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EVIDENCE BY INTERACTION: A NEW ROLE OF CASPASE-2

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Evidence by interaction: a new role of caspase-2

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“The gem cannot be polished without friction, nor man perfected without trials.”
— Chinese proverb

Till Julia

ABSTRACT

Caspase-2 is the best conserved member of the caspase family. Although being known to mediate cell death following DNA damage, many studies have implicated that this protease regulates a variety of cellular processes. Despite this, few well-defined and mechanistically explained functions of caspase-2 has been described. In an attempt to shed light on this enigma, we performed a yeast two-hybrid screen, searching for interaction partners of caspase-2 which could explain many of the reported observations. From the screen two hits stood out, relating to the proteins RFXANK and FAN. In the first study we investigated the relationship between caspase-2 and RFXANK, a protein known for regulating expression of MHC class II genes. The interaction between the two proteins was confirmed to take place primarily in the cytoplasm of cells. Caspase-2 was able to bind to a construct resembling the four ankyrin repeats of RFXANK, indicating that this is the region important for the interaction. Cells lacking caspase-2 contained higher total levels of MHC II, thereby suggesting that caspase-2 suppresses normal expression of the complex. Surprisingly, antigen-presenting cells from *caspase-2*$^{-/-}$ mice did not display any differences in surface distribution of the MHC II, indicating that the transport of MHC II from the cell interior to the exterior was somehow impaired. In the second study, we were interested in the understanding of the relationship between caspase-2 and FAN. Like in the first study, the interaction between the two proteins was confirmed by methodologies separated from yeast two-hybrid. FAN is a protein which has been reported to regulate a wide range of processes. We, therefore, systematically evaluated how a lack of caspase-2 would affect cells, while comparing to what is known about FAN-deficiency. Interestingly, we found that loss of caspase-2 caused the same outcomes as has been described for cells which have lost FAN. Notably, the ability to secrete IL-6 was greatly impaired in caspase-2-deficient cells, when comparing with relevant controls. Furthermore, enzyme-deficient cells took longer time to repopulate cell-free areas, indicating hampered cell motility. Although FAN is known to also regulate ceramide production, we did not observe any differences in sphingolipid contents when removing caspase-2. We did, however, observe that caspase-2-deficient cells contained abnormally enlarged vesicular/lysosomal structures, similar to what has been described following loss of FAN. In the third study we investigated the potential involvement of caspase-2 in the cell death process induced by Gemtuzumab ozogamicin (GO; Mylotarg®). This was based on the fact that GO causes DNA damage, a cause known to activate the proapoptotic function of caspase-2. We found that inhibition or removal of caspase-2 protected AML cells from GO-induced apoptosis. Strikingly, caspase-2 appeared to be involved in the processing of BID, but not the activation of BAX following treatment with GO. This may imply that the protease acts in parallel with GO, mainly by increasing the effect of the drug rather than being vital for the drug toxicity.

Taken together we describe novel interaction partners to caspase-2 and highlight how the protease may regulate processes which are not necessarily tied with apoptosis.

LIST OF SCIENTIFIC PAPERS

I. Forsberg J, Li X, Akpinar B, Salvatori R, Ott M, Zhivotovsky B, Olsson M (2018). **A caspase-2-RFXANK interaction and its implication for MHC class II expression**. Cell Death and Disease, 9: 80

II. Forsberg J, Li X, Zamaraev AV, Panaretakis T, Zhivotovsky B, Olsson M (2018). **Caspase-2 associates with FAN through direct interaction and overlapping functionality**. Biochemical and Biophysical Research Communications, 499: 822-828

III. Hååg P, Lagergren Lindberg M, Forsberg J, Olsson M, Zielinska Chomej K, Zong D, Kanter L, Stenerlöw B, Lewensohn R, Viktorsson K, Zhivotovsky B, Stenke L. **Caspase-2 is a mediator of apoptotic signaling in response to gemtuzumab ozogamicin in AML** (manuscript, in revision).

