003). Cultured cerebellar granule neurons from Bax deficient mice are not sensitive to potassium deprivation (Miller et al. 1997) and cortical cells to glutamate induced exitotoxicity (Xiang et al. 1998).

Bax deficient mice show less dramatic reduction in apoptosis in some areas of the cerebellum and hippocampus, where specific defects may become visible in postnatal life (Deckwerth et al. 1996; White et al. 1998; Fan et al. 2001). It has been shown that Bax inactivation disrupts apoptosis of cerebellar granule neuron progenitors (CGNPs), which may result in CGNPs transformation and formation of medulloblastoma in postnatal period (Garcia et al. 2013). Bax-dependent apoptosis regulates also adult neurogenesis in the dentate gyrus (DG) of the hippocampus and in the subventricular zone (SVZ) on the lateral walls of the lateral ventricle (Petreanu and Alvarez-Buylla 2002; Sun et al. 2004; Kim et al. 2007; Kim et al. 2011a; Kim and Sun 2011). Here, Bax deficiency results in a progressive accumulation of newly produced neurons in the adult hippocampus, severely impairing synaptic plasticity and associative memory (Sun et al. 2004; Kim et al. 2009). Excess neurons in the SVZ, however, cause only limited consequences to the olfactory function in Bax deficient adult mice brain (Kim et al. 2007)

Some developing neuronal subpopulations in the mouse dentate gyrus, cerebellum and most neurons in the striatum and cortex seem to remain unaffected by Bax deficiency (White et al. 1998; Fan et al. 2001; Gibson et al. 2001; Jung et al. 2008; Kim et al. 2009), indicating that Bax is not an obligate participant in PCD in these cell populations (Fan et al. 2001). As Bax and Bak have redundant roles in regulating cell death in mitotic cells, the same phenomenon has been suggested to occur during the

development of these neuronal populations (Gavalda et al. 2008). It is, howeve, not thoroughly studied whether Bak or other Bcl-2 family members could compensate for Bax deficiency in neurons *in vivo*. Moreover, there is no unequivocal evidence that full-length Bak is expressed in post-mitotic neurons. Instead, there is evidence that many types of neurons exclusively express N-Bak, which is a neuron-specific splice variant of Bak (Sun et al. 2001; Uo et al. 2005) (see below). N-Bak mRNA has capacity to encode a protein that lacks the characteristic multi-domain structure of Bak (Sun et al. 2001). It thus appears that Bax may serve as the only multidomain pro-apoptotic Bcl-2 family member available in the neurons. These data provide one explanation to the results demonstrating the central role of Bax in mediating apoptosis in post-mitotic neurons (Deckwerth et al. 1996; Miller et al. 1997; White et al. 1998; Bar-Peled et al. 1999; Cregan et al. 1999). It also supports the views that other PCD mechanisms or even non-death-related processes may be involved to allow functionally optimal cell numbers and connectivity in the Bax-deficient brain (Hotchkiss et al. 2009; Kim and Sun 2011). The potential compensatory mechanisms have remained however poorly characterized. Alternatively, several brain areas may express only Bax, whereas some may express Bax and full-length Bak together. However, most neurons in both PNS and CNS however show little or no Bak immunoreactivity (Krajewski et al. 1996; Krajewska et al. 2002). Previous observations also indicate that the functional redundancy or compensation among pro-apoptotic Bcl-2 family members is not guaranteed even then they seem to be co-expressed in neuron (Putcha et al. 2002; Engel et al. 2011).

Several studies have investigated the fate of supernumerary neurons in Bax deficient mice further and found that excess neurons often fail to integrate to the neural network and undergo atrophy (White et al. 1998; Kim et al. 2011b; Kim and Sun 2011). For instance, Bax deficient sensory and motor neurons fail to execute apoptosis during development, but the motor and sensory functions are virtually normal in Bax deficient mice (Sun and Oppenheim 2003). The extra motoneurons that survive developmental PCD are progressively excluded from the circuit and they fail to grow and elongate axons (Sun and Oppenheim 2003; Kim and Sun 2011). Consequently, there are similar numbers of functionally significant motoneurons in wild-type and Bax deficient mice. Other studies have found that Bax deficient mice show impaired neuronal migration of surplus neuroblasts from the SVZ to the olfactory bulb, thus compensating the failure of PCD in the adult brain SVZ (Kim et al. 2007). The removal of neurons in development may involve also the death-receptor mediated pathway or non-apoptotic pathways (Clarke 1990; Kuan et al. 2000b; Roth et al. 2000a; Yu et al. 2003b; Yuan et al. 2003; Yu et al. 2008; Hotchkiss et al. 2009). Hence multiple mechanisms may compensate failed apoptosis in the nervous system (Kim and Sun 2011) further indicating that Bax and Bak redundancy might be eliminated in post-mitotic neurons (Wright and Deshmukh 2006).

Consistent with its role in regulation of neuron cell death in CNS and PNS development, Bax is expressed in various parts of the nervous system, particularly during developmental period when post-mitotic neurons undergo PCD (Krajewski et al. 1994; White et al. 1998). In the adult brain Bax expression is significantly decreased, remaining high only in few neuronal populations (Vekrellis et al. 1997; Polster et al. 2003). Bax

expression is often upregulated in response to injury and in pathological conditions associated with the neurodegenerative diseases (Raghupathi et al. 2003; Vila and Przedborski 2003).

There is also evidence that Bax can function as pro-survival factor in certain subtypes of developing neurons as well as under pathological situations. Middleton and Davies (1996) found that overexpressed murine Bax rescues chicken sensory and parasympathetic neurons in culture form trophic factor deprivation-induced apoptosis (Middleton et al. 1996). However, Bax effect on neuronal survival was transient or less effective than Bcl-2 or Bcl-xL (Middleton et al. 1996). Further studies suggested that Bax could have protective function also in mouse trigeminal ganglion neurons *in vivo* (Middleton and Davies 2001).

Infection of CNS with Sindbis alphavirus (SV), which causes acute encephalomyelitis and mortality in young mice, induces diverse cell death pathways in different neuronal populations, resulting in apoptosis but also excitotoxic cell death, that could affect also bystander cells (Levine 2002; Irusta and Hardwick 2004). Lewis and co-workers have demonstrated that young *Bax* deficient mice are significantly more susceptible to fatal SV infection than their wild-type littermates (Lewis et al. 1999), suggesting that endogenous Bax may protect mice from SV-infection-induced mortality (Lewis et al. 1999). Overexpression of Bax in SV infected wt or Bax deficient mice, rescues these mice from lethal infection (Lewis et al. 1999). The protective effects of Bax could be reproduced also in hippocampal slice cultures but not in dissociated DRG neuronal cultures (Lewis et al. 1999), indicating that Bax-mediated inhibition of SV-induced cell death is highly dependent on neuronal subtype. Although intriguing, the mechanism by which Bax may promote survival in some neuronal populations is not clear.

Mice lacking Bak appear developmentally normal (Lindsten et al. 2000). Bak deficient neurons exhibit no protection from major developmental death stimuli (Deckwerth et al. 1996; White et al. 1998; Putcha et al. 2002), supporting the notion that Bax is the main Bcl-2 family pro-apoptotic regulator during neuronal development. Overexpression of Bak, however, can accelerate trophic factor withdrawal-induced apoptosis in sympathetic neurons (Farrow et al. 1995; Kiefer et al. 1995; Sun et al. 2001), showing that Bak may modulate cell death pathways in neurons.

Analysis of cell death properties in Bak null mutant mice using a variety of assays related to pathological neuronal death suggests that Bak may have either pro-or antiapoptotic functions in the neurons (Lamb and Hardwick 2010). For instance, Bak deficient mice are resistant to dopaminergic neurodegeneration induced by the neurotoxic agricultural pesticide paraquat (PQ), but are significantly more vulnerable to MPTP toxicity than wild-type mice, indicating that endogenous Bak could mediate both, dopaminergic neuron death and survival *in vivo* depending on the death stimulus (Fannjiang et al. 2003; Fei et al. 2008). However, direct biochemical evidence for Bak expression and activity in the dopaminergic neurons is lacking. Consistent with its expected pro-apoptotic function, Bak has been suggested to promote cell death in the stroke/ischemic brain injury model, affecting survival of the cells in adult mouse striatum and cortex (Fannjiang et al. 2003).

Juvenile Bak deficient mice are shown to be more vulnerable to SV infection mediated mortality than their wild-type littermates, but this phenotype is reversed in more mature animals (Fannjiang et al. 2003). Targeted overexpression of Bak in the nervous tissue of young *bak* null mice reduces the SV infection-induced mortality rates, but increases cell death in older mice (Fannjiang et al. 2003). These results suggest that Bak function in apoptosis regulation may change during neuronal maturation. Hippocampal organotypic cultures form Bak deficient mice are, irrespective of age, significantly more sensitive to SV infection-induced cell death compared to the control cultures, whilst Bak overexpression rescues this phenotype (Fannjiang et al. 2003). Age-dependent differences are evident in spinal cord slice cultures, where Bak overexpression only partially rescues the phenotype (Fannjiang et al. 2003), indicating that Bak function may be also cell-type specific.

Treatment of rodents with potent neuroexcitatory amino acid kainate at high doses induces limbic motor seizures and subsequent excitotoxic cell death in the hippocampus (Fannjiang et al. 2003). Injection of Bak deficient mice with kainate results in a greater hippocampal neuron loss than in control mice and similar results have been obtained from kainite treated hippocampal slice cultures (Fannjiang et al. 2003). Restoration of Bak expression in Bak deficient hippocampal slice cultures protects neurons from kainate-induced cell death but also from seizures, suggesting that Bak may modulate both neuronal excitability and cell death pathways in the brain (Fannjiang et al. 2003).

Indeed, it appears that Bak null mice have more severe seizures than their heterozygous or wildtype littermates following kainate treatment (Fannjiang et al. 2003). The seizure phenotype occurs already within minutes of treatment in Bak null mutant mice. That is days before any neuronal death becomes detectable in the brain tissue, suggesting that Bak deficiency alters the intrinsic excitability of neurons in such a way that it gives rise to seizure. Electrophysiological studies have confirmed alterations in spontaneous and evoked activities in acute brain slices prepared from Bak deficient mice (Fannjiang et al. 2003). It has been therefore suggested that the brains of Bak-deficient mice might be somehow differently wired compared to normal mice (Fannjiang et al. 2003; Lamb and Hardwick 2010). Whether this effect is caused by deletion of Bak during the brain development or represents its neurophysiologic function in the brain is currently unknown. Similar questions arise when interpreting neurophysiological phenotypes of other Bcl-2 family members (Henshall and Engel 2013). It is also not well understood to what extent such neurological phenotypes may reflect the dysfunction of non-neuronal cells.

Immunohistochemical analysis in human autopsied CNS and PNS tissue samples have found only little or no Bak immunostaining in the CNS, whereas sympathetic and DRG neurons and their axons were immunopositive for Bak (Krajewski et al. 1996). Occasional CNS neurons with morphological evidence of ischemic degeneration show increased levels of Bak immunoreactivity which may represent post-mortem change (Krajewski et al. 1999). Bak immunoreactivity during murine nervous system development is low or restricted focally to some brain areas or group of cells, but the exact cell types showing Bak immunoreactivity remains indistinguishable (Krajewska et

al. 2002). Similar studies have not been performed in Bak null mutant mice, leaving the specificity of these staining results open. As already discussed, full-length Bak is proposed to be expressed only in non-neuronal cells in the nervous system as its exclusively expressed splice variant N-Bak replaces it in several types of differentiated neurons (Sun et al. 2001; Uo et al. 2005). Hence, several of the abovementioned suggestions about Bak function and expression require re-evaluation to elucidate role of *bak* gene in neuronal apotosis.

Altogether, these data indicate that the role of *bax* and *bak* in neuronal apoptosis may be developmental stage, neuronal subtype and stimulus-specific. It also appears that connectivity of neurons in the experimental setting can significantly influence the function of these two proteins following the insults (Lamb and Hardwick 2010), as dissociated and plated hippocampal neurons are highly susceptible to Bax or Bak-overexpression induced death, but exhibit improved survival in the culture conditions where neuronal connections have been preserved (Lewis et al. 1999; Fannjiang et al. 2003; Lamb and Hardwick 2010). Whether unusual antiapoptotic effects of Bak and Bak are related to some alternative functions in the cell remains to be assessed. More comprehensive analysis is required to clarify the key molecular mechanisms that help to explain pro-or anti-apoptotic effects of Bak and Bax in natural and pathological neuronal death.

2.4.6 Neuronal Bak

Sun and colleagues (2001) found that Bak pre-mRNA is subjected to alternative splicing in postmitotic neurons, resulting in a neuron-specific variant of Bak, termed N-Bak (Sun et al. 2001).

N-Bak mRNA expression is detectable already in eary developing nervous tissue in mouse peaks at perinatal stage, thereafter it persists at least until adulthood (Sun et al. 2001). In adult mouse brain, N-Bak was found to be expressed in all brain regions and similar results were found in human samples (Sun et al. 2001).

N-Bak mRNA has been shown to express in a variety of neuronal subtypes, including in the cultured sympathetic, cortical, hippocampal and cerebellar granule neurons (Sun et al. 2001; Uo et al. 2005). In purified neuronal and glial cell cultures, it was convincingly demonstrated that N-Bak is expressed exclusively in neurons, whereas Bak is expressed only in non-neuronal cells (Sun et al. 2001). Whether N-Bak is expressed in all types of neurons is currently unknown. Mutually exclusive expression patterns of N-Bak and Bak in neurons and glia however imply that N-Bak expression in brain is likely restricted to neurons, whilst Bak expression in brain likely originates from glial cells (Sun et al. 2001).

The neuron-specific splicing of Bak pre-mRNA involves the inclusion of novel 20 nucleotide long cassette exon (exon N) (Sun et al. 2001). Consequently, the coding sequence of N-Bak is prematurely terminated, resulting in capacity to encode only a BH3-only variant of Bak. Putative N-Bak protein, however, contains a novel C-terminal

sequence that has no homology with any known proteins (Sun et al. 2001). The unique C-terminal stretch was shown to direct overexpressed N-Bak protein into intracellular membranes (Sun et al. 2001; Sun et al. 2003).

Overexpression of N-Bak was found to attenuate apoptosis in NGF deprived sympathetic neurons *in vitro* but to promote apoptosis in non-neuronal cells (Sun et al. 2001; Sun et al. 2003). The molecular mechanism of N-Bak-mediated delay in NGF-deprived sympathetic neuron death is not resolved. Mutagenesis analysis has revealed that both, the C-terminal membrane binding domain and functional BH3 domain were important for its "neuroprotective" function in *in vitro* studies (Sun et al. 2003). Another study has shown that overexpression of N-Bak results in a Bax dependent apoptosis in cortical neurons, which is consistent to general models for BH3-only protein activity in cells (Uo et al. 2005). It was also demonstrated that overexpressed N-Bak could interact with Bcl-xL, thus functioning as a sensitizing BH3-only protein (Uo et al. 2005). Nevertheless, anti-apoptotic properties of overexpressed N-Bak protein in neurons challenge the conventional models explaining the BH3-only protein function in the regulation of multidomain Bcl-2 family members.

However, the observations that BH3-only proteins might act in anti-apoptotic manner have been reported. Moran et al., (2013) demonstrated that classical BH3-only protein Bmf may protect neurons against seizure-induced neuronal death *in vivo* (Moran et al. 2013). Other studies have shown that BH3-only protein Bad exerts pro-survival functions in the cell prior to activation of its pro-apoptotic function (Datta et al. 1997; Condorelli et al. 2001; Seo et al. 2004). These studies also highlight the possibility that the functions, previously attributed to Bak in postmitotic neurons (Fannjiang et al. 2003) may, in part, reflect N-Bak function in post-mitotic neurons.

Currently there is a significant controversy in detecting the expression of endogenous N-Bak protein in neurons. The expression of N-Bak protein has been detected in cultured cortical neurons, where it has been also shown to be upregulated to contribute to the DNA-damage induced cell death in a Bax dependent manner (Uo et al. 2005). The presence of endogenous N-Bak protein in these studies was demonstrated by immunoblot analysis. Others have used immunohistochemical approach with Bak-specific antisera and found that most CNS neurons and glial cells lack detectable Bak immunoreactivity, but occasional neurons or cell groups in the mouse brain show detectable Bak signal (Krajewski et al. 1999; Krajewska et al. 2002). The specificity of these results needs to be confirmed on Bak null mutant tissue samples. Moreover, N-Bak and Bak share the N-terminal epitope, where the recognition site of used Bak-specific antibodies has been mapped, thus further complicating the conclusions of N-Bak protein expression in neurons. The majority of the studies have not found evidence for endogenous or induced expression of N-Bak protein in post-mitotic neurons (Sun et al. 2001; Putcha et al. 2002; Sun et al. 2003; Kole et al. 2011b). It indicates that N-Bak protein may not be constitutively expressed in neurons.

Discordant expression levels of N-Bak mRNA and protein in neurons suggest post-transcriptional regulation. Recently Kole et al. (2011b) discovered that in mature cortical neurons the expression of several BH3-only proteins is down-regulated by post-

transcriptional regulatory mechanism involving a miRNA mediated translational inhibition of respective mRNA transcripts. Among others, N-Bak mRNA was shown to be supressed by miRNA (Kole et al. 2011b). The molecular regulation of N-Bak mRNA translation in young cortical neurons has been not addressed. Whether N-Bak protein expression is regulated by additional post-transcriptional mechanisms remains to be studied and is the subject of current thesis.

2.5 Insights to post-transcriptional gene expression regulation

This part of the review provides insights into the selected post-transcriptional mechanisms that control mRNA stability, localization and translation in mammalian cells. Current mechanistic concepts are only briefly discussed.