Additional publications (not included in the thesis)

Olsson M, Forsberg J, Zhivotovsky B (2015)
Caspase-2: the reinvented enzyme
Oncogene, 34: 1877–1882

Forsberg J, Zhivotovsky B, Olsson M (2017)
Caspase-2: an orphan enzyme out of the shadows
Oncogene, 36: 5441–5444

CONTENTS

LIST OF ABBREVIATIONS

ADC	Antibody-drug conjugate
AML	Acute myeloid leukemia
APC	Antigen-presenting cell
APP	β-amyloid precursor protein
ATG	Autophagy-related
atm	Ataxia telangiectasia mutated (gene)
ATM	Ataxia telangiectasia mutated (protein)
atr	ATM and Rad3-related (gene)
BAK	BCL-2 homologous antagonist / killer
BAX	BCL-2-associated X protein
BCA	Bicinchoninic acid
BCL-2	B-cell lymphoma 2
bcl9l	B-cell lymphoma 9-like protein (gene)
BCL9L	B-cell lymphoma 9-like protein
BCL-XL	B-cell lymphoma-extra large
BEACH	Beige and Chediak-Higashi
BID	BH3 interacting-domain death agonist
BIM	BCL-2-interacting mediator of cell death
BLS	Bare lymphocyte syndrome
CIITA	Class II transactivator
CAM	Calmodulin
CaMKII	Calcium/calmodulin-dependent kinase II
CARD	Caspase activation and recruitment domain
CD	Cluster of differentiation
CDK1	Cyclin-dependent kinase 1
CED-3	Cell death protein 3
CHK1	Checkpoint kinase 1
CoA	Coenzyme A
CTL	Cytotoxic T-cell
CXCL2	Chemokine (C-X-C motif) ligand 2

DD	Death domain
DED	Death effector domain
DEN	Diethylnitrosamine
DFF45	DNA fragmentation factor subunit alpha
DISC	Death-inducing signaling complex
DNA	Deoxyribonucleic acid
E1A	Adenovirus early region 1A
ECL	Enhanced chemiluminescence
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
FACS	Fluorescent-activated cell sorting
FADD	Fas-associated protein with death domain
FAN	Factor associated with neutral sphingomyelinase activation
FLICE	FADD-like IL-1β-converting enzyme (a.k.a. caspase-8)
G6P	Glucose-6-phosphate
GATA-1	Erythroid transcription factor
GFP	Green fluorescent protein
GO	Gemtuzumab ozogamicin
GSH-Px	Glutathione peroxidase
HPLC-MS	High-performance liquid chromatography - Mass spectrometry
HRP	Horseradish peroxidase
ICAD	Inhibitor of caspase-activated DNase
ICE	Interleukin-1β-converting enzyme (a.k.a. caspase-1)
ICH-1	ICE and CED-3 homolog (a.k.a. caspase-2)
IFN-γ	Interferon gamma
IL	Interleukin
iPLA2	Calcium-independent phospholipase A2
IRE1α	Inositol-requiring enzyme 1 alpha
LCFA	Long-chain fatty acid
MCL-1	Myeloid cell leukemia 1

MDM2	Mouse double minute 2
MEF	Mouse embryonic fibroblast
MMTV	Mouse mammary tumor virus
mRNA	Messenger RNA
miRNA	MicroRNA
NCCD	Nomenclature Committee on Cell Death
nedd	NPC-expressed, developmentally downregulated (gene)
NEMO	NF-κB essential modulator
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NGF	Nerve growth factor
NK	Natural killer
NO	Nitric oxide
NPC	Neural precursor cell
NPM1	Nucleophosmin
NSD	nSMase activation domain
nSMase	Neutral sphingomyelinase
PBS	Phosphate-buffered saline
PCD	Programmed cell death
PFA	Paraformaldehyde
PFGE	Pulsed field gel electrophoresis
PFT	Pore-forming toxin
PGE2	Prostaglandin E2
PH	Pleckstrin homology
PIDD	p53-induced death domain protein
PP1	Protein phosphatase-1
PUMA	p53-upregulated modulator of apoptosis
RAIDD	RIP-associated ICH-1/CED-3-homologous protein with death domain
RANKL	Receptor activator of nuclear factor kappa-B ligand
RFP	Red fluorescent protein