The activity of a protein-coding gene depends on combined actions of cellular machinery that execute and regulate transcription, translation but also mRNA and protein decay. These fundamental processes are interconnected in the cell (Maniatis and Reed 2002), but can be dissected into the layers to better explain the multistep nature of gene expression regulation (Bentley 2014). Post-transcriptional gene expression control refers to the mechanisms that regulate mRNA life cycle from synthesis to decay (Figure 10). Such mechanisms influence mRNA processing, modification and packaging in the nucleus, but also its transport into the cytoplasm, where mRNA stability, localization and association with the ribosomes can be further controlled. All these steps are regulated with high spatiotemporal precision and ultimately determine which mRNAs are translated into a protein.

Key factors mediating mRNA post-transcriptional regulation are diverse groups of RNA-binding proteins (RBPs) and non-coding RNAs that bind to characteristic *cis*-acting sequences or structural elements in the target mRNA. The best characterized class of non-coding RNAs are micro RNAs (miRNAs), that serve as negative regulators of gene expression in a variety of cellular systems (reviewed in Kloosterman and Plasterk 2006; Valencia-Sanchez et al. 2006; Kiebler et al. 2013) (Figure 10).

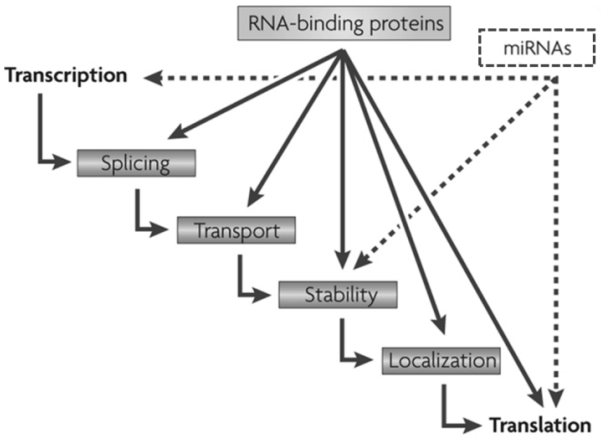


Figure 10. Post-transcriptional control involves mRNA regulation by RNA-binding proteins and microRNAs. In eukaryotic cells, RNA-binding proteins and small non-coding RNAs influence critical steps in mRNA life- cycle and are therefore regarded as key regulators of mRNA post-transcriptional regulation**.** miRNAs, microRNAs. Adapted from Keene (2007)

Functional mRNA is generated from primary transcript (pre-mRNA) through multiple processing steps, which include 5'-cap methylation, splicing and 3´-end polyadenylation. The splicing reaction occurs in the large macromolecular complex, a spliceosome that is composed of five small nuclear ribonucleoprotein particles and many auxiliary proteins. All these factors are required for sequential transesterification reactions, which excise the introns and join the exons in precise order, resulting in mature mRNA formation. Also, subsets of mRNAs are further modified through methylation or editing to ensure their proper expression and regulation in the cell (Licht and Jantsch 2016).

Mature mRNA consists of an open reading frame (ORF), which is flanked by the untranslated regions (UTRs) at the 5' and 3' ends. Both UTRs, but also an ORF region influence the mRNA stability, localization, and translation in the cell. In addition, cap structure at the 5′ end and poly (A) tail at the 3′ end of an mRNA are crucial in many steps of mRNA life-cycle regulation.

The best characterized regulatory elements found within the mRNA 5´-UTRs are upstream open reading frames (uORFs), upstream initiation codons (uAUGs) (Barbosa et al. 2013) and higher order structures, such as internal ribosome entry sites (IRES) (Pickering and Willis 2005) and G-quadruplexes (Bugaut and Balasubramanian 2012). mRNA 3´-UTR may contain alternative polyadenylation elements, AU-, GU-, U-rich sequences, different mRNA localization zipcodes or distinct binding sites for other regulatory factors, including miRNAs (Lee and Gorospe 2011).

mRNA can also be generated alternatively by joining the different combinations of exons together in a process called alternative splicing (Black 2003). Other mechanisms that generate alternative transcript isoforms include the usage of alternative transcription start sites or polyadenylation sites, respectively extending or shortening mRNA 5´-and 3´ends (Chen 2015). Importantly, pre-mRNA alternative processing may change the posttranscriptional regulation of resulting mRNA in the cell (Hamid and Makeyev 2014; Chen 2015).

It is estimated that in human cells, up to 95% of the intron-containing genes are alternatively spliced, resulting in the generation of multiple, distinct mRNA isoforms

from a single gene (Pan et al. 2008; Hamid and Makeyev 2014; de Klerk and t Hoen 2015). Alternative splicing may affect mRNA translational efficiency and protein expression levels by introducing or deleting upstream initiation codons or open reading frames in the 5'-UTRs and microRNA targeting sites at the 3'-UTRs (Chen 2015) (Figure 11). It can also introduce a premature termination codon (PTC) to the mRNA coding sequence, hence targeting that transcript for degradation by nonsense-mediated mRNA decay (NMD) (Lewis et al. 2003; Lejeune and Maquat 2005) (Figure 11). Furthermore, correlative transcriptome and proteome analyses indicate that not all alternatively spliced transcripts produce functional proteins. Therefore, it has remained challenging to dissect the functional consequences of alternative splicing in the mRNA metabolism. Global transcriptome analyses have revealed that neurons have the highest frequency of alternative splicing events compared to other cell types. This feature is probably required to maintain their highly specialized structures and functions (Black and Grabowski 2003; Licatalosi and Darnell 2010).

Regulation of mRNA stability, localization and translational status is crucial to control a variety of cellular processes. Therefore, even subtle disturbances in mRNA metabolism accompany many human diseases.

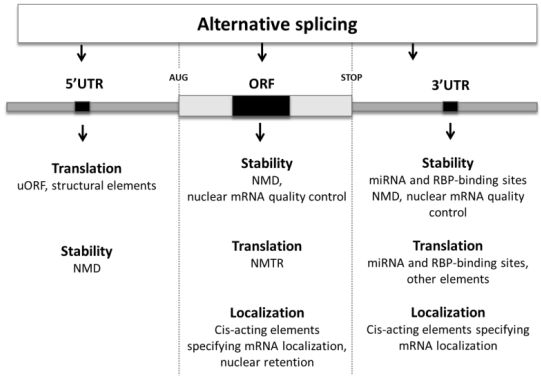


Figure 11. The impact of pre-mRNA alternative splicing on mRNA stability, translatability and sub-cellular localization. Schematic representation of mature mRNA, containing an open reading frame (ORF) from initiator codon AUG to the STOP-codon, and flanking untranslated regions (UTRs), respectively at the 5'-and 3' ends. Black boxes in outlined mRNA regions denote alternative splicing generated changes, which may cause different outcomes at posttranscriptional level. uORF, upstream open reading frames; NMD, nonsense-mediated decay; NMTR, nonsense-mediated translational repression. RBP, RNA-binding protein. Adapted from Mockenhaupt and Makeyev (2015).

2.5.1 Principles of miRNA regulation

While RBPs are essential factors in mRNA metabolism, it is only recent years when the better characterization of various groups of non-coding RNAs has started to highlight an extra layer of complexity in the gene expression regulation (Fatica and Bozzoni 2014). MicroRNAs (miRNAs) are the class of short (~20 nucleotides long) non-coding RNA molecules that alter gene expression at post-transcriptional level. miRNAs are initially

synthesized as long primary transcripts (pri-miRNAs) that mature through extensive processing steps in the cell nucleus and cytoplasm. Mature miRNAs are generally incorporated into the RNP complexes, also referred as miRNA-containing RNA-induced silencing complex (miRISC), that contain a member of Argonaute ribonuclease family protein (AGO1–4 in mice and humans) as well as other protein factors (Huntzinger and Izaurralde 2011) that are critical for their biological functions. An essential feature that directs miRISC silencing complex to the target mRNA is the miRNA ability to bind with full or partial complementarity to the mRNA molecule. mRISC binding to the target mRNA results in either target mRNA translational repression and degradation depending on the Argonaute protein function. Some studies report miRNA-mediated translational increase, during specific cellular states (Vasudevan et al. 2007; Saraiya et al. 2013). While the mechanisms that mediate such biological functions are poorly understood, it is accepted that miRNAs can act cooperatively on the target mRNAs. Their cooperation with the RBPs has also been suggested. Altogether, it is estimated that human genome contains more than 1000 miRNAs, although this number may be larger (Broderick and Zamore 2011). Current miRNA prediction algorithms suggest that miRNA binding sites are located on the mRNA 3´-UTR. However, other parts of an mRNA, although less frequently, can be targeted as well. Bioinformatic predictions and ample experimental data suggest that single miRNA may have multiple target mRNAs, and a single mRNA may have multiple miRNA binding sites within the transcript (Bartel 2004; Rajewsky and Socci 2004; Lewis et al. 2005; Baek et al. 2008; Selbach et al. 2008; Friedman et al. 2009; Biggar and Storey 2015). Therefore, aberrant regulation of miRNAs can have a profound effect on the expression patterns of several hundred mRNAs. miRNA-mediated regulation has a significant impact on the key biological processes, including cell proliferation, differentiation and apoptosis (Reddy 2015) and can be thus regarded as a major component of gene expression regulation.

2.5.2 RNA binding proteins in post-transcriptional regulation

RNA-binding proteins (RBPs) are recognized as central regulators of mRNA metabolism (Glisovic et al. 2008). In mammals, more than 2500 RBPs are estimated to interact with RNA (Moore 2005; Hogan et al. 2008; Castello et al. 2012). Of those, nearly 1300 have been experimentally proven to bind to RNA in human cells (Cook et al. 2011).

There are classes of general mRNA-binding proteins, such as poly (A)-binding proteins, serine-arginine rich (SR) proteins and heterogenous nuclear ribonucleoproteins (hnRNPs) that interact with almost all mRNAs, and more specific RBPs that recognize only certain elements in a subset of mRNAs.

Most characteristic binding sites for RBPs are the short, single-stranded RNA sequences or structured RNA elements (Ellis et al. 2007; Ray et al. 2009). Sequence-specific associations between RBPs and their RNA targets are typically mediated by one or more RNA-binding domains (RBDs), of those RNA recognition motif (RRM) and hnRNP K-homology (KH) domains are prevailing (Cook et al. 2011; Ray et al. 2013). Approximately 30% of RBPs contain also intrinsically disordered regions, and a

significant fraction of all RBPs carry short linear motifs or low complexity sequence elements that are crucial for their reversible aggregation into dynamic RNA granules (Reijns et al. 2008). It has been suggested that sequence disorder is a key feature for flexible regulation of RBP networking activity (Calabretta and Richard 2015). Some other RBDs, however, are still poorly characterized. Therefore, the information of their distribution across the RBP subclasses is incomplete.

In principle, one specific RBP can bind hundreds of different mRNAs (Darnell 2013) and analogously to the transcription factors, can RBPs act in a cooperative or competitive manner (Ray et al. 2013). RBPs may also compete or cooperate with other RNA-binding factors such as miRNA to regulate the expression of target genes (Abdelmoshen 2012). It is thought that mRNAs encoding functionally related proteins are co-regulated by a common set of RNA-binding proteins (Keene 2007; Hansen et al. 2015). Recent advances in RNA interactome characterization, particularly RBP cross-linking and immunoprecipitation approach combined with transcriptome-wide high-throughput sequencing has enabled elucidation of the core binding sites for several individual RBPs. However, due to the varied sequence preferences of RBPs and highly dynamic nature of RNP complexes, it is still challenging to define all the components that modulate particular mRNA life-cycle in the cell.

The mRNA is decorated with various classes of RBPs throughout its life-cycle. Protein complexes, deposited onto mRNA during the splicing of pre-mRNA facilitate its export to the cytoplasm. Furthermore, mRNA "nuclear processing history" has also key importance in determining mRNA fate through a variety of surveillance pathways. Cytoplasmic mRNA and protein assemblages are termed as messenger ribonucleoprotein particles (mRNPs) (Keene 2010). Those structures are constantly remodeled and modified depending on the physiological context of the cell (Huttelmaier et al. 2005; Fu and Ares 2014).

The best-characterized RBP that has specialized functions in regulating mRNA stability or translational activity belong to the large group of heterogeneous nuclear ribonucleoprotein (hnRNP) and Hu protein families (Abdelmoshen 2012). Several other mRNA-binding proteins, including the T-cell intracellular antigen 1 (TIA-1), TIA-1-related (TIAR), tristetraprolin (TTP), polypyrimidine tract-binding protein (PTB) are involved in regulation of mRNAs stability, but also translational dynamics in cells (Abdelmoshen, 2012). Some RNA binding proteins, such as Staufen1 and 2, zipcode binding protein 1 (ZBP1), heterogeneous nuclear ribonucleoprotein A2 (hnRNP A2) and Fragile X mental retardation protein (FMRP) have obvious roles in mRNA transport to distinct neuronal compartments. Additionally, they also repress the translation of their target mRNAs (Hamilton et al. 1999; Laggerbauer et al. 2001; Huttelmaier et al. 2005; Welshhans and Bassell 2011). As RBPs fulfill many versatile roles in the regulation of core cellular functions, it is not surprising that defects in the formation or the composition of RNPs can often lead to diseases, including cancer and neurodegeneration.

2.5.3 mRNP granules

Perhaps most remarkable feature regulating mRNA distribution, translation, and degradation in the cell is the coordinated assembly of ribonucleo-protein (mRNP) complexes into the higher-order structures, also referred as RNP granules. These structures have diverse composition and function and their assembly has been shown to integrate many aspects of cellular metabolism with mRNA processing, transport, translation and degradation (Ohn et al. 2008; Keene 2010). Proper functioning of RNP granules is crucial for the normal physiology of the cell and disturbances in RNP granule assembly or function are related to several diseases.

Particularly well characterized RNP granules are transport-RNP granules, processing bodies (P-bodies or PBs) and stress granules (SGs), but also other types of granules exist in eukaryotic cells. For example, neurons and germ cells contain cell-type specific granules whose functions are poorly characterized.

The emerging phase transition model posits that RNP granules are nucleated through multiple low-affinity interactions between RNA-binding proteins that lead to the formation of stable or more dynamic aggregates in discrete cytoplasmic loci (Li et al. 2012; Kedersha et al. 2013; Calabretta and Richard 2015). Such mechanism for mRNA storage /stabilization may facilitate rapid changes in protein synthesis under certain conditions (Kedersha et al. 2013).

2.5.3.1 Stress granules

Stress granules (SGs) are dynamic cytoplasmic structures that form transiently to reprogram RNA translation under stressful conditions (Anderson and Kedersha 2009; Ash et al. 2014). In mammalian cells, SG formation is predominantly initiated by stimuli leading to eIF2a phosphorylation and global translational arrest (Ohn et al. 2008; Anderson and Kedersha 2009). Translationally silenced mRNAs are necessary for SG formation and together with the classic SG marker proteins, such as cell intracellular antigen 1 (TIA-1), tristetraprolin (TTP), and Ras-GTPase activating protein SH3-domain-binding protein (G3BP), lead to primary nucleation of SGs (Gilks et al. 2004). SGs also contain non-translating mRNAs, translation initiation components and RBPs involved in mRNA translation control and stability. These include Fragile X mental retardation protein (FMRP), survival of motor neuron protein (SMN), and cytoplasmic polyadenylation element binding protein (CPEB), but also microRNAs and a number of disease-linked proteins that all localize to SGs in stressed cells. SGs also mature with time and may associate with proteins that have no direct functions in mRNA translation and decay (Ohn et al. 2008; Anderson and Kedersha 2009; Ash et al. 2014). Altogether, more than one hundred proteins may regulate SG dynamics in mammalian cells (Ohn et al. 2008).

RBPs, responsible for SG assembly, contain low complexity regions and poly-glycine rich domains, which have tendency to aggregate (Gilks et al. 2004). Those domains are sensitive to post-translational modifications and thus may integrate stress-

related signals to control mRNA metabolism during stress (Gilks et al. 2004; Kedersha et al. 2013).

SG biology also modulates cell survival signaling cascades. For example, sequestering of receptor for activated C kinase 1, RACK1 and Rho-associated, coiled-coil containing protein kinase 1, ROCK1 at SGs inhibits the c-Jun N-terminal kinase (JNK) signaling cascade to trigger apoptosis (Kedersha et al. 2013). The sequestration of pro-apoptotic molecule TRAF2 (TNF receptor associated factor 2) prevents the caspase activation upon ER-stress induction. It is therefore suggested that SG assembly may serve as a state of emergency that is crucial for cell survival until stress is resolved.

SGs dismantle along with the stress recovery, but the process is poorly understood**.**

2.5.3.2 Processing bodies

Processing bodies (PBs) are dynamic cytoplasmic RNA granules that are thought to serve as main sites for mRNA decay (Cougot et al. 2004; Parker and Sheth 2007; Cougot et al. 2008; Zeitelhofer et al. 2008). The key components enriched in PBs are mRNA decapping enzymes (Dcp1, Dcp2), exonuclease (Xrn-1), RNA helicase (Dhh1p/rck/p54 protein), Lsm1–7 and Hedls/GE-1 complexes and deadenylases (Pan2, Pan3, Caf1), which are all involved in mRNA 5´ -3´degradation (Sheth and Parker 2003; Parker and Sheth 2007; Zheng et al. 2008). Many other proteins involved in NMD, mRISC, and ARE-mediated decay pathways have also been found in PBs (Lykke-Andersen and Wagner 2005; Parker and Sheth 2007). NMD factors, however, associate with P-bodies only transiently, suggesting their role in only delivering PTC-containing mRNAs to the site of degradation (Sheth and Parker 2006).

Frequent observations of mRNAs harboring stalled translation complexes in P-bodies support the notion that P-bodies could accommodate also translationally repressed mRNAs. It has remained unclear whether mRNAs are stored for later translation or await activation of the decay machinery in P-bodies (Eulalio et al. 2007). Interestingly, some transcripts can exit P-bodies and re-enter translation when environmental conditions change (Ohn et al. 2008). Studies in neurons indicate that PB dynamics is controlled by neuronal activity, thus suggesting their role in regulating local protein synthesis at certain cellular compartment (Thomas et al. 2011).

While biochemical studies have revealed variety of pathways for P-body assembly (Kedersha et al. 2005; Zheng et al. 2008), it is clear that the assembly of mammalian P-bodies does not depend on tress-induced phosphorylation of eIF2α (Kedersha et al. 2005)

Although SGs and PBs form independently and are differently regulated, they may interact with each other and share several protein components (Kedersha et al. 2005; Aizer et al. 2008). Therefore, PBs and SGs are often seen as 'triage centers' that sort, remodel and export specific mRNP complexes for translation, decay or storage. Such sorting seems to serve as an efficient mechanism to control the mRNA fate depending on environmental conditions and to allow optimal translation of stress-responsive anti-apoptotic mRNAs.

2.5.4 mRNA translational control: focus on upstream open reading frames

mRNA translation is a three-step process, comprising of initiation, elongation and termination steps that are followed by ribosome recycling (Sonenberg and Hinnebusch 2009). In eukaryotic cells, the synthesis of most proteins is driven by cap-dependent mRNA translation and is primarily regulated at the initiation step (Sonenberg and Dever 2003; Pavitt 2005; Sonenberg and Hinnebusch 2007). Translation initiation factors assamble onto the mRNA 5´cap structure and mediate series of steps which leads to the recruitment of so-called 43S preinitiation complex (43S PIC) to the mRNA (Aitken and Lorsch 2012) (Figure 12). 43S PIC, consisting of GTP- bound eIF2 and initiator methionyl methionine-tRNA (Met-tRNA$_i^{Met}$) ternary complex, along with other initiation factors and the ribosomal 40S subunit, scans along the 5'-UTR to find appropriate sequence context for the translation initiation (Figure 12). Selected initiation codon base-pairs with Met-tRNA$_i^{Met}$ in the P site of the small ribosomal subunit and establishes the frame for the proper protein synthesis. Joining of 60S ribosomal subunit leads to the formation of an 80S ribosome that is ready to proceed with protein synthesis (Figure 12). The interaction between the large scaffolding protein eIF4G at the mRNA 5´ end and poly(A)-binding protein (PABP) at the 3´ poly(A) tail results in a closed loop structure that stabilizes the complex and facilitates several rounds of translation from mRNA.

The most efficient site for translation initiation is an AUG codon in the context of the consensus sequence, first defined by M. Kozak, consisting of a purine residue at position −3 relative to the +1 adenosine of the AUG and a guanosine at position +4 (Kozak 1986). Such context supports the initiation of virtually all scanning ribosomes and is therefore classified as strong (both critical residues match the consensus sequence), as adequate (either residue -3 or +4 matches) or as weak (neither residue matches) (Kozak 1986). The precise molecular mechanism underlying the mRNA 5′ untranslated region scanning by the 43S PIC is not resolved. However, recent structural studies have suggested that initiation factors are key components in examining the start codon context during scanning (Lomakin and Steitz 2013; Erzberger et al. 2014; Hussain et al. 2014).

Protein translation may also be initiated in a cap-independent manner by using complex RNA structural elements, called internal ribosomal entry sites (IRES), which provide an alternative mode for the ribosome to bind to the mRNA and initiate translation. IRES elements provide structurally similar context for the ribosome as canonical initiation factors, hence enabling translation initiation even in a variety of physiological and pathological (stress) situations where the availability of canonical initiation factors is compromised. Examples of the genes regulated by such mechanisms include apoptosis and stress-related factors (Coldwell et al. 2000; Henis-Korenblit et al. 2000).

The term translational control refers to a wide array of mechanisms that affect the quantity of completed protein product synthesized from a given amount of mRNA (Farabaugh 2000). Such control can be achieved globally by affecting the core components of the translation machinery, or selectively thought the mRNA-specific features that ensure that only a defined subset of mRNAs respond to the specific cellular

stimuli. For example, multiple signaling pathways, activated by DNA damage, ER stress, a viral infection or disruptions in metabolism, may trigger a global translational repression in the cell via inhibitory phosphorylation of the eukaryotic translation initiation factor 2 (eIF2) or eIF4E subunits (Pavitt 2005; Jackson et al. 2010). Transcript-specific translational regulation may involve a wide variety of protein factors or non-coding RNAs that allow controlling the timing, duration, and location of protein synthesis, depending on the extra-and intracellular stimuli.

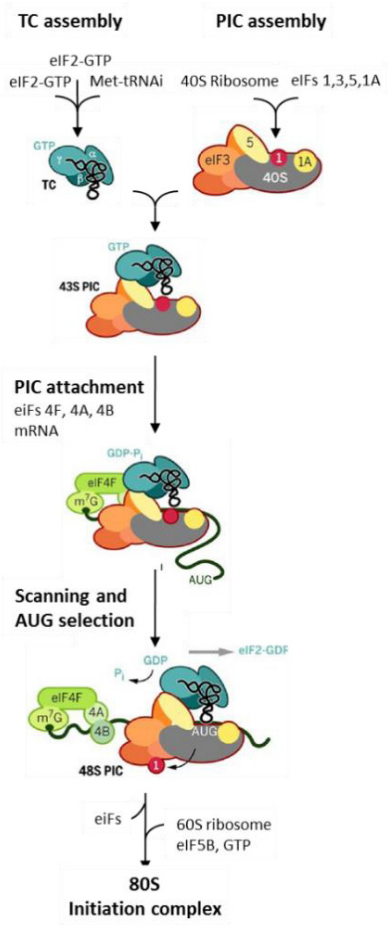


Figure 12. Scematic owerview of mRNA translation in eukaryotes. Translation initiation requires that ribosomal pre-initiation complex (PIC), assembles with the eIF2 ternary complex (TC). The resulting 43S preinitiation complex (43S PIC) attaches with additional eIFs at mRNA cap-structure and starts scanning along mRNA leader sequence to the start codon. It results in precise placement of an initiation codon into the ribosome, thereafter the ribosomal 60S subunit join the mRNA and translation preceeds with the 80S ribosome. Adapted from Hinnebusch et al., (2016)

2.5.5 Upstream open reading frames in gene expression regulation

Upstream open reading frames (uORF), defined as initiation and stop codon delimited sequences beginning upstream of the main coding sequence initiation codon, are frequently present cis-acting elements within the mRNA 5´-UTRs. uORFs may modulate the translational efficiency of the transcript main ORF (Araujo et al. 2012; Chew et al.

2016). Validation of uORFs functional activity by luciferase reporter assays has demonstrated mostly inhibitory effects on downstream ORF translation. uORFs can also mediate the induction of downstream protein translation, especially during the changes in global translational conditions (Wethmar et al. 2014). The latter is most thoroughly explained in the model of yeast Gcn2 mRNA, where the translation, induced in stressed cells, allows fast responses to nutritional stress (Mueller and Hinnebusch 1986; Hinnebusch 2005). Similar mechanisms control translation of stress response related activating transcription factor-4 (ATF4) and -5 (ATF5), but also CCAAT/enhancer binding proteins A and B transcripts in mammalian cells (Calkhoven et al. 2000; Harding et al. 2000; Zhou et al. 2008). There are also examples where efficient translation from the main ORF can be controlled by the proliferative status of the cell (Schleich et al. 2014). The functional importance of uORF-mediated translation regulation in an animal model has been reported for CCAAT/enhancer binding protein β in mouse, in which uORF controls the expression ratio of functionally distinct protein isoforms (Wethmar et al. 2010a).

It has been estimated that almost 13% of yeast, 49% of human, 44% of mouse and 65% of zebrafish mRNA transcripts contain uORFs (Iacono et al. 2005; Matsui et al. 2007; Calvo et al. 2009; Wethmar et al. 2014; Chew et al. 2016), among those, only a limited number of uORFs have been functionally characterized. The ribosome profiling studies have confirmed that over 50% of human transcripts show translation initiation events at upstream translational initiation sites (uTISs) (Ingolia et al. 2011; Lee et al. 2012). Those studies have also uncovered extensive translational changes upon the initiation of differentiation and stress responses (Ingolia et al. 2011; Gerashchenko et al. 2012). It suggests that alternative translation initiation has a widespread physiological role in gene expression regulation. How largely uTIS selection expands the proteome diversity, for instance by extending the N-terminal part of the protein, has remained largely elusive (Wethmar 2014).

Intriguingly, uORFs have mostly sub-optimal initiation contexts and are therefore thought to be poorly translated (Chew et al. 2016). Also, many near-cognate triplets, such as triplets differing in one base from the classical AUG, may act as alternative start codons for initiating uORF translation (Touriol et al. 2003). Most surprisingly, uORF length and the position in the 5' UTR, as well as the number per transcript can remarkably vary (Matsui et al. 2007; Calvo et al. 2009). Genome-wide comparisons of human and mouse sequences argue that although AUG is the best-conserved nucleotide triplet within the transcript leader sequences (Calvo et al. 2009), there is a tendency for selective evolutionary loss of nonfunctional uORF start sites (Iacono et al. 2005). It indicates the functional importance of uORFs in gene expression regulation (Lee et al. 2012).

Bioinformatics analyses have dissected variables that correlate with strong repression of main coding sequence translation (Figure 13). These include long 5´ cap-to-uORF distance, proximity of the uORF to the main coding sequence initiation site, uORF length, multiplicity of uORFs, conservation among species, and initiation sequence context (Calvo et al. 2009; Wethmar et al. 2014). Also, the involvement of trans-acting factors and cooperation between other elements residing in main ORF and even in the 3´-

UTR has started to emerge as important modulators of uORF-mediated effects on mRNA expression (Medenbach et al. 2011). Many studies confirmed that uORF repressiveness is modulated by the combination of various features that are present in the transcript (Matsui et al. 2007; Calvo et al. 2009; Araujo et al. 2012; Chew et al. 2016).

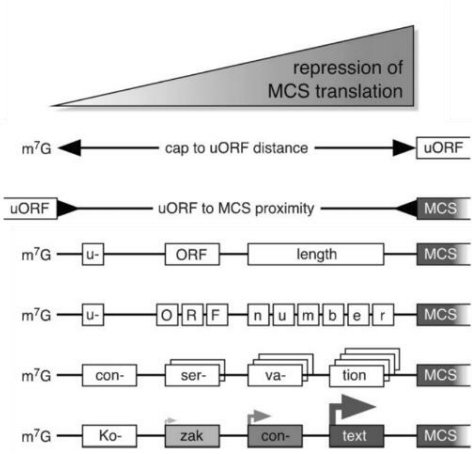


Figure 13. **Schematics of varying structural properties that may affect the regulatory potential of uORFs.** Variables most often correlating with enhanced repression of MCS translation are following: i) increased m7 G to uORF distance, ii) uORF to MCS proximity, iii) uORF length, iv) number and conservation among species, and v) an increasingly favorable uORF initiation context. m7G, mRNA cap structure; uORF, upstream open reading frame; MCS, main coding sequence. Modified from (Wethmar et al. 2010b)

Altogether, uORF utilization in different transcripts is diverse and current models outline only well-established mechanisms. Those include i) blocking of the ribosome by the cis-acting peptide products or specific termination context, that can cause ribosome stalling, ii) provoking of transcript instability through prematurely terminated translation iii) failure to reinitiate translation at the downstream ORF (Gaba et al. 2001; Oyama et al. 2004; Neafsey and Galagan 2007; Barbosa et al. 2013; Somers et al. 2013). Accordingly, mass spectrometry analyses have confirmed the expression of small peptides from the uORFs (Oyama et al. 2004; Slavoff et al. 2013). Few typical examples demonstrating the functionality of uORF encoded peptide involve the eukaryotic arginine attenuator peptide (AAP) and carnitine palmitoyltransferase 1C (CPT1C) gene regulation. The peptides encoded from their uORFs reduce the initiation of translation of the downstream ORF through ribosomal stalling at the termination codon of respective gene uORF (Oyama et al. 2004). Carnitine palmitoyltransferase 1C controls metabolism in the brain and its mRNA translation occurs in response to glucose deprivation and other stress stimuli. It emphasizes that gene expression and repression occurs in cycles, under influence of cellular responses to metabolic challenges.

The extent to which uORFs may induce mRNA decay has not been fully clarified. Bioinformatics analysis has revealed that uORF containing transcripts have lower levels and shorter half-lives than transcripts lacking the uORFs (Matsui et al. 2007). However, translation efficiencies may vary, and it probably influences these estimations. uORF translation can terminate in a premature context, suggesting that a fraction of those transcripts may be targeted for degradation by nonsense-mediated mRNA decay pathway (Mendell et al. 2004; Yepiskoposyan et al. 2011).

There are also a few mechanisms that overcome uORF-mediated translational repression. Among those, leaky scanning, translation re-initiation following the

termination of the uORF, ribosomal shunting or the presence of an internal ribosome entry site are most thoroughly characterized (Wethmar 2014). Some genes are regulated by combined effects of leaky scanning and reinitiation events. The best-studied examples of such mechanisms include the yeast and insect model systems.

In principle, leaky scanning allows ribosomal scanning complexes to bypass the uAUG or uORF and continue scanning for the next AUG, whereas ribosomal shunting enables skipping of parts of he mRNA leader sequences and initiation of translation at downstream uAUG. The capacity to reinitiate depends on cis-activating sequences or various trans-acting factors that support the ribosome to resume scanning after termination and find appropriate initiation codons downstream. Ribosome re-initiation efficiency decreases with the increased length of the uORF. For example, the capacity for re-initiation at the main downstream ORF can be compromised when uORF is longer than 35 amino acid residues (Kozak 2002). It is continuously emphasized that uORFs functionality is the result of the combined effects of many regulatory elements and cooperation of those elements, may depend on particular context.

An increasing number of studies link mutations that eliminate, create or modify uORFs to the development of human disease (Wethmar et al. 2010b; Barbosa et al. 2013). It is an especially important field to study, as uORFs are abundant and relatively actively translated. In addition, acquired genetic lesions may affect the initiation events at uORFs or the main coding sequence and lead to protein synthesis from the genes, which are supposed to be expressed in a dose-dependent manner. Such alterations have been confirmed to cause some inherited syndromes and contribute to the development of malignancies, metabolic or neurologic disorders (Wethmar et al. 2010b; Barbosa et al. 2013; Wethmar 2014). Around 500 single-nucleotide polymorphisms (SNPs) have been only recently found to create or delete uORFs (Calvo et al. 2009; Araujo et al. 2012). It was experimentally confirmed that some polymorphisms, reduced the protein levels and were responsible for disease phenotype (Calvo et al. 2009). However, many other uORF altering polymorphisms await experimental validation. Altogether, uORFs may influence protein expression in several ways and any alteration in the uORF-mediated gene expression control can cause changes in the homeostasis and/or be involved in the pathophysiology of various disorders.

2.5.6 Nonsense-mediated mRNA decay pathway

Nonsense-mediated mRNA decay is an evolutionarily conserved cytoplasmic mRNA quality control pathway that promotes rapid degradation of premature termination codon-containing (PTC^+) mRNAs (Maquat 2002). It was initially recognized as a mechanism that can suppress the synthesis of aberrant or C-terminally truncated polypeptides and thus, to protect the cell from their possible deleterious effects (Maquat 2004). Subsequently, it was found that NMD can also limit the expression of physiological transcripts that contain NMD activating features (Neu-Yilik et al. 2004; Giorgi et al. 2007; Nicholson et al. 2010; Colak et al. 2013; Hamid and Makeyev 2014; Mockenhaupt and Makeyev 2015). NMD targets broad classes of transcripts, synthesized from protein-

coding genes in eukaryotic organisms, as different as yeast and human. Current estimates suggest that depending on the organism or cell type, approximately 5% to 20% of transcripts are directly or indirectly regulated by NMD (He et al. 2003; Mendell et al. 2004; Rehwinkel et al. 2006; Wittmann et al. 2006; Weischenfeldt et al. 2008; Chan et al. 2009; He and Jacobson 2015). Increasing evidence also indicates that substantial amount of PTC^+ transcripts may evade NMD in a regulated manner (Stockklausner et al. 2006; Toma et al. 2015). It suggests that NMD itself is also the subject of regulation (Karam et al. 2013). Indeed, the founding studies on NMD demonstrated its role in many congenital diseases. Subsequent molecular and genetic studies have demonstrated its wide impact on modulating genetic disease phenotypes, as well as its importance in organismal homeostasis, cell growth and development (Frischmeyer and Dietz 1999; Miller and Pearce 2014).

The key factors carrying out NMD in mammalian cells are a set of up-frameshift suppressor (UPF) family proteins, SMG (suppressor of morphological defects in the genitalia) proteins and core factors of the exon junction complex (EJC).

EJC is a protein complex that marks most of the spliced exon-exon junctions in eukaryotic mRNAs (Le Hir et al. 2000a; Chang et al. 2007; Sauliere et al. 2010). EJCs are loaded onto mRNA approximately 20-24 nucleotides upstream of the mRNA exon-exon junctions (Le Hir et al. 2000a; Ballut et al. 2005) in a splicing-dependent, but sequence - independent manner (Chang et al. 2007). The core components of the EJC are the ATP-dependent RNA helicase eIF4A3 and its regulators MAGOH, Y14 and Barentsz/MLN51. These proteins interact with other accessory factors and assist mRNA during its journey from the nucleus to the cytoplasm. EJC core constitutes a binding platform for the factors that execute NMD in mammalian cells (Le Hir et al. 2000a; Le Hir et al. 2000b; Kim et al. 2001; Le Hir et al. 2001; Lykke-Andersen et al. 2001; Gehring et al. 2003; Gehring et al. 2005; Kashima et al. 2010).