RFX	Regulatory factor X
RFX5	Regulatory factor X 5
RFXANK	Regulatory factor X-associated ankyrin-containing protein
RFXAP	RFX-associated protein
RIP	Receptor interacting protein
RIP1	Receptor interacting protein 1
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
shRNA	Short hairpin RNA
SOD	Superoxide dismutase
TNF-α	Tumor necrosis factor alpha
TNFR1	Tumor necrosis factor receptor 1
TRADD	TNFR1-associated death domain protein
TRAF2	TNF receptor-associated factor 2
TRAIL	TNF-related apoptosis-inducing ligand
UPR	Unfolded protein response
WD	Tryptophan-aspartic acid
wt	Wild type (gene level)
Y2H	Yeast two-hybrid
YPD	Yeast extract peptone dextrose

1 INTRODUCTION

The reciprocal relationship between life and death

Since the dawn of medical research, the goal has been to understand the physiology and pathophysiology of humans, in order to prevent, treat and abolish ailments, diseases and disorders. This seemingly never-ending quest has resulted in improved life conditions, which in turn has extended the average human life span. Despite this, it remains difficult to define what life is in a precise, unquestionable and undeniable term, whether from a theological or scientific viewpoint. What most people agree on is that there are certain attributes which living beings fulfill. Based on this, one could define life as being a state in which an entity independently is able to interact with its own environment and/or other entities, in order to maintain its own existence and/or that of another. Consequently, the understanding of death, *i.e.* the cessation of life, becomes fundamental in order to achieve new medical breakthroughs. This became notably apparent upon the realization that cells may die in a non-accidental, programmed fashion as a result of both normal and abnormal causes. Cell death has been reported as far back as in 1842 by Carl Vogt, who observed dying cells in the notochord and cartilage of metamorphic toads. Vogt sadly failed to realize the importance of this naturally occurring cell elimination observed in his model. Likewise, throughout the following decades, cell death during insect metamorphosis was either overlooked or misinterpreted (Clarke and Clarke, 1996). However, in present times, the phenomenon of controlled cell death has become recognized as being vital for an organism's health and development. Conversely, dysregulation of controlled cell death can give rise to many diseases and disorders. Philosophically, this signifies the intricacy between life and death, as well as health and disease. It is, therefore, not without irony that mankind, in an attempt to extend life, inevitably has to study death.

Cell death

Cells in our bodies have a finite life span. Similarly to how old or broken parts of a machine eventually need to be replaced, so do the cells. A cell which has sustained damage typically attempts to repair itself. The cellular response towards the injury depends on the cause and severity. For example, DNA-damage following radiation normally activates various DNA repair systems (Norbury and Hickson, 2001), while damaged or unneeded proteins are degraded to avoid a buildup which could potentially become harmful for the cell. Should the extent of an injury exceed the cell's capability of self-repair, it triggers a "self-destructing" signal, leading to its breakdown and removal. Likewise, senescent cells have reached a point to where telomere shortening triggers the DNA damage response, causing the same outcome. This sacrificial programming is important for multi-cellular organisms in order to prevent abnormal and malfunctioning cells from replicating, which could otherwise give rise to severe pathological conditions. Besides from maintaining the cellular homeostasis, cell death also plays a vital role during the developmental stages of multicellular organisms (Elmore,

2007, Fuchs and Steller, 2011). For instance, cell death is the underlying mechanism that removes interdigital webs in higher vertebrates, thereby forming the organism's digits (Lindsten et al, 2000). Similarly, it is essential for formation of the neural tube during development of the central nervous system (Glücksmann, 1951).