UPF1 is a group I RNA helicase and RNA-dependent ATPase that is an essential component of the NMD machinery. Its recruitment to the translation termination complex is a prerequisite step for NMD initiation (Chang et al. 2007). Subsequent interactions with other family members, such as the UPF2 and UPF3 are required for NMD progression (Le Hir et al. 2001). In particular, UPF1, UPF2 and UPF3 assemble into a trimeric complex that stabilizes UPF1 interactions with the terminating ribosome and stimulate its ATPase and helicase activity (Chamieh et al. 2008; Chakrabarti et al. 2011). Many PTC-containing mRNAs escape NMD in UPF1-deficient HeLa cells (McGlincy et al. 2010). Similarly, mutation or silencing of UPF factors in yeast cells may selectively stabilize mRNAs that are regulated by NMD (He et al. 1997). UPF1 phosphorylation/dephosphorylation status determines its interactions with other NMD factors (Karam et al. 2013).

SMG 5-6 proteins are essential for exo- and endo-nucleolytic degradation of NMD substrates. SMG-1 is a key protein kinase that regulates UPF1 phosphorylation and dephosphorylation cycles in the cell (Yamashita et al. 2001).

Current consensus is that NMD is triggered when ribosomes terminate translation prematurely (Kervestin and Jacobson 2012). In addition, recognition of PTCs requires

active translation (Maquat 2004). How cells discriminate between the normal and premature termination events has not been entirely elucidated. It is suggested that the context of the stop codon dictates whether NMD will be triggered or not.

Classical model describing the NMD in mammalian cells posits that EJC positioned more than 50-55 nucleotides downstream of the termination codon can trigger NMD (Le Hir et al. 2000a; Chang et al. 2007). Generally, EJCs are believed to be displaced by the translocating ribosome during the first round or "pioneer" round of translation (Lejeune et al. 2002). As typical mammalian mRNAs lack the splice junctions downstream of the canonical termination codon, they avoid the NMD. However, when one or more EJC are recognized at a distance of 50-55 nucleotides downstream of the termination codon, the NMD can be triggered. These observations have laid the foundation for the so-called "55-nucleotide rule of NMD activation" (Nagy and Maquat 1998; McGlincy et al. 2010).

Recent evidence indicates that the length and composition of the 3′ UTR are also critical for defining termination events as aberrant (Toma et al. 2015). Global studies have shown enrichment of mRNAs containing long 3′ UTRs among endogenous NMD substrates (Mendell et al. 2004; Hurt et al. 2013). Spatial relationship between the stop codon and poly(A) tail bound cytoplasmic poly-A binding protein (PABP) has been also implicated as a conserved feature for NMD induction in mammalian cells (Amrani et al. 2004; Behm-Ansmant et al. 2007; Eberle et al. 2008; Ivanov et al. 2008; Singh et al. 2008; Rebbapragada and Lykke-Andersen 2009; Hogg and Goff 2010; Fatscher et al. 2014; Toma et al. 2015).

Most common substrates for NMD are the classes of mRNAs which contain spliced introns downstream of the termination codon (Nagy and Maquat 1998), or long 3′ UTRs (Kebaara and Atkin 2009)), and in some cases uORFs (Ruiz-Echevarria and Peltz 2000; Silva et al. 2006).

The biochemical basis of NMD induction relies on the interactions between the ribosome termination complex and the NMD machinery (Singh and Lykke-Andersen 2003; Schweingruber et al. 2013). Eukaryotic ribosome release factors eRF1 and eRF3 recruit the SMG1 and UPF1 to the stalled ribosomes (Figure 14). This interaction stimulates the UPF1 phosphorylation and results in the formation of the transient protein complex, also referred as SURF (SMG1-UPF1-eRF1-eRF3) that then associates with EJC via the adaptor protein UPF2 and induces a UPF1-UPF2-UPF3 surveillance complex, which is then activate NMD (Rebbapragada and Lykke-Andersen 2009).

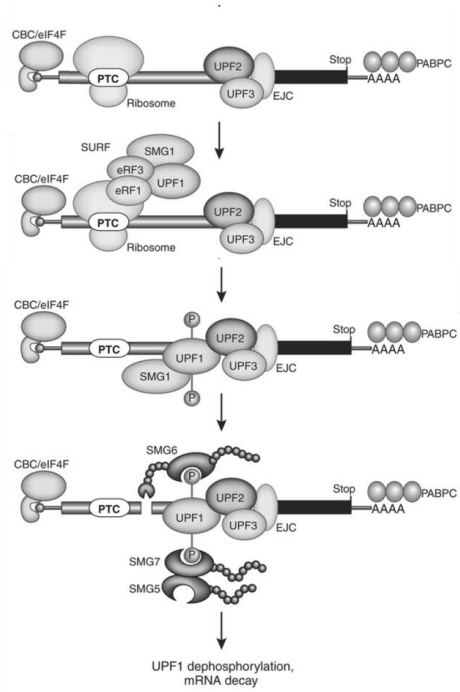


Figure 14. Nonsense-mediated decay pathway in mammalian cells. Schematics showing a ribosome at a PTC and the EJC, associated with NMD factors (UPF3 and UPF2) at the exons-exon junction (gray and black rectangles). NMD targets the mRNPs irrespective of whether the cap is protected with the nuclear cap-binding complex (CBC) or with the eIF4F complex. A premature translation termination event leads to the assembly of a SURF complex, consisting of SMG1, UPF1, eRF1 and eRF3. This complex interacts with UPF2, UPF3 and core EJC proteins at the downstream exon-exon boundary. This interaction triggers UPF1 phosphorylation and the dissociation of eRF1 and eRF3. Phosphorylated UPF1 recruits the nucleases (SMG5–SMG7 and SMG6) which promote mRNA degradation. SMG5–SMG7 and SMG6 also promote UPF1 dephosphorylation and recycling of NMD factors. Adapted from Hentze and Izaurralde (2013)

It is now well- accepted that NMD regulates the expression of variety of physiologic transcripts (Mendell et al. 2004). By current estimates, up to 10 % of normal physiological mRNA variants, encoding cell differentiation and cellular stress- or maturation related proteins, are targeted by NMD in human cells (Kurosaki and Maquat 2016). Different developmental stages or cellular contexts can modulate NMD activity, especially through the targeting of the phosphorylation/dephosphorylation cycle of Upf1 (Karam et al. 2013). For example, NMD prevents inappropriate activation of the unfolded protein response (UPR) and promotes timely termination of the UPR to protect cells from apoptosis. Most of the key UPR components—ATF3, ATF4, ATF6, FSD1L, HERP, IRE1α, PERK, PRDG1, TNRC1, and TRAF2—are NMD substrates (Karam et al. 2015). NMD inhibition, in turn may upregulate the cellular stress response pathways (Gardner 2008).

Bruno and colleagues have demonstrated (2011) that NMD activity is downregulated during neuronal development in mice. In particular, miR-128 is highly expressed during the brain development and neuronal maturation. It supresses the expression of core NMD factors UPF1 and Mln51, leading to the elevated expression of NMD targets that encode neuronal factors (Bruno et al. 2011). *Upf1, Upf2* and *Smg1* deficiency leads to early embryonic lethality and marked developmental abnormalities in mice (Medghalchi et al. 2001; Weischenfeldt et al. 2008; McIlwain et al. 2010). Decreased functionality or loss of mutations in key NMD factor genes cause various forms of neuro-developmental conditions and congenital anomalies in humans. It shows that NMD is involved in proper development and functioning of nervous system (Karam and Wilkinson 2012).

Selected groups of potential NMD substrates, such as uORF-containing mRNAs and transcripts with the long 3´-UTRs, may escape NMD and can be actively translated (Zhao et al. 2010; Toma et al. 2015). In addition, many mRNAs appear as NMD targets, even if they lack the canonical NMD activating features (Hurt et al. 2013). It suggests that transcript-specific regulatory features can likely influence NMD.

2.5.6.1 NMD coupling with alternative splicing

Differential pre-mRNA processing and alternative usage of gene promoters can naturally generate PTC-containing transcripts. Some PTC-generating or deleting features are associated with conserved elements that are used in an autoregulatory manner (Ni et al. 2007; Jangi et al. 2014). For example, core spliceosomal factors and large groups of RBPs can autoregulate their expression levels through the inclusion or exclusion of ultraconserved or so-called "death exons" (Lareau et al. 2007; Ni et al. 2007). Such mechanism, also referred as alternative splicing-coupled NMD or regulated unproductive splicing and translation, has been now shown to modulate gene expression in organisms as different as yeast and human (Mockenhaupt and Makeyev 2015). It is suggested that coupling of alternative splicing and NMD may significantly contribute to the establishment and maintenance of developmental stage and cell type-specific mRNA profiles, mostly by shifting alternative splicing events in co-expressed sets of transcripts towards the desired expression patterns (Licatalosi and Darnell 2010; Mockenhaupt and Makeyev 2015). NMD evasion

Despite the recognition that transcripts with long 3´UTRs are common substrates for NMD, there are examples of their full or partial escape from NMD. It, therefore, suggests that NMD evasion is another strategy to fine-tune gene expression control.

Toma and colleagues (2015) have shown that a subset of mRNAs with long 3′ UTR (>1250 nt) may evade NMD through the mechanism involving the A/U rich *cis*-acting elements positioned in the first 200 nt, downstream of the PTC (Toma et al. 2015). Also, the polypyrimidine tract binding protein (PTB) has been found to protect subsets of long 3´-UTR containing human transcripts from destruction by NMD (Ge et al. 2016). This activity was associated with a reduction in Upf1 binding to transcripts 3'-UTRs (Ge et al. 2016). However, other NMD-evading elements or even structural arrangements, such as positioning of PABP in spatial proximity to the termination codon, may also be involved in regulating mRNA escape from NMD (Eberle et al. 2008).

The functional relevance of temporary NMD evasion in gene expression regulation has been elegantly demonstrated in the study by Colak et al., (2013), where naturally expressed NMD-candidate mRNA for axon guidance receptor Robo 3.2 was found to escape NMD through translational repression, specifically in the navigating commissural axons (Colak et al. 2013). Robo 3.2 translational de-repression occurs only in the axons that have crossed their intermediate target in the spinal cord floor plate, thus enabling neurons to choose the correct navigating trajectories in the spinal cord. However, the expression of Robo 3.2 isoform in post-crossing axon terminals is limited due to the rapid destruction of its mRNA by co-translational NMD mechanism. It ultimately results in expression of only controlled amounts of the protein product from every mRNA molecule.

A similar regulation has also been reported for *Arc* gene expression in dendrites (Giorgi et al. 2007). It is therefore suggested that temporary NMD evasion and translational control allows cells to express certain proteins only as a controlled bursts depending on the cellular context.

Conditionally regulated NMD has also a role in apoptosis regulation. It has been reported that an alternatively spliced, PTC-containing mRNA for caspase-2 short isoform is a substrate for NMD, but it is also translated at low quantities in healthy cells (Wang et al. 1994; Solier et al. 2005). The resulting protein, caspase-2s, is able to counteract the function of the conventionally expressed major pro-apoptotic isoform, caspase-2_L but its anti-apoptotic effect is not considerable. In a variety of cancer cells, the caspase-2s mRNA escapes NMD resulting in higher protein levels and changes in the caspase-2s/2L ratio. It therefore shows that alternative splice variants may be selectively regulated by the NMD mechanism.

2.5.6.2 Nonsense-mediated translational repression-NMTR

NMD evasion seems to be closely related to so-called nonsense-mediated translational repression (NMTR), may induce translational repression and stability within the subset of PTC-containing mRNAs. NMTR molecular mechanism is poorly characterized. NMTR was initially described in tumor cells where a PTC-containing mRNA variant for transforming growth factor-beta receptor type 2 (TGFβR2) was shown to escape NMD due to translational repression (You et al. 2007; Lee et al. 2010). In this study, an EJC downstream of premature translation termination codon was shown to serve as a key determinant that inhibits translation after formation of 80S ribosome complex (Lee et al. 2010).

NMTR could also depend on repressive *cis*-acting elements in the mRNA 3′-UTR sequence, as evidenced by Morita et al (2014), showing that smooth muscle–specific PTC-containing mRNA splice variant of *arp5* may be degraded by NMD but also translationally repressed by NMTR (Morita and Hayashi 2014).

Currently, it is not clear whether NMTR represents an “incompletely executed” NMD program (Mockenhaupt and Makeyev 2015) or provide a mechanism to protect PTC-containing mRNA isoforms from NMD under certain cellular conditions. Therefore, additional studies are required to uncover precise molecular features regulating NMTR in mammalian cells.

3 AIMS OF THE STUDY

This study aims to elucidate unique molecular features controlling apoptotic programs in the post-mitotic neurons.

Our previous results have shown that all tested types of post-mitotic neurons do not express the mRNA for Bcl-2 family key apoptosis regulator protein Bak, but instead, it's alternatively spliced BH3-only isoform N-Bak. Yet, further studies have shown controversial results in detecting endogenously expressed N-Bak protein in neurons. While the expression of endogenous N-Bak protein has been demonstrated in cortical neurons, where it has been shown to be further induced in response to etoposide induced DNA damage, there is a number of other studies indicating that N-Bak protein remains undetectable by classical biochemical methods. We set to clarify whether such discrepancy has biological or experimental origin.

N-Bak mRNA and protein expression levels are discordant, suggesting that endogenous N-Bak protein expression might be post-transcriptionally regulated. Our next aim was to characterize underlying molecular mechanisms that may regulate N-Bak expression in neurons, particularly at the post-transcriptional level.

The specific aims were:

- to clarify whether endogenously expressed N-Bak protein is detectable in the healthy, stressed or apoptotic neurons.
- to analyse whether the abundance N-Bak protein in neurons is controlled by the proteasomal degradation mechanism.
- to find out whether premature translation termination within the N-Bak mRNA 3´-UTR has importance in determining N-Bak mRNA stability and translational status in neurons. In particular, to investigate if N-Bak mRNA is rapidly degraded by nonsense-mediated mRNA decay (NMD) pathway.
- to characterize the regulatory elements within N-Bak mRNA 5'-UTR contributing to its translational regulation in neurons.
- to characterize the N-Bak mRNA localization in the healthy and apoptotic neurons.

4 MATERIALS AND METHODS

Materials, methods and technical equipment used in this study have been described in detail in the original articles (I, II). Summary of the methods used in this study with the references to the original articles are also presented in the Table 2.

Table 2. Methods used in this study.

Methods used in this study	used in
Cloning of reporter plasmids and sequencing	I,II
RNA isolation	I,II
Reverse transcription	I,II
Polymerase chain reaction (PCR)	I,II
Quantitative PCR (qPCR)	I,II
Statistical methods	I,II
Cell cultures	I,II
In vitro splicing block assay	I
Cell culture transfections	I,II
Neuronal microinjections	I,II
Fluorescent *in situ* hybridization	II
Immunostaining	II
Confocal fluorescent microscopy	II
Computer assisted image processing	I,II
Protein extractions	I,II
In vitro translation	I
Polyacrylamide gel electrophoresis	I,II
Immunoblotting	I, II
Coomassie gel stainings	II
Mass spectrometry	II
Luciferase reporter assays	I,II
Metabolic labelling	I

4.1 Overview of methods used in this study

4.1.1 Cloning of reporter plasmids

Detailed cloning strategies are described in original papers (I, II). Mouse Bak 5'UTR and 3'UTRs were amplified from mouse embryo embryonic day (E) 10.5 cDNA or genomic DNA with respective primer pairs (Table 3) using polymerase chain reaction (PCR). Respective PCR fragments were digested with appropriate restrictases, purified and cloned into firefly luciferase reporter vector pGL 4.13 (Promega, Madison, WI, USA). The fragment encompassing the highly conserved putative miRNA binding sites in N-Bak 3´-UTR reporters was replaced with heterologous fragment from pSIREN-dsRED DNR vector (Clontech, Mountain View, CA, USA). Mutated 5'-UTR was constructed by deleting a 38bp fragment containing initiation codon for the longest uORF. All constructs were

verified by sequencing. The cloned expression constructs are presented schematically in article II, Figure 1.

The expression plasmids for N-Bak, Bak, N-Bak-L76E and Bcl-xL are published elsewhere (Sun et al. 2001). pEGFPF-C1-Dcp1α plasmid is a kind gift from J. Peränen (University of Helsinki, Finland, unpublished). Renilla luciferase reporter pGL4.73 is from Promega and enchanced green fluorescent protein reporter p-eGFP-N1 is from Clontech.

4.1.2 Reverse transcription, polymerase chain reaction (PCR), quantiatative PCR

Total RNA was isolated from P0 mouse brain and liver tissue, whole mouse embryos (E10,5) or from the cultured cells. Isolated RNA was dissolved in the RNase-free water and treated with DNAse I. RNA concentration was measured by NanoDrop spectrophotometer and 1-2 µg of total RNA with oligo (dT) or random hexamer primers (Roche Diagnostics, Mannheim, Germany) was used for cDNA synthesis (I, II). The PCR conditions are described in papers I and II.

PCR primers were designed with Primer3 software. Quantitative PCR (qPCR) primers were designed using either Primer Express v2.0 software (Applied Biosystems) or Roche Universal Probe Library Assay Design center (Roche Diagnostic). All primers were analysed *in silico* with OligoAnalyzer software (eu.idtdna.com) to exclude primer pairs with tendency to form homo-or heterodimers. PCR primers used for cloning are listed in Table 3; for RT-PCR in Table 4 and for qPCR in Table 5.

Table 3. Primers used for cloning.

PCR primers used in cloning			
PCR primers	**orientation**	**sequence**	**used template**
N-Bak-5'UTR	forward	5'-GATTAAGCTTGGAGCTGGGACCTCCTCTATG-3'	mouse (E10.5) cDNA
	reverse	5'-ACTTAA GCTTGAATTCACCACCGTCACTTGTCAC-3'	
N-Bak-3'UTR	forward	5´-TTCATCTAGAGGTTTGACCGGCTTCCTG-3'	mouse (E10.5) cDNA
	reverse	5´-ATACGGATCC GCTGGGATTACAGCAGTGTG-3´	
N-Bak-EJC-3'UTR	forward	5´-TTCATCTAGAGGTTTGACCGGCTTCCTG-3´	mouse genomic DNA
	reverse	5´-ATACGGATCCGCTGGGATTACAGCAGTGTG-3´	
Bak-3'UTR	forward	5-CGTATCTAGACAGATTCTTCAGATCATGACTCC-3'	mouse genomic DNA
	reverse	5´-ATACGGATCCGCTGGGATTACAGCAGTGTG-3´	

Table 4. Promers used for RT-PCR analysis.

Primers used in RT-PCR analysis			
PCR primers	**orientation**	**sequence**	**amplicon length**
flanking eej N/6	forward	5´- TTCATCTAGAGGTTTGACCGGCTTCCTG -3´	processed eej 450 nt
in N-Bak 3'UTR	reverse	5´- ACCATGCAATGTTGGGGTAT -3´	unprocessed 581 nt
flanking exon N	forward	5'-TTGCCCAGGACACAGAGGAGGT-3'	N-Bak 530 nt
in N-Bak mRNA	reverse	5'-GAATTGGCCCAACAGAACCACACC-3'	Bak 510 nt

Table 5. Primers used for quantitative PCR.

PCR primers used in quantitative PCR		
q-PCR primers	**orientation**	**sequence**
mouse 7SL ncRNA	forward	5'-CGA TCG GGT GTC CGC ACT AAG T-3'
	reverse	5'-TGG TCG TTC ACC CCT CCT TAG G-3'
rat 7SL ncRNA	forward	5'-GTTTCCGACCTGCGCCGGTTC-3'
	reverse	5' -TATGCCGATCGGGTGTCCGCAC-3'
Rat 18S rRNA	forward	5'-GCACGGCCGGTACAGTGAAACT-3'
	reverse	5'-AGCGCCCGTCGGCATGTATTAG-3'
mouse N-Bak 1	forward	5'-TGCTGATGGCAACTTCAACTG-3'
	reverse	5'-AGCCAAAGCCCAGGAGAG-3'
mouse N-Bak 2	forward	5'-CAGCAACATGCACAGCCTAT-3'
	reverse	5'-CTGTGCATGTTGCTGCTGG-3'
rat N-Bak	forward	5'-ATGGAATCCTGTGGCATCCAT-3'
	reverse	5'-GTGCATGCCGCTGCTGCTG-3'
murine Bax	forward	5' -TTGCTGATCTTGTCGTCGGACTGT-3'
	reverse	5'-CAGCCCATGATGGTTCTGATC-3'
ENO2	forward	5'-CTGCAGTCCAAGAGCATCGA-3'
	reverse	5'-ATGAGCTGGTTGTACTTCGCCAGA-3'
NEF-M	forward	5'-GCTACGACACGGAGTTCCAGA-3'
	reverse	5'-ACTGCTGGATGGTGTCCTGG-3'
Actb	forward	5'-ATGGAATCCTGTGGCATCCAT-3'
	reverse	5'-CCACCAGACAACACTGTGTTGG-3'
Cyclophilin G	forward	5'-CAATGGCCAACAGAGGGAAG-3'
	reverse	5'-CCAAAAACAACATGATGCCCA-3'
Fluc	forward	5´-TGCAAAAGATCCTCAACGTG-3´
	reverse	5´-AATGGGAAGTCACGAAGGTG-3´
RLuc	forward	5´-CCTGATCAAGAGCGAAGAGG -3´
	reverse	5´-GTAGGCAGCGAACTCCTCAG -3´

The MIQE guidelines were followed in qPCR assay design and analysis. The levels of the endogenous reference genes in treated and untreated sample groups were validated by the 2^{-CT} method, *p* values were calculated using Student's *t*-test. The changes in the mRNA levels following the treatments were analyzed by relative expression software tool REST as presented (I).

4.1.3 Cell cultures

The cortical neurons from the embryonic day 15-16 mice and neonatal sympathetic SCG neurons were isolated from NMRI strain as published (I, II). Cortical neurons were grown on the polyornithine-coated plastic dishes (Becton Dickinson, Franklin Lakes, NJ, USA) and sympathetic neurons were cultured on polyornithine-laminin (Sigma, St. Louis, MO, USA) coated dishes or glass coverslips. Neurons were cultured in the Neurobasal medium (Invitrogen, Carlsbad, CA, USA) containing 2% of B27 serum supplement (Invitrogen), 0.5 µM L-Glutamine and 100 µg/ml Primocin (InvivoGen, San Diego, CA, USA). Sympathetic neurons were also supplemented with mouse 2.5 S NGF for survival or deprived of NGF for 24 or 48 h by adding the function-blocking anti-NGF antibodies with the fresh culture medium to the cells. Neurons were cultured for 5-6 days *in vitro* (DIV) before subjection to microinjection or NGF deprivation (sympathetic neurons) (I, II); treated with different chemicals as indicated in original papers or left untreated (I, II). Where necessary,

caspase inhibitors were used to avoid cell death. All chemicals added to culture media are listed in (Table 6). Vivo-Morpholino oligonucleotides were applied to cortical neurons for 24-48h (Table 6 and 7).

Mouse primary glia cell cultures were established by cultivating cells from dissociated SCG in uncoated cell culture dishes for 4h, thereafter the unattached sympathetic neurons were removed and fresh growth medium was added to the attached glia cells.

Human cervical carcinoma HeLa, human neuroblastoma Neuro-2a, human embryonic fibroblast NIH-3T3 and mouse primary glia cells were grown in uncoated plastic dishes in DMEM (Invitrogen) supplemented with 10% fetal bovine serum (HyClone, Thermo Scientific, UK) and 100 µg/ml Normocin (InvivoGen). Rat pheocromocytoma PC6 cells were grown in DMEM with 10% of horse serum (PAA Laboratories, Pasching, Austria), 5% of fetal bovine serum and 100 µg/ml of Normocin. Cells, at 60-70% confluency were treated with different chemicals (Table 6), transfected with expression plasmids or left untreated before sample collection. All experiments were repeated on independent cultures 3-5 times.

Table 6. Reagents and their end concentrations used in cell culture media.

Reagents added to cell culture media	Concentration	Source
Pan-caspase inhibitor boc-aspartyl(OMe)-fluoromethylketone (BAF)	25 µg/ml	Calbiochem/EMD Biosciences
function-blocking anti-NGF antibodies	2 µg/ml	EMD Millipore
Cycloheximide	20 mg/ml	Sigma
Actinomycin D	5 mg/ml	Sigma
proteasome inhibitor MG-132	10 mg/ml	Sigma
Etoposide	4µg /ml	Sigma
Vivo-Morpholino oligonucleotides*	4 or 8 mg/ml	GeneTools, LLC
Pan-caspase inhibitor Q-VD-OPh	5 µM	R&D Systems
Thapsigargin	10 nM	Molecular Probes
mouse 2.5 S NGF	30 ng/ml	Promega

Table 7. Vivo-Morpholino oligonucleotide sequences.

***Vivo-Morpholino oligonucleotides**

	oligonucleotide sequences
target	5'-ACCTGTGCATGTTGCTGCTGGCCT -3'
(scrambled) control	5'-AGCGCACGATCAACATGCAGACGG-3'

4.1.4 Transfection and microinjection

The cell lines were transfected with the expression constructs by using Lipofectamine 2000 reagent (Invitrogen). Different pGL4.13 based 5'UTR or 3'UTR (Fluc) and pGL4-73 (Rluc) reporter plasmids were transfected at 50:1 ratio as described in (II).

Nuclear microinjection of cultured SCG neurons was performed as published (I, II). The neurons were microinjected with different 5'UTR or 3'UTR FLuc reporter plasmids together with RLuc and p-eGFP-N1 reporter plasmids. About 220-300 neurons were injected with each plasmid combinations. About 50-70 neurons were injected with pEGFPF-C1-Dcp1α plasmid and neurons were maintained with pan-caspase inhibitor Q-VD-OPh (R&D Systems, Minneapolis, MN, USA) to avoid cell death (II).

4.1.5 Immunoblotting

Protein samples from cultured cells, in vitro translated protein samples, mouse brain and liver samples were collected, lysed and homogenized in SDS sample buffer or lysis buffer as described (I, II). Protein samples were separated in SDS-PAGE and blotted onto nitrocellulose membranes. Antibodies used for immunoblotting are listed in Table 8. Enhanced chemiluminecence reagent (Pierce) and Luminescent Image Analyzer 3000 (Fujifilm) were used to detect the immunoblot signals.

Table 8. Primary antibodies used in this study.

Primary antibodies used in this study					
raised in	**antigen**	**source**	**catalogue nr.**	**dilution**	**publication**
mouse	**α-tubulin***	Sigma	#T9026 (DM 1A)	1:5000	I
	Bak*	EMD Millipore	#AM04, Ab-2 (TC-102)	1:200	I
	β-actin*	Sigma	A5316 (AC-74)	1:5000	I
	Dcp1α**	Abnova	#H00055802-M06 (3G4)	1:1000	II
	Hsp70*	Stressgen Biotechnologies	#ADI-SPA-810 (C92F3A-5)	1:1000	I
rabbit	**Bak***	Santa Cruz Biotechnology	#sc-832 (G-23)	1:1000	I
	Bak*	Upstate/EMD Millipore	#06-536	1:1000	I
	Bak*	Sigma	#B-5897	1:2000	I,II
	P-eIF2α*	Cell Signaling	#S 3398 (D9G8)	1:1000	II
sheep	**digoxigenin**	Roche Applied Science	#11207733910	1:800	II

used in *immunoblot, ** *in situ* hybridization

4.1.6 Fluorescent *in situ* hybridization and immunostainings

Detailed description of in situ hybridization and subsequent immunostaining are presented in original paper (II). mRNA *in situ* hybridization (ISH) was performed in NGF maintained or deprived sympathetic neurons with 5' and 3' digoxigenin-labelled antisense or sense Locked Nucleic Acid (LNA) based DNA oligonucleotides (Table 8). Primary ISH signal was amplified with biotinyl tyramide and detected with fluorocrome conjugated streptavdin. In detection of endogenous or overexpressed Dcp1α, the primary antibody signal was not amplified.

The fluorescent images were acquired using the confocal microscope Leica TCS SP5. The images were deconvoluted and identically processed with ImageJ and Photoshop CS5 (Adobe Systems) programs (II).

4.1.7 Mass spectrometry analysis

Detailed overview of mass spectrometry analysis used in this study is presented in original paper (II). The protein lysates were separated by SDS-PAGE followed by fixation and Coomassie staining, thereafter gel pieces, surrounding 20 kDa region, but avoiding, 15kDa, 25kDa areas and neighbouring lanes, were cut out and collected to separate tubes with storage buffer. Further treatments and proteome analysis were performed at Max Planck Institute of Biochemistry, Microchemistry Core Facility, Mass Spectrometry Service, Martinsried, Germany.

4.1.8 Luciferase reporter assay

Luciferase reporter assay was performed 48 hours after cell culture transfections or neuronal microinjections (I, II). The relative luciferase activity (ratio of Fluc/Rluc) was

determined with GloMax 20/20 luminometer (Promega). Successfully injected, eGFP-positive neurons were counted before lysis and the ratio of Fluc/Rluc was normalized to the number of eGFP-positive neurons. The experiments were repeated three to six times on independent cultures. Statistical analysis was performed by using GraphPad InStat 3 program (GraphPad Software, Inc., CA, USA).

4.1.9 Metabolic labelling

To optimize the CHX inhibition rate to the global protein translation in cultured SCG neurons, the metabolic labelling assay was used (I). The incorporation of [^{35}S]-labeled amino acids into the total cellular protein pool was measured in the presence or absence of protein translation inhibitor CHX. After 4h of labelling, the proteins were precipitated by trichloro-acetic acid, bound to the glass microfiber filter disks and the extent of labeling was measured in scintillation beta-counter.

RESULTS

4.2 N-Bak mRNA is highly expressed in mouse brain tissue and cultured neurons (I)

Earlier studies have demonstrated that Bak pre-mRNA is alternatively spliced in neurons, resulting in the expression of exclusively neuronal isoform, N-Bak, with a capacity to encode a putative BH3-only protein in neurons (Sun 2001).

Unpublished data from U. Arumäe´s lab has revealed that alternative splicing of *Bak* pre-mRNA starts to produce N-Bak mRNA when mouse neuronal progenitors initiate cell cycle exit and give rise to first post-mitotic neurons (Sun and Arumäe unpublished data, not shown).

We have also found that *in vitro* cultivated mouse neuronal progenitors express only Bak, whereas along with their *in vitro* neuronal differentiation they start to express N-Bak (not shown) (Jakobson and Arumäe unpublished data).

The current study demonstrated that N-Bak mRNA is highly expressed in the newborn (P0) mouse brain tissue and in the cultures of cortical and sympathetic neurons. Bak expression was detected at much lower level in these samples, most probably originating from the non-neuronal cells that are always present in the brain tissue and neuronal cultures (Sun 2001). From newborn mouse liver tissue and mouse fibroblast cells (NIH-3T3), only Bak but not N-Bak transcripts were detected. Rat pheochromocytoma PC6 and mouse neuroblastoma Neuro2a cells expressed exceptionally both isoforms of *bak*, but the level of N-Bak mRNA was very low.

4.3 N-Bak protein is not expressed in healthy neurons (I, II)

In current study, two commercially available anti-Bak antibodies (EMD Millipore and Sigma B-5897) were shown to specifically recognize about 25 kD Bak and about 20 kD N-Bak proteins from *in vitro* translated or ectopically expressed protein samples. These two antibodies did not detect endogenous N-Bak protein from any of the samples obtained from newborn mouse brain tissue or cultured sympathetic and cortical neurons, as demonstrated by protein immunoblot analyses. The presence of Bak protein, shown to be expressed only in non-neuronal cells (Sun et al, 2001), was always detected.

Notably, a band of about 21 kD was inconsistently observed in some samples of cultured cortical neurons but not in sympathetic neuronal culture samples. This band resembles the endogeneous N-Bak claimed by another group in the healthy and apoptotic cortical neurons, using different anti-Bak antibody, Ab-2 (EMD Millipore/Calbiochem (Uo, 2005). No N-Bak specific signal with Ab-2 was detected in our experiments.

Quantitative mass spectrometry analysis confirmed independently the absence of N-Bak protein in the healthy SCG and cortical neurons whereas the peptides of N-Bak were clearly identified in the positive control samples. The observed protein of about 21 kD in the cortical cultures was therefore not N-Bak, as claimed by others (Uo et al, 2005), but appeared to be non-specifically recognized by the anti-Bak antibodies.

4.4 N-Bak protein is not rapidly degraded by proteasome (I)

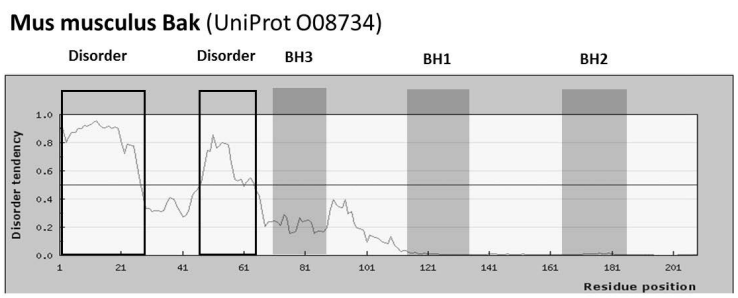
A potential signal for accelerated intracellular proteolysis**,** known as PEST sequence, encompasses the residues 12-33 of mouse Bak and N-Bak protein as predicted by computational analysis with the Mobyle program. Immunoblot analysis revealed no detectable endogenous N-Bak protein in any of the protein samples, obtained from the cultured cortical neurons subjected to proteasome inhibition with MG-132 for 16 or 24 hours, in presence or absence of pan-caspase inhibitor BAF. Control heat shock protein (Hsp70) was expectedly upregulated in the samples treated with MG-132. Thus, no evidence was found for rapid proteasome-mediated degradation of endogenous N-Bak protein in neurons.

4.5 N-Bak protein may contain low-complexity and disordered regions (unpublished)

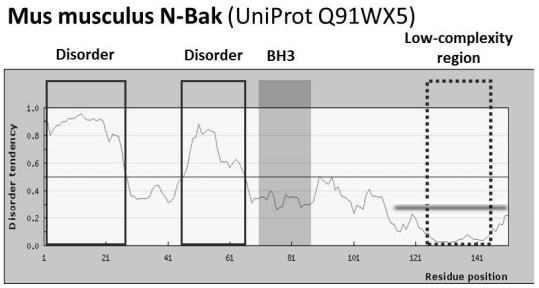
Disorder-to order transitions are common in BH3-only protein subgroup playing central role in their dynamics and post-translational regulation. Low complexity sequences enable transient, but specific interactions between multiple binding partners allowing proteins to assamble into signaling hubs. We analyzed the presence of ordered/disordered and low-complexity regions within the mouse Bak and N-Bak protein sequences using IUPred bioinformatics tool and eukaryotic linear motifs (ELM) database search (Dosztanyi et al. 2005; Dinkel et al. 2012). IUPred is designed to predict of intrinsically unstructured regions within the proteins based on the difference in sequence characteristics that are present in well-characterized folded and disordered proteins and the estimated interaction energies to form stabilizing contacts at the local sequential neighbourhood (Dosztanyi et al. 2005). ELM prediction algorithms enable to find putative linear motifs in user-submitted protein sequence based on experimentally validated linear motifs (Dinkel et al. 2012).