Defective cells often suffer from faulty cell death machineries. This may allow cells to either escape elimination or, at least, require higher degrees of stimulus in order to accomplish cell death. This is most notably the case for tumorigenic cells. In contrast, too much cell death may cause a loss of vital cell types, as well as abrogated tissue functions (King and Cidlowski, 1998), which is the case of many neurodegenerative disorders (Lang-Rollin et al, 2003). The term "cell death" encompasses several different modalities by which cells are eliminated. Based on the various biochemical and morphological features displayed by dying cells, the Nomenclature Committee on Cell Death (NCCD) proposed recommendations for accurate classification of the many modes of cell death (Kroemer et al, 2005). Arguably the most studied and best described modes include apoptosis, autophagy and necrosis.

Apoptosis

The term "programmed cell death" (PCD), introduced by Lockshin and Williams in 1965, is commonly used while referring to the process known as apoptosis (Lockshin and Williams, 1965, Kerr et al, 1972, Elmore, 2007). This is one of the most studied subjects within the field of cell biology (Wong, 2011). Hallmarks of apoptosis include protein cleavage, DNA fragmentation and morphological changes such as cell shrinkage and the formation of apoptotic bodies with an undamaged membrane. These changes culminate in the removal of the dying cell(s) by phagocytic cells (Elmore 2007, Hengartner, 2000), recruited and activated by "eat me" signals in the final stages of the apoptotic process. The key mediators of apoptosis are members from a family of cysteine proteases called caspases. However, not all caspases are pro-apoptotic. For instance, caspase-1, -4, -5 and -11 all play various roles in inflammatory responses (Jiménez Fernández and Lamkanfi, 2015), while caspase-14 is important for keratinocyte differentiation (Rendl et al, 2002). Apoptotic caspases are classified into two groups, initiator and effector caspases. The initiator caspases (caspase-2, -8 and -9) generally become recruited to larger complexes as zymogens, leading to their proximity-induced activation. Subsequently, the initiators may bring about the cleavage and activation effector caspases (caspase-3, -6 or -7) in turn. The pro-apoptotic caspases cleave specific substrates at aspartate residues, causing the breakdown of cellular structures, ultimately resulting in the shrinkage of the dying cell. Recent experimental evidence suggests that caspase substrates may include up to 1300 different human proteins (Crawford et al, 2013). Some of these caspase-targets are proteins which are protecting cells from apoptosis. An example of this is Inhibitor of caspase-activated DNase/DNA fragmentation factor subunit alpha (ICAD/DFF45), which becomes cleaved by caspase-3, thereby allowing DNA fragmentation to commence (Thornberry and Lazebnik, 1998). Similarly, caspase-mediated cleavage of the anti-apoptotic protein B-cell lymphoma 2 (BCL-2) releases the repression of apoptosis, thereby causing cell death (Cheng et al, 1997). Other targets cleaved by caspases

include lamins and cytoskeletal-regulatory proteins (Takahashi et al, 1996, Kothakota et al, 1997).

Apart from caspases, other important regulators of apoptosis include members of the BCL-2 family. These proteins are divided into three groups, depending of their individual composition of up to four different BCL-2 homology (BH) domains (Adams and Cory, 1998). Pro-survival members, such as BCL-2, BCL-XL and MCL-1, contain all four variants of the BH domains. Pro-apoptotic members contain either BH1-3, such as BAX and BAK, or only the BH3 domain. BH3-only proteins include BIM, BID, NOXA and PUMA (Adams and Cory, 1998, Czabotar et al, 2014). BAX and BAK form pores in the mitochondrial outer membrane upon activation, while BH3-only proteins (with the exception of BID) generally inhibit anti-apoptotic proteins in order to promote BAX and BAK (Westphal et al, 2011, Shamas-Din et al, 2011).