IUPred prediction showed that two regions within the Bak and N-Bak N-terminal part, spanning the amino acid residues 1-25 and 47-64, are likely disordered (Figure 15 a-b) (Jakobson and Arumäe, unpublished data). ELM prediction showed that the C-terminal part of N-Bak (amino acid residues 127-140) may contain low-complexity region (Figure 15b) (Jakobson and Arumäe, unpublished data). The latter resides within the sequence that is unique for N-Bak, and thus is not present in Bak (Figure 15 a, b). Neither disordered regions nor low-complexity region overlaps with classical BH domains when compared in BLAST sequence analysis tool. Combined data from bioinformatics predictions results are presented in the Figure 15. These results suggest that N-Bak is partially disordered protein, which may require more structured partners for its stability. The C-terminal low-complexity region may mediate uniqe interactions with multiple proteins.

a



b



c

	Motif	Position(s)	Length in aa	Feature identifier
Bak	disorder	1-25	~25	IUPred
	disorder	47-64	~17	IUPred
	BH3	72 – 86	15	Blast
	BH1	115 – 134	20	Blast
	BH2	167 – 182	16	Blast

	Motif	Position(s)	Length in aa	Feature identifier
N-Bak	disorder	1-25	~25	IUPred
	disorder	47-64	~17	IUPred
	BH3	72 – 86	15	Blast
	Low-complexity region	127-140	~13	ELM

Figure 15. Combined graphical outputs of IUPred and ELM searches, showing the prediction results for ordered/disordered and putative low complexity regions within mouse Bak (a) and N-Bak (b) protein sequences. IUPred classifies a sequence region as either structured or disordered. Locally stabilizing interaction values higher than the cutoff of 0,5 correspond to disorder and are shown above the threshold line in the output graph. Lower values indicate structured regions and are displayed below the threshold line. Boxes with thick black lines mark the disordered regions in Bak and N-Bak N-terminal part. A box with a dashed line indicates the position of low-complexity region in the C-terminal unique part of N-Bak protein. Boxes, shaded with dark grey show well-defined BH (Bcl-2 homology) domains in Bak (BH1-3) (a) and N-Bak (BH3)(b) as obtained from a BLAST sequence analysis.

4.6 N-Bak protein expression was not induced in the apoptotic or stressed neurons (II)

Mitochondrial apoptosis was induced in cortical neurons with the inhibitor of topomerase II, etoposide and in SCG neurons by NGF deprivation. Neither condition led to expression of N-Bak protein as shown by immunoblot analysis.

Mild ER-stress leads to translational reprogramming through increased phosphorylation of general translation initiation factor 2 (eIF2α). Cultured cortical neurons, subjected to thapsigargin-induced endoplasmic reticulum stress showed maximal level of eIF2α phosphorylation after 2h of treatment. However, endogenous N-Bak protein was not detected in the neurons under thapsigargin-induced stress, as demonstrated by immunoblot analysis

Also, quantitative mass spectrometry analysis did not reveal endogenous N-Bak protein from the protein samples derived from NGF-deprived sympathetic neurons or

from cortical neurons subjected to etoposide-induced intrinsic apoptosis, thapsigargin-induced cell stress (II) or hypoxia (not published).
These data demonstrated that N-Bak protein expression was not induced in the apoptotic or stressed neurons.

4.7 N-Bak mRNA escapes nonsense-mediated decay and is stable in neurons (I, II)

N-Bak mRNA meets the criteria to be a substrate for translation dependent cellular quality control pathway, termed nonsense-mediated decay (NMD). Translation inhibitor cycloheximide (CHX) allows accumulation of the NMD substrate mRNAs in cells.

Quantitative RT-PCR analysis revealed that CHX treatment of cultured cortical neurons led to small but significant increase of N-Bak transcripts by 8 hours of treatment whereas during 4 hours no significant accumulation occurred. In rat pheocromocytoma PC6 cells, increase in the N-Bak mRNA level was observed by 4 hours of treatment. In both cell types the increase of N-Bak mRNA levels was however completely blocked by co-treatment with transcriptional inhibitor ActD. Thus, previously detected increase of N-Bak mRNA levels in the CHX treated cells was caused by increased transcription, not by decreased degradation. The mRNA for pro-apoptotic Bcl-2 family member Bax that lacks premature termination context did not show any accumulation under any conditions, showing the specificity of our assay. In summary, N-Bak mRNA is not a constitutive substrate for NMD but rather belongs to a class of NMD-escape mRNAs.

4.8 N-Bak mRNA is stable in neurons (I)

Endogenous N-Bak mRNA stability was studied in the cortical neuron cultures and rat pheocromocytoma PC6-3 cell line, subjected to transcriptional inhibition with RNA pol II inhibitor ActD. To that end, temporal dynamics of N-Bak and, for comparison, Bax mRNA degradation was determined by qRT-PCR. The data were normalized to RNA polymerase III transcribed noncoding RNAs that are insensitive to ActD mediated transcriptional inhibition.

N-Bak mRNA appeared to be remarkably stable in the cultured cortical neurons, whereas the levels of Bax transcripts decreased in time, as expected. In the ActD-treated PC6 cells, N-Bak and Bax transcripts decreased with the same rate in time. Our data do not support hypothesis of accelerated turnover of N-Bak mRNA in these cells.

4.9 Prevention of alternative N exon splicing and PTC did not lead to translation of resulting Bak mRNA in neurons (I)

The endogenous Bak pre-mRNA splicing was interfered in cultured neurons to study if the absence of exon N and PTC affect the resulting Bak mRNA translation in neurons.

Vivo-Morpholino (vMO) oligonucleotides, designed to block inclusion of exon N and thus revert the Bak type of splicing in the neurons were applied to cell culture media.

RT-PCR analysis showed that application of the exon N-specific oligonucleotides to cortical neurons lead to significant appearance of Bak mRNA with concomitant disappearance of N-Bak mRNA, whereas scrambled control oligonucleotides had no effect.

Immunoblot analysis with anti-Bak antibodies (06-536, EMD Millipore) showed no increase of Bak protein in any cortical cultures forced to express Bak mRNA. It shows that prevention of alternative splicing does not lead to Bak protein translation in neurons. Bak protein levels were also not increased in vMO treated cultures simultaneously treated with caspase inhibitor BAF or proteasome inhibitor MG-132. In summary, our data suggested that Bak and N-Bak mRNAs are translationally supressed in neurons.

4.10 N-Bak mRNA UTRs contain translationally repressive regulatory elements (I, II)

Potential translationally repressive regulatory elements in the 5'- or 3'-UTRs of N-Bak mRNA were studied using Dual Luciferase Reporter assay using reporter constructs where UTRs with wild-type or deleted candidate control regions of the N-Bak mRNA were fused upstream or downstream of Firefly luciferase (Fluc) coding sequence (Figure 16). In neuroblastoma Neuro-2a cells, Fluc activities were measured and normalized to Rluc activities. In neurons, FLuc/Rluc ratio was normalized to the number of injected neurons. Reporter mRNA levels were analyzed in parallel from Neuro-2a samples with qRT-PCR.

Transcript levels for all used reporters were similar, showing that measured relative luciferase activities reflect rather the differences in translation efficiency of the reporter mRNAs than differences in reporter mRNA turnover rates. Mouse Bak and N-Bak transcripts share common 253 nucleotides long 5'UTR with two upstream open reading frames (uORFs). First, 189 nucleotide long uORF (uORF1) with initiator AUG in the context of Kozak consensus sequence and second (uORF2) with no clear Kozak sequence, laying within the first uORF (Figure 16). Human *bak* transcripts have also similar buildup in the 5´-leader region (Figure 16), although the distance of uORF1 initiation codon from the cap is longer than that in the mouse transcripts and the nucleotide context surrounding the uORF2 start codon is not optimal (Table 9). In both species the uORF1 lenghts and their stop codon distances to the respective main ORF initiation codon were similar (Table 9), whereas this feature was not characteristic to uORF2 (Table 9). However, the distance between uORF1 and uORF2 start sites is 59nt in both species.

In sympathetic neurons the presence of mouse full-length N-Bak 5'UTR significantly decreased the relative luciferase activity, showing 2.6 fold decreased activity compared to control Fluc values. Deletion of 38 nt from the proximal segment of 5'-UTR, including the initiation codon of the first uORF (Figure 16), markedly increased the translation efficiency of the reporter mRNA compared to full-length 5'UTR reporter. These results demonstrated the inhibitory role of the first uORF in (N-)Bak 5-'UTR.

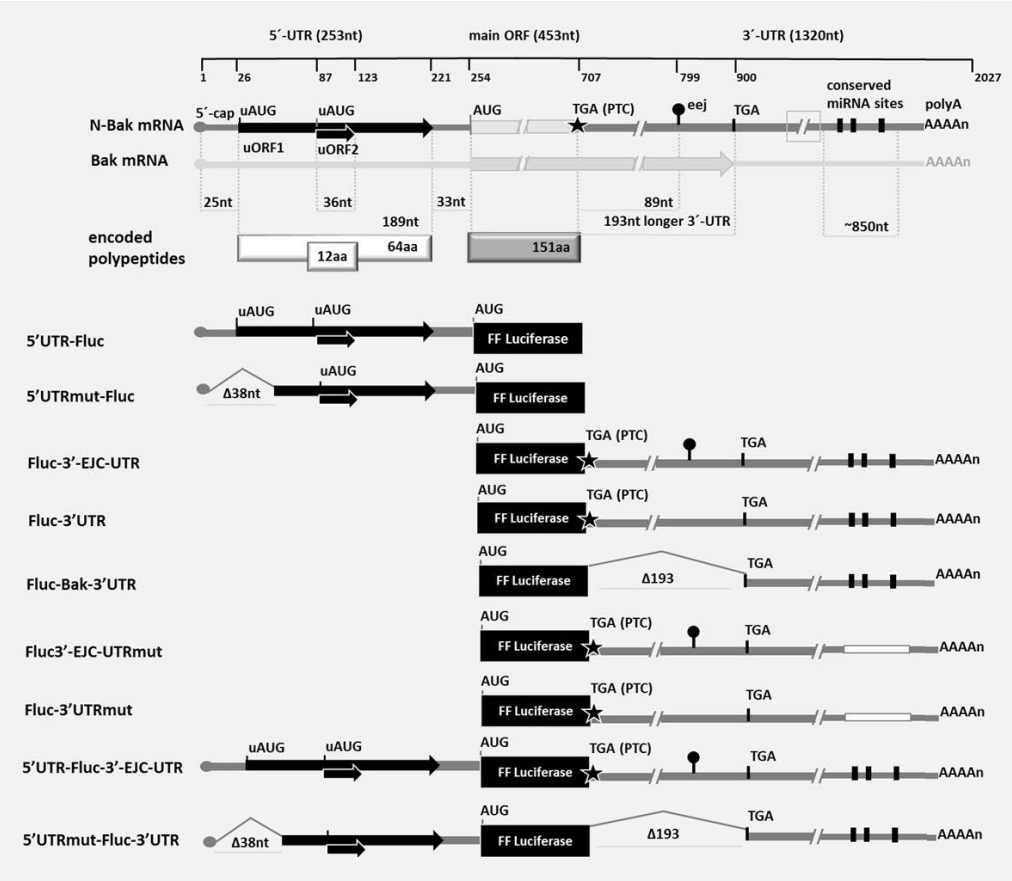


Figure 16. Scematics of N-Bak and Bak mRNA structure and luciferase reporter (Fluc) constucts used in this thesis. Shown are the positions and lenghts of the uORF1, uORF2 and main coding sequence (main ORF) of N-Bak and Bak mRNA. The lengths of the predicted polypeptides, encoded from uORFs are indicated by white boxes, whereas polypeptide encoded by main ORF is shown as a gray box. Firefly luciferase reporter gene is depicted as black rectangles. Intact or variously modified N-Bak and Bak 5´-and 3´-UTRs are shown in the left and right side of the Firefly luciferase reporter. AUG-translation initiation codon; eej-exon-exon junction; TGA-termination codon; TGA (PTC)-premature stop codon; uAUG-upstream AUG; uORF-upstream open reading frame; Δ-deletion.

Table 9. Comparison of caracteristic features found in mouse and human (N)-Bak 5´-UTR.

Sequence features found in N-Bak mRNA	5'-UTR				CDS	
	uORF1		uORF2		genic ORF	
	mouse	human	mouse	human	mouse	human
ORF length (nt)	192	198	36	15	453	459
encoded polypeptide length (aa)	64	66	12	5	151	153
initiation codon distance from cap (nt)	25	74	84	135	245	300
Translation initiation context (5´-3´)	TCT**ATG**G	TCT**ATG**A	ACC**ATG**A	CAG**ATG**C	AAA**ATG**G	AAA**ATG**G
uORF STOP codon distance to mORF ATG (nt)	33	27	159	147		
uORF STOP codon distance to next eej (nt)	< 50	< 50	> 50	> 50	>50	>50

The 3'-UTR of N-Bak mRNA harbors premature termination codon (PTC) at its second last exon, followed by the last exon-exon junction 89 nucleotides downstream of PTC (Figure 16). The effect of such premature termination context on mRNA translation was studied with luciferase reporters carrying wild-type (i.e. with an added intron) and intronless N-Bak 3'UTRs (Figure 16). Reporter construct with wild-type Bak 3'UTR, starting from its natural stop codon on the last exon, was used as control. Correct splicing of intron containing N-Bak 3'-UTR reporter was verified by RT-PCR analysis with primers flanking addressed exon-exon junction sequence. Thus, it represents the wild-type N-Bak mRNA with PTC and EJC downstream it.

Despite differences within the most proximal part of their 3'-UTRs, intronless 3'-UTR reporters of N-Bak and Bak did not show significant differences in the luciferase activities in either cell type. Thus, no repressive elements were found in the sequence between the PTC and downstream stop codons. The spliced N-Bak 3'-UTR reporter demonstrated significantly reduced luciferase activity compared to the intronless reporters in both cell types. Thus, repressive effect was found only in the sequence context which most closely mimicked the premature termination context of the N-Bak mRNA.

Luciferase reporters, flanked with full-length N-Bak 5'-UTR and its intron containing 3'-UTR showed dramatically decreased expression when compared to the reporter construct where uORF1 initiation site and premature termination context were deleted (Figure 16). It suggests that both elements can synergistically contribute to the endogenous N-Bak suppression.

Possibility of the mRNA translational repression, mediated by miRNA binding sites, was studied with modified wild-type and intronless N-Bak 3'UTR reporters, where the 3'UTR region encompassing all three predicted microRNA sites was replaced with the heterologous sequence about the same length (Figure 16). Evolutionarily conserved binding sites for conserved miRNAs, miR-29abcd (UGGUGCUA), miR125ab-5p (CUCAGGGA) miR26ab (UACUUGAA) on the 3'-UTR of Bak mRNA were found from predictions of TargetScan 5.1 program (Table 10). The replacement of miRNA binding region increased the luciferase activity in a small but significant scale only in the context resembling spliced wild-type N-Bak 3'-UTR reporter (Figure 16) in both cell types whereas intronless 3'-UTR reporter (Figure 16) did not show a significant difference in luciferase activity values.

Table 10. Conserved miRNA binding sites of conserved miRNA families found in (N)-Bak 3´-UTR.

3'-UTR	
Conserved sites for conserved miRNA families	
mouse	**human**
mmu-miR-29a-c	hsa-miR-29a-c
mmu-miR-125a,b-5p	hsa-miR-125b/hsa-miR-125a-5p
mmu-miR-351	hsa-miR-27a,b
mmu-miR-26b	hsa-miR-26a,b/hsa-miR-1297

In summary, two translationally repressive regulatory elements, an inhihitory uORF region in the 5'UTR and exon-exon junction downstream of PTC in the 3'UTR, were found.

4.11 N-Bak mRNA has granular pattern in the SCG neurons (II)

Intracellular localization of endogenous N-Bak mRNA was studied in the NGF maintained or deprived mouse sympathetic neurons—by *in situ* hybridization, utilizing LNA oligonucleotide probes designed specifically to detect a 20 nucleotide exon N within the N-Bak mRNA. Antisense probe-hybridized neurons exhibited dense granular pattern, located mostly in the soma, whereas the nuclei were free of the signal. Similar granular pattern for N-Bak was also detected in NGF deprived apoptotic neurons. The sense probe for exon N was used as a negative control, demonstrating faint and disperse pattern in healthy neurons. Non-neuronal cells, lacking N-Bak mRNA did not show specific signal in any hybridization. Pyknotic dying neurons could non-specifically bind both, antisense and sense LNA probes.

N-Bak mRNA association with mRNA storage and processing granules, such as P-bodies, was studied by combining in situ hybridization and immunostaining in sympathetic neurons using LNA probes for N-Bak mRNA and antibodies for P-body component Dcp1α detection. First, anti-Dcp1α antibodies were demonstrated to specifically recognize ectopically expressed GFP-Dcp1α fusion protein in sympathetic neurons. The N-Bak mRNA- granular signal and endogenous Dcp1α -positive signal did not co-localize. Thus, N-Bak mRNA is associated with the granules whose identity remained elusive.

5 DISCUSSION

Aberrant regulation of cell death accompanies various human diseases and is therefore in a focus of extensive biomedical research today. Several lines of evidence now suggest that apoptotic pathways in neurons are more tightly refined than in most of the dividing cell types (Kole et al. 2013). Many of those molecular mechanisms are shared with other postmitotic cell types and may contribute to apoptosis resistance in cancer cells. Therefore, better characterization of cell- and tissue-specific features that regulate cell death programs may provide new insights to understand developmental and pathological processes.

Recent studies suggest that neurons frequently govern their highly specialized structures and functions through a variety of RNA regulation modes (Licatalosi and Darnell 2010; Scheckel and Darnell 2015). In general, such mechanisms are part of the post-transcriptional control which plays critical roles in the actualization and fine tuning the genetic programs in eukaryotic cells (Di Liegro et al. 2014). It suggests that more thorough understanding of various post-transcriptional mechanisms may lead to potential new strategies to modulate apoptotic cell death machinery in injured or diseased neurons. It may also improve our knowledge of other cellular contexts, such as tumorigenesis, where better cell survival is achieved through the strict control of apoptosis machinery.

The current study was initiated to examine the role of *bak* gene alternative splicing in neurons, and particularly focused on the molecular mechanisms that regulate the expression of its exclusively neuronal isoform N-Bak. Our findings reveal that multiple post-transcriptional regulatory mechanisms repress endogenous N-Bak protein expression in neurons. Collectively our results underline the importance to further study various post-transcriptional control mechanisms in the regulation of key apoptosis factors.

5.1 General considerations of Bak and N-Bak function in nervous tissue

Previous studies have demonstrated that mRNA for Bak is ubiquitously expressed in several non-neuronal cell types, whereas its alternative isoform N-Bak can be found in the nervous tissue. Results presented in this thesis confirm the original findings by Sun and colleagues (2001) that the expression of N-Bak mRNA is exclusively restricted to neurons, at least in neuronal populations studied. It suggests that the *Bak* mRNA isoforms are expressed in a specific temporal order and mutually exclusive manner in the developing nervous system.

We also found that both, the length and the position of exon N between constitutively spliced exons 4 and 5 of the *Bak* gene are evolutionally conserved in mammals, suggesting that N-Bak-type of splicing may be a pan-neuronal phenomenon that is critical for neuron´s physiology. Whether it's the evasion of Bak or presence of N-

Bak that has a functional relevance in neuronal apoptosis raises several interesting questions.

We find it intriguing to speculate that post-mitotic neurons do not tolerate full-length Bak expression and thus replace its transcript to a novel isoform that eliminates the possibility to express multidomain Bak protein in neurons. This idea is in line with evidence implying that apoptotic pathways undergo maturation-dependent changes (Kole et al. 2013), resulting in more stringent control over the apoptotic pathways. It appears to be a rather general feature also in other post-mitotic cell types (Kole et al. 2011b; Gama et al. 2014). In mouse neurons, cytochrome c is targeted for proteasome-mediated degradation (Gama et al. 2014), caspases are shown to be strongly associated with inhibitory XIAP protein, and in the adult brain, the expression of Apaf-1 and caspase-3 are down-regulated (Yakovlev et al. 2001; Kole et al. 2013). These mechanisms are suggested to support neuronal survival, predominantly to resist mitochondrial damage and decrease any risk of apoptosis caused by the accidental release of cyt *c* (Gama et al. 2014). Neuronal apoptosis pathways can be exploited also in a stimulus-specific manner (Selznick et al. 2000; Yu et al. 2003b). Several studies highlight the sole importance of Bax in mediating MOMP in neuronal apoptosis, (Deckwerth et al. 1996; Miller et al. 1997; Deshmukh and Johnson 1998; White et al. 1998; Xiang et al. 1998; Cregan et al. 1999; Lindsten et al. 2000; Fan et al. 2001; Putcha et al. 2002; Wright and Deshmukh 2006; Kole et al. 2013), further indicating that prevention of Bak expression may be critical for the long-term survival of the neurons. Therefore, removal of full-length Bak expression in neurons through its pre-mRNA alternative splicing may be one of the first brakes for the neurons to avoid accidental apoptosis.

Putative protein encoded by N-Bak mRNA retains the BH3 death domain but lacks the other characteristic BH domains of the Bak, thus representing a BH3-only isoform of Bak. Whether N-Bak accounts for a novel, functionally relevant BH3-only protein *in vivo* in neurons is another intriguing question waiting to be answered.

Previous studies analyzing the expression of endogenous Bak in nervous tissue (Krajewski et al. 1996; Krajewski et al. 1999; Krajewska et al. 2002) have used antibodies which do not distinguish between the Bak isoforms in immunocytochemical experiments. In addition, the specificity of these antibodies has not been tested in *Bak*-deficient tissues and thus, cannot be considered as compelling evidence for Bak expression in the neurons. Consequently, alternative explanations, such as unspecific binding or detection of N-Bak signal instead of the Bak, should also be considered when interpreting immunocytochemical staining results.

The studies addressing the physiology of Bak-deficient neurons have concluded that *bak* deficiency does not protect neurons from major developmental death stimuli (Deckwerth et al. 1996; White et al. 1998; Putcha et al. 2002). However, overexpressed Bak may modulate cell death pathways in the brain and dissociated primary neuron cultures (Farrow et al. 1995; Kiefer et al. 1995; Sun et al. 2001; Fannjiang et al. 2003). While *bak* deficiency protects some type of neurons from pathological insults, such as stroke and Sindbis virus infection-induced cell death (Fannjiang et al. 2003; Fei et al. 2008), this effect may change during neuronal maturation (Fannjiang et al. 2003). To

what extent *bak* deficiency in glial cells contributes to these effects has not been thoroughly studied.

Both, overexpressed Bax and Bak have been implicated to act as pro-survival proteins in certain types of developing neurons, as well as under some pathological conditions (Middleton et al. 1996; Middleton and Davies 2001; Fannjiang et al. 2003; Fei et al. 2008). It indicates that their role in neuronal apoptosis may be developmental stage, neuronal subtype and stimulus-specific. However, biochemical evidence for endogenous Bak expression in neurons has remained non-conclusive and currently there are no mechanistic insights to explain the anti-apoptotic activity for Bax and Bak in certain neuronal populations. The study by Fannjiang and colleagues (2003) also suggests that Bak may modulate neuronal excitability in the brain. Whether such phenotype reflects Bak function in neurons or is a result of neurodevelopmental defect or dysfunction in non-neuronal cells in the brain of the *bak* null mice is uncertain (Henshall and Engel 2013).

Given that N-Bak is expressed in various types of neurons, it is likely that some of the roles attributed to Bak in neurons may refer to the function of N-Bak instead. To date, no studies have addressed this possibility in detail. Contrary to the classical BH3-only proteins, overexpressed N-Bak appears to be anti-apoptotic in sympathetic neurons, while it is pro-apoptotic in other cell types (Sun et al. 2001; Uo et al. 2005). The level at which overexpressed N-Bak executes its anti-apoptotic effects in neurons is, however, not clear. It also remains unknown whether the effects observed in previous *in vitro* experiments contribute to apoptosis regulation *in vivo* in neurons. Likewise, the underlying mechanisms for the different functions of overexpressed N-Bak in neuronal and non-neuronal cells remain to be studied. To date, N-Bak function in neurons physiology *in vivo* can be only indirectly inferred from studies that have characterized *bak* deficiency in neurons (Fannjiang et al. 2003; Henshall and Engel 2013). Altogether, these open questions will probably drive further research efforts in future.

5.2 Detection of endogenously expressed N-Bak protein in healthy neurons

Previous studies, attempting to detect endogenously expressed N-Bak protein, have shown controversial results. In several experimental settings the expression of endogenous N-Bak protein has remained undetectable (Sun et al. 2001), whereas Uo and colleagues (2005) have demonstrated that N-Bak is expressed at the noticeable level, at least in cortical neurons. This conundrum let us set up the biochemical analyses, in particular complementing immunoblot and mass spectrometry analyses, to find out whether the endogenous N-Bak protein is detectable in nervous tissue. Several antibodies, generated to recognize the N-terminal region of Bak, could be used to study N-Bak protein expression from samples derived from nervous tissue or cultured neurons. Our comparison of four commercially available anti-Bak antibodies resulted in the selection of two antibodies that recognized overexpressed and in vitro translated N-Bak from the positive control samples. Contrary to the report of Uo et al., (2005) we found

that Ab-2 (EMD Millipore/Calbiochem) antibody is not specific to detect N-Bak in immunoblots. While our results demonstrate that N-Bak mRNA is exclusively expressed in neuronal tissue and primary cultured neurons, we did not detect any endogenous N-Bak protein expression in healthy neurons with none of the used antibodies.
However, sole immunoblot analysis did not exclude the possibility that the N-terminal part could be somehow modified in endogenous N-Bak, thus remaining undetectable in immunoblots. An interesting feature found in the N-terminal part of N-Bak protein sequence by bioinformatics prediction was the region enriched in proline, glutamic acid, serine and threonine residues, termed as PEST signal, which commonly marks the protein for accelerated proteasomal degradation (reviewed in Rechsteiner and Rogers 1996). Targeting BH3-only proteins to the proteasomal degradation pathway is a common mechanism to control their levels in healthy cells. The presence of PEST sequence in N-Bak may, therefore, decrease its stability and result in virtually undetectable levels in the cells. Our analysis of proteasome inhibitor-treated cortical neurons did not reveal any signal from endogenous N-Bak protein indicating that its absence cannot be explained by accelerated proteasome degradation.

In recent years, it has been increasingly acknowledged that Bcl-2 family proteins contain intrinsically disordered regions, important for their transient interactions with the membranes or other proteins (Hinds and Day 2005; Kvansakul and Hinds 2013). BH3-only proteins, in particular, appear to lack a well-defined tertiary structure in their native, functional state and are therefore viewed as intrinsically disordered proteins. Intrinsically disordered regions are enriched with short linear motifs, which are composed of short stretches of adjacent amino acids that interact with linear motif binding protein domains (Dinkel et al. 2012; Yang et al. 2016). It has been also recently suggested that BH3 motifs should be redefined as short linear motifs (Aouacheria et al. 2015), as it would help to explain the biology of expanding list of non-canonical BH3-only proteins. Nevertheless, presence of short linear motifs and disordered regions provide a wide range of functionality to proteins (Davey et al. 2011; Van Roey et al. 2014) and are therefore primary targets for the post-translational modifications that result in the changes in protein activity, localization or stability within the cell (Yin 2006).

N-Bak mRNA is generated via alternative splicing of *Bak* pre-mRNA. Its putative N-terminal structure derives from exons encoding the most unstructured part of Bak, whereas its C-terminal part is completely unique. We found that this part contains also a low-complexity region. These observations raise a possibility that N-Bak is an unstable protein that requires stabilization through some posttranscriptional modification. Therefore, its common epitope with Bak may be modified or inaccessible for the used antibodies. Our complementary quantitative mass-spectrometry analysis, however, confirmed that no N-Bak specific peptides were present in samples obtained from the cultured neurons. Thus, we concluded that N-Bak is not constitutively expressed in healthy neurons.

5.3 Induction of N-Bak in apoptotic or stressed neurons

Whether the N-Bak expression is induced upon some physiologically relevant trigger was next in our focus. It is well established that challenging metabolic condition or harmful agents elicit various signaling pathways in the cells that enable to adjust the stress response and maintain homeostasis. Due to the implications that BH3-only proteins sense different stress signals and are intimately involved in apoptosis signaling, induction of N-Bak might be expected. The expression of classical BH3-only proteins can be induced transcriptionally (e.g. Noxa and Puma) and through the variety of post-translational mechanisms releasing them from the sequestering protein complexes. For instance, induction or activation of some proapoptotic BH3-only proteins is sensitive to the metabolic status of the cell (Coloff et al. 2011; Altman and Rathmell 2012), whereas others respond to the irradiation or trophic factor deprivation. It has been suggested that varied induction and activation of BH3-only proteins allows cells to integrate complex death signals more accurately (Aouacheria et al. 2013).

The previous study by Uo and colleagues (2005) demonstrated increased N-Bak expression in the etoposide-treated apoptotic cortical neurons due to the transcriptional upregulation. Our immunoblot analysis, however, showed that endogenous N-Bak protein expression was not induced in the etoposide-treated apoptotic neurons. We also found that the N-Bak protein was not induced in classical mitochondrial apoptosis elicited in NGF-deprived sympathetic neurons, as well as in stressed neurons, where thapsigargin treatment induces an ER- stress response. The latter leads to the increased phosphorylation of eIF2a, resulting in translational reprogramming and increased protein synthesis from several mRNAs, which encode stress-related proteins. The findings that N-Bak is not induced in apoptotic or stressed neurons were further confirmed by quantitative mass spectrometry analysis.

Of note, we observed a signal in our immunoblots, which was comparable to the size of N-Bak in positive control samples. The signal of this band changed under some experimental conditions, but it was observed inconsistently. Therefore, it is likely that it may derive from the non-neuronal cells that are present in varying numbers in our primary cell cultures. We suggest that the same non-specific band was reported earlier by others (Uo et al. 2005) as N-Bak in the cortical cultures explaining the discrepancy between different studies.

We concluded that endogenous N–Bak protein is not expressed in apoptotic nor stressed neurons and thus does not contribute to the initiation of classical mitochondrial apoptosis. However, we cannot exclude that some other pro-apoptotic stimuli may induce its expression in neurons.

Several aspects of the biology of classical Bcl-2 family proteins are only now starting to be uncovered. Hence, it is also likely that N-Bak mRNA may respond to some cues, which may be not related to apoptosis but challenge neuronal physiology in general. Additional roles of Bcl-2 family members in cell involve several homeostatic functions related to mitochondrial dynamics, energy metabolism, Ca^{2+} homeostasis and autophagy (Vander Heiden and Thompson 1999; Rong and Distelhorst 2008; Danial et al. 2010;

Luciani et al. 2013). For example, a BH3-only protein Bad has a physiological role in glucose metabolism (Danial et al. 2008). Another BH3-only family member Bid contributes to the pathways controlling genomic stability and mitosis (Kamer et al. 2005; Zinkel et al. 2005). It remains to be studied whether N-Bak is required for any physiological function in the cell. The presence of unique SLiM at the C-terminal part of N-Bak suggests its role in mediating some isoform-specific interactions of N-Bak. Recent global study assessing an AS mediated changes in protein interaction capabilities, found that alternative inclusion or exclusion on interacting linear motifs or other domains could significantly modulate protein-protein interactions (Yang et al. 2016). Consecuently, it was concluded that alternative isoforms tend to behave like distinct proteins rather than as minor variants of each other (Yang et al. 2016). Therefore, it is likely that putative N-Bak protein is functionally more diverse from Bak than it could be expected and its expression is not related to apoptosis signalling.

Based on our results of analyzing endogenously expressed N-Bak protein in neurons, we concluded that the lack of concordance between N-Bak mRNA and protein levels in neurons reflects post-transcriptional regulation rather than technical constraints.

5.4 Post-transcriptional regulation in N-Bak expression

Gene expression regulation at the post-transcriptional level has a central importance in maintaining cellular function and homeostasis (Lee and Gorospe 2011; Kiebler et al. 2013). Alternative mRNA processing, and AS in particular is a powerful mechanism that can significantly modulate the transcriptome and proteome diversity in the eukaryotic cells. According to general estimate, nearly 21,000 human genes may give rise more than 80,000 differentially spliced mRNA species that may encode different protein isoforms with different properties and functions (de Klerk and t Hoen 2015; Mockenhaupt and Makeyev 2015). However, alternative mRNA transcripts could also be differentially regulated in the cell. All these features might differ in a cell type- or developmental stage-specific manner.

N-Bak is an alternatively spliced variant of *bak* pre-mRNA. It suggests that its changed composition may have key importance in determining its mRNA post-transcriptional fate in the cell, including its stability, localization and ultimately its translatability into a functional protein. We found that N-Bak mRNA possesses several compositional features that are known to be involved in post-transcriptional control. Those are the uORFs, located in the N-Bak mRNA 5'-UTR, and conserved miRNA binding sites in the N-Bak mRNA 3´-UTR, that might potentially decrease its stability or suppress its translation in neurons. Also, we observed that N-Bak mRNA main ORF stop codon is followed by markedly similar context that is frequently observed in the transcripts susceptible to nonsense-mediated decay (NMD) (Isken and Maquat 2008).

5.5 uORFs in repressing N-Bak mRNA translation

Several cis-acting regulatory elements in the mRNA 5´-UTR, including uORFs, uAUGs and secondary structures may contribute to the mRNA translational repression. We found that *bak* transcripts contain two uORFs, (designated as uORF1 and uORF2 in this thesis).

There is no significant conservation between the human and mouse uORF in *bak* leader sequences, raising questions about their functional relevance on *bak* gene regulation in different organisms. However, bioinformatics studies suggest that many other features, such as the uORF translation initiation context as well as its length, AUG position relative to 5´cap and proximity to the main coding sequence can contribute to the functional activity of uORFs in translational repression (Iacono et al. 2005; Calvo et al. 2009). Furthermore, only 38% of uORFs are conserved among human rat and mouse mRNA transcripts (Iacono et al. 2005). It is also continuously emphasized that structural properties of uORFs are transcript-specific and their regulatory functions are highly variable (Wethmar 2014). The presence of more than one uORF further complicates validating uORF-mediated translational regulation as uORFs may combine their effects on main ORF translation regulation often in a context-specific manner (Wethmar et al. 2010b).

We found that initiation codon of mouse uORF1 resides in moderate Kozak´s context, whereas its initiation context in the human transcript is weak. Initiation context was also weak in mouse and human uORF2, which resides within the uORF1. It is not surprising, as bioinformatics studies indicate that uORFs do not have distinct initiation sequence contexts that promote their translation (Chew et al. 2016). It is different from main ORFs, where up to 90% of initiation codons are embedded into either strong or adequate initiation context (Iacono et al. 2005).