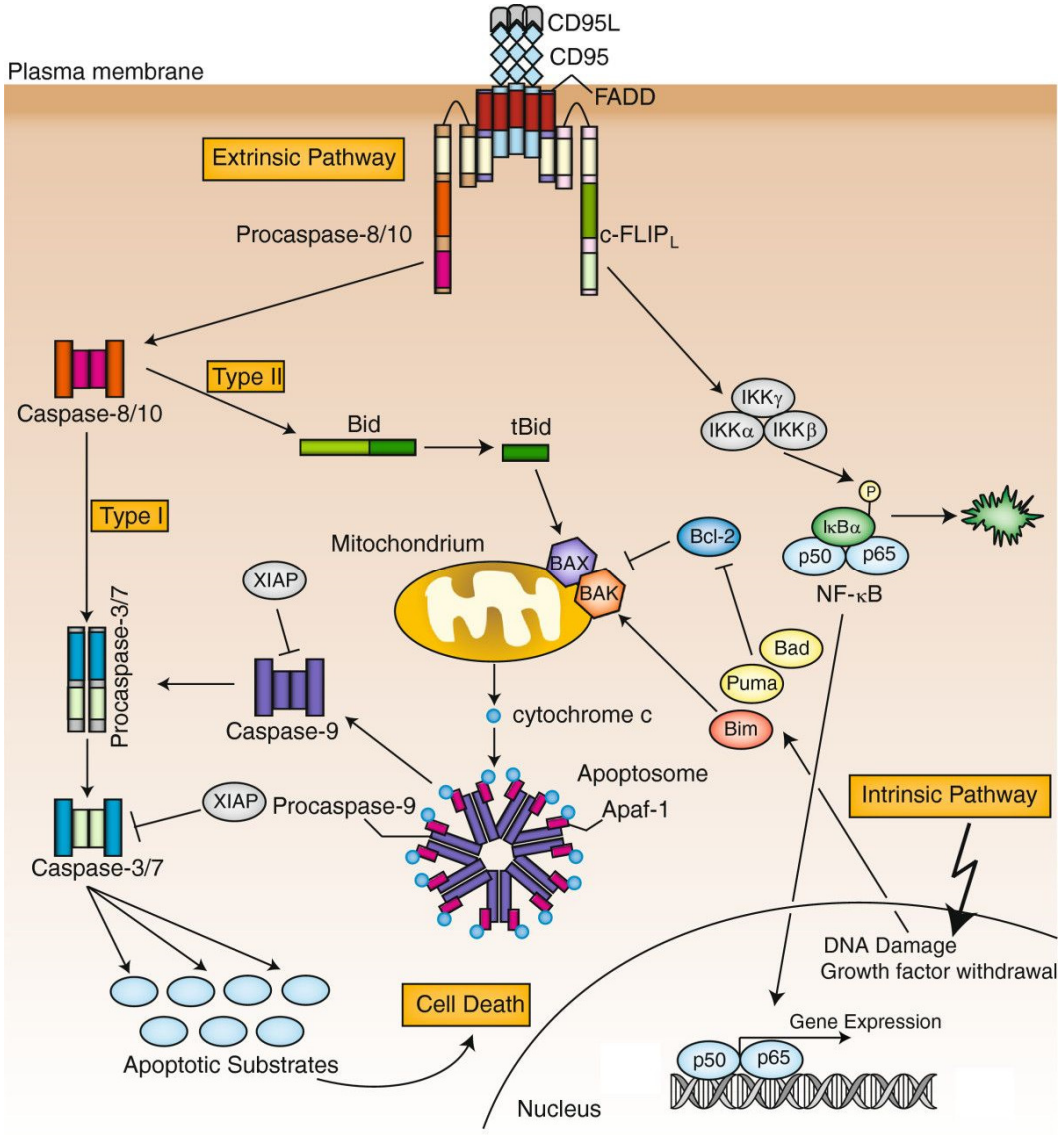

Figure 1. An overview of apoptotic processes.

Image reused with permission from BioMed Central Ltd (Schleich and Lavrik, 2013).

Several different apoptotic molecular routes are known, namely the extrinsic, intrinsic and perforin/granzyme B pathways. In addition, a separate, caspase-2-mediated pathway exists, which is initiated following DNA damage. Although initially being triggered by different causes, the pathways converge upon the activation of caspase-3 (Elmore, 2007). The extrinsic pathway starts at the cell surface and involves death receptor-mediated signaling following stimulation with cognate ligands such as FasL, Tumor necrosis factor alpha