Interestingly, the lengths of human and mouse *bak* uORF1 were very similar, encoding respectively putative peptides with the lengths of 64 and 66 amino acid residues. Also, the distance from uORF1stop codon to the downstream genic ORF initiation codon was similar, being in mouse and human transcripts respectively 33 and 27 nucleotides long. Since both, uORF encoded peptides, as well as uORF termination context, may modulate the translational activity of downstream ORFs (Chew et al. 2016), it would be interesting to investigate whether these features have a role in N-Bak and Bak mRNA translational control.

We demonstrated that intact uORFs present within *bak* 5´-UTR are able to decrease luciferase reporter protein expression significantly in sympathetic neurons and neuroblastoma cells. Our quantitative real-time PCR results suggest that reporter mRNA synthesis or decay rates were not increased. It let us conclude that uORFs may contribute to endogenously expressed N-Bak mRNA translational repression. However, Bak and N-Bak mRNAs probably share the uORFs in 5´-UTR, suggesting that uORFs may have a universal role in regulating translation of both *bak* isoforms. Indeed, our 5´-UTR reporter constructs did not discriminate between N-Bak and Bak transcripts and we detected reporter mRNA translational repression also in non-differentiated Neuro-2a cells, which express both *bak* isoforms. However, only Bak protein is detectable in Neuro-2a cells

whereas N-Bak is not, suggesting that *bak* uORFs can only partially hinder the protein expression of *bak* transcripts. It is in agreement with the general observation that multitude of factors can modulate the regulatory impact of uORFs (Wethmar 2014). By current estimate, uORFs reduce protein translation from the downstream ORF by 30-80% (Calvo et al. 2009). Therefore, it is likely that differential translation of *bak* transcripts may depend on transcript-specific features and/or cellular factors associating with them.

Notably, our results revealed that luciferase reporter activity was even more decreased when both, the 5′-UTR and the N-Bak mRNA 3′-UTR were present in the reporter construct. It suggests that N-Bak mRNA 5'- and 3'-UTRs may cooperate to repress its translation. Indeed, uORF-mediated translational control can involve the interactions with trans-acting factors or other sequence elements from other parts of the mRNA. Such cooperation may be cell type or context specific and thus modulate the functional activity of uORFs in translational repression. For example, human epidermal growth factor receptor 2 (Her-2) mRNA contains uORF that negatively controls its translation in normally dividing cells (Child et al. 1999). However, Her-2 mRNA is efficiently translated in cancer cells (Mehta et al. 2006). This effect is driven by mRNA 3'UTR that counteracts the inhibitory effect of uORF, resulting in more efficient translation of Her2 main ORF in cancer cells (Mehta et al. 2006).

We found that naturally spliced exon-exon junction downstream of the N-Bak PTC represses luciferase reporter translation. Spliced exon junctions are marked with exon junction complexes, which are remodelled during the mRNA transport and translation. Although the addressed exon-exon junction (eej) in N-Bak mRNA is also present in Bak mRNA, it is a part of the main coding sequence of Bak and is probably removed by the translating ribosome. On the contrary, in the N-Bak mRNA, the eej associated proteins may remain bound with the 3′-UTR and may, therefore, recruit regulatory proteins that execute translational repression either alone or in cooperation with 5′-UTR. Interestingly, the role of ECJ downstream of PTC has been also implicated in NMTR (Lee et al. 2010), where it plays an inhibitory role in translational repression. It is however not addressed in earlier studies, whether NMTR regulated transcripts contain uORFs or not. Characterization of molecular mechanisms underlying uORFs function in 5′-UTR and their possible cooperation, for instance with the NMD activating features at the transcript 3′-UTR, is beyond the scope of this thesis and requires more thorough investigation in future.

It is also possible that 5′-uORFs function in Bak and N-Bak mRNA are differently regulated by cell type-specific factors. For example, recently discovered trans-acting protein complex, DENR–MCT-1 can selectively modulate translation re-initiation rates in higher eukaryotic cells. (Schleich et al. 2014). It was demonstrated that unlike in stress-related GCN/ATF4 or ATF5 regulation paradigm, DENR–MCT-1 controls translational status of uORFs containing mRNAs depending on the cellular proliferation status. Proliferating cells have high DENR–MCT-1 activity, resulting in efficient translation of those mRNAs, whereas DENR activity is low in non-proliferating cells, leading to the inhibition of translation of proliferation-promoting mRNAs in quiescent cells. It suggests

that non-proliferating cells might control translational reinitiation of certain mRNAs differently, to control their proliferation status. A Recent genome-wide study in mouse ES cells demonstrated that translation from uORFs is decreased upon cell differentiation (Ingolia et al. 2011). Our unpublished data suggest that N-Bak is expressed in neurons that have exit the cell cycle. Whether it is a proliferative status of the cell that determines the differential translation of *bak* transcripts in different cell types remains to be studied. As well, the differential requirement for trans-acting factors in Bak and N-Bak mRNA translation regulation is an important question to answer in future studies.

5.6 The role of NMD in N-Bak expression

Regulatory elements that modulate mRNA fate in the cytoplasm can be found in all parts of the mRNA molecule, in the 5′- and 3′- untranslated regions (UTRs) and the main coding sequence (CDS) (Zurla et al. 2016). Nonsense-mediated mRNA decay (NMD) pathway is an evolutionarily conserved post-transcriptional surveillance mechanism that is responsible for the rapid degradation of transcripts harboring a premature stop codon or carry features that define translation termination premature. This mechanism is classically considered to suppresses the synthesis of C-terminally truncated polypeptides and protects the cell from their possible deleterious dominant-negative effects (Maquat 2004). However, the role of NMD is not restricted to protect cells from unintended errors in gene expression, but plays important roles in regulating normal gene expression as well. For example, naturally occurring coupling between alternative splicing and NMD is a common mechanism to down-regulate the expression of particular gene under desired conditions (Neu-Yilik et al. 2004; Giorgi et al. 2007; Lareau et al. 2007; McGlincy and Smith 2008; Nicholson et al. 2010; Colak et al. 2013; Hamid and Makeyev 2014; Mockenhaupt and Makeyev 2015). Splicing factors can autoregulate their expression level through regulated unproductive splicing and translation strategy that relies on feedback loops involving alternative splicing-coupled NMD mechanism (Ni et al. 2007; Jangi et al. 2014). It is also widely used strategy to control the expression of stress-related genes. It suggests that changes in protein sequences may be less important than changes in the mRNA structure (Mockenhaupt and Makeyev 2015).

RUST mechanism would provide an attractive explanation for the absence of N-Bak protein in neurons. In this scenario, activation of a conserved 20nt exon in *bak* pre-mRNA could lead to the elimination of conventional Bak expression in neurons whereas resulting neuronal transcript for N-Bak contains PTC, which induces its degradation via NMD. It would suggest that alternative splicing generates N-Bak transcript only to destruct it in the following downstream surveillance pathways. We found, however, that NMD inhibition did not lead to significant accumulation of endogenous N-Bak mRNA in primary neuronal cultures. It indicates that NMD is not constitutively used strategy to control N-Bak expression in neurons. This suggestion is further supported by our findings that the luciferase reporter transcripts carrying N-Bak mRNA 3´UTR sequence with classical NMD activating features were as stable as corresponding controls we used in our study. We also found that endogenous N-Bak mRNA exhibits stability that is above the

average of the mRNA half-lives determined in the mammalian cells (Yang et al. 2003; Sharova et al. 2009; t Hoen et al. 2011), and consequently its mRNA is easily detectable by conventional PCR approach. This data collectively suggested that endogenous N-Bak mRNA is not constitutively targeted to the degradation by NMD machinery, but instead may be stabilized in neurons. Based on our findings that N-Bak mRNA is relatively abundant and very stable, but its protein is undetectable in neurons, we concluded that N-Bak mRNA might belong to the group of mRNAs that naturally escape NMD via translational repression.

Recently described post-transcriptional control mechanism, NMTR, is tightly related to NMD and involves translational repression but not degradation of certain PTC-containing transcripts (You et al. 2007). Although mechanistically not well understood, it is known that both, the phosphorylation status and levels of a key NMD protein, Upf1, may initiate translational repression in PTC-containing transcripts (Isken et al. 2008; Lee et al. 2010). NMTR could also depend on repressive *cis*-elements in the 3′UTR sequence (Morita and Hayashi 2014). NMD is inhibited in stressed cells, leading to translation of stress responsive genes (Wengrod et al. 2013), suggesting its broader role in physiological processes. Currently, it is not understood whether NMTR represents an unfinished NMD or exist as an independent branch of gene expression regulation. The former is supported by some experimental data indicating that NMD of certain alternatively spliced isoforms is conditional (Solier et al. 2005) and that some stabilized NMD targets may be translated (Barberan-Soler et al. 2009). N-Bak mRNA encodes a putative BH3-only variant of Bak. It suggests that such protein product may be used in some physiological condition in the cell. As a BH3-only protein, N-Bak is expected to act upstream of multidomain Bcl-2 family apoptosis regulators. Our studies, however, suggest that classical apoptosis signalling does not elicit N-Bak mRNA translational de-repression in neurons. As discussed before, it is possible that N-Bak mRNA translation is induced in some other physiologically relevant condition.

It remains to be studied whether there are some conditions when N-Bak mRNA is translated in neurons. We suggest, however, that N-Bak mRNA translational de-repression may be coupled with NMD. An interesting new perspective demonstrating the biological relevance of such mechanism was recently published by Colak and colleagues (2013), showing that AS-coupled NMD regulation in neurons allows cells to control protein abundances under desired conditions. This study demonstrated that translational silencing protects Robo-3.2 splice variant form NMD mediated destruction. However, Robo3.2 translational repression is transient, as changed guidance cue signaling milieu leads to the translation of Robo3.2 mRNA, specifically in navigating commissural axon growth cones. Robo3-2 mRNA translational de-repression leads to the rapid decrease of its mRNA via NMD, probably limiting its protein numbers to one copy from each mRNA (Colak et al. 2013). Further investigation also revealed that NMD machinery is predominantly localized to the axon tips in various types of neurons. However, AS-coupled NMD mechanism could limit protein abundances also in some other neuronal compartments. For example, translation of the NMD-sensitive mRNA isoform for Arc-1 leads to the destruction of its mRNA in dendritic spines (Giorgi et al. 2007).

Nevertheless, these data imply that PTC-containing mRNA may escape from NMD until it is translationally repressed. However, translational de-repression may destroy the transcript via NMD pathway, resulting in only limited amounts of translated protein product in the cell. Therefore, it is also likely that N-Bak mRNA translational de-repression coincidently leads to the destruction of its mRNA, thus allowing only controlled bursts of N-Bak protein in the cell.

5.7 Other molecular insights into N-Bak translational repression: miRNA regulation

We found that the suppression of exon N inclusion to *Bak* mRNA sequence in neurons, using morpholino oligonucleotides, did not de-repress the translation of resulting Bak mRNA, indicating that some cis-or trans-acting factors significantly contribute to the translational repression of N-Bak mRNA in neurons.

One group of candidate molecules that may functionally cross-talk with RBPs and determine target mRNA translatability in the cell are microRNAs (miRNAs). miRNAs are the best-characterized class of small non-coding RNAs that can negatively regulate gene expression by altering their target mRNA translation or stability. MiRNAs are now known to functionally participate in a wide variety of physiological or pathological processes and also interfere with apoptosis.

In silico miRNA binding site prediction algorithms suggest that *bak* transcript can be targeted by various miRNAs, most notably by three evolutionarily conserved miRNA families- miR-26, miR-125, miR-29, that include multiple members. Each of those conserved miRNA families have a single recognition site at evolutionarily conserved positions (Schickel et al. 2008). Almost all miRNAs can participate in distinct cellular pathways, and may have, for instance, depending on the cell type and expression pattern, different effects on cell survival, growth and proliferation (Reddy 2015). It thus remains to be studied whether and in which physiological context the miRNA sites found in the *bak* gene transcripts are targeted by the predicted miRNAs. miR-26b has been shown to be upregulated in Alzheimer's disease, contributing to aberrant cell cycle entry and apoptosis in postmitotic neurons (Absalon et al. 2013). In recent past, miRNA-125b has been experimentally proven to target *Bak* mRNA in breast and prostate cancer cells (Zhou et al. 2010; Wang et al. 2012). Although miRNA 125b is also abundantly expressed in developing and adult nervous system (Krichevsky et al. 2003), its role in *bak* gene expression regulation in nervous tissue has not been demonstrated. Kole and colleagues recently demonstrated that microRNA-29b targets the mRNA 3'-UTRs of five different BH3-only family members, including N-Bak (Kole et al. 2011a). It was demonstrated that miRNA-29b mediated translational repression has functional importance in protecting mature but not young sympathetic neurons from apoptosis (Kole et al. 2011a). It thus suggests that targeting multiple BH3-only members by the same microRNA family members may serve as a mechanism to circumvent a functional redundancy that different BH3-only members have in the cells and hence provide another mechanism for the neurons to control their apoptotic programs more tightly, particularly at mature stages.

We found that N-Bak mRNA 3`-UTR, containing intact miRNA sites, reduces luciferase reporter mRNA expression in cultured sympathetic neurons, indicating that miRNAs might contribute to N-Bak translational suppression in young neurons. Whether or not other miRNAs contribute to N-Bak translational suppression in mature neurons awaits further study, as does the role of N-Bak-targeting miRNAs during neuronal development.

5.8 Translationally repressed N-Bak mRNA localization in the sympathetic neurons

Like many other aspects of RNA metabolism, the mRNA decay and translational repression does not occur uniformly throughout the cytoplasm. Stress granules form to temporarily arrest mRNA translation in stressed cells, but dismantle and allow translation to continue when stress is relieved. Processing-bodies (P-bodies) are other cytoplasmic sites to prime and execute mRNA degradation, but may also function as storage sites for translationally repressed mRNAs (Bhattacharyya et al. 2006). Also, P-bodies may serve as dedicated sites where aberrant, PTC-bearing mRNAs are recruited and degraded by NMD (Sheth and Parker 2006). However, entry into the P-body is not sufficient to initiate mRNA decay, as only concerted action of additional RBPs, particularly UPF family members is required. In addition, there is evidence that certain mRNAs can also exit P-bodies if an appropriate stimulus is received (Bhattacharyya et al. 2006), suggesting that P-bodies may have a role in mRNA sorting prior to translation.

We characterized the subcellular localization of N-Bak mRNA, using locked nucleic acid (LNA) based oligonucleotides (Vester and Wengel 2004), which were designed to recognize only the short 20 nt exon (exon N) of N-Bak mRNA, by *in situ* hybridization. LNAs are nucleic acid analogs that are often used when targeting sequences are short or very similar. Incorporation of one or more LNA-nucleotides into a DNA or RNA oligonucleotide sequences increases the stability of the resulting nucleic acid duplex in hybridization and consequently allows using substantially higher hybridization temperatures compared to conventional hybridization probes with the same length (Hummelshoj et al. 2005). Using this approach, we found that N-Bak mRNA localizes into the granular structures in neurons. Our results, however, revealed that N-Bak mRNA does not colocalize with the P-body or stress granule marker proteins. We find it challenging to identify the exact nature of these granules. Together with our results that N-Bak mRNA is stable and appears to be not constitutively translated, we concluded that N-Bak mRNA is stored in yet unidentified cytoplasmic granules that probably contribute to its translational repression and stabilization.

Translational de-repression of mRNAs involves remodeling their bound RBP content in various granules. We did not observe significant changes in N-Bak mRNA-associated granular structures between healthy, stressed or apoptotic neurons. However, mRNA ISH is performed on fixed samples and intrinsically lacks a temporal dimension. Therefore, analysis of N-Bak mRNA localization dynamics will require technically more advanced approaches. For example, mRNA dynamics can be followed indirectly by using fluorescently tagged bacteriophage MS2 coat protein (MCP), which binds to a unique RNA

hairpin sequence (MS2-binding site-MBS) that can be cloned into the mRNA of choice (Bertrand et al. 1998; Buxbaum et al. 2015). Co-expressing MCP-fluorescent protein and MS2-tagged mRNA in cells enables to assess mRNA kinetics and localization in live cells. More advanced mRNA particle tracking methods (Chenouard et al. 2014), following mRNA local diffusion properties or its interactions with fluorescently labeled RBPs *in vitro* and *in vivo* may provide more conclusive understanding about N-Bak mRNA dynamics in the cells.

6 CONCLUSIONS

This thesis provides new insights into the molecular mechanisms that control neuronal Bak expression in neurons. , We outline that *bak* expression in neurons is controlled by multiple post-transcriptional mechanisms. In particular, in neurons, *bak* pre-mRNA undergoes alternative splicing, resulting in the neuronal isoform, N-Bak, with altered molecular composition enabling targeting by the NMD pathway. N-Bak mRNA, however, escapes NMD, exhibits high stability and appears to be deposited into granular structures in the cell. Based on these data and our biochemical and proteomic analyses, we conclude that N-Bak mRNA is translationally repressed in neurons. N-Bak mRNA 5´-and 3´-UTR regions were found to harbor several regulatory elements that contribute to its translational repression. Although these elements may synergistically suppress N-Bak mRNA translation, that the exact molecular details, as well as the importance of such complex regulation, remain unclear. Whether N-Bak expression is induced in some physiological condition, as well as its function in neurons would require further investigation.

Our study is among the first to thoroughly characterize of apoptosis related gene regulation at post-transcriptional level. Alternatively processed mRNA isoforms likely will continue to provide surprising functional outcomes to apoptosis regulation and are therefore intriguing topic for further studies.

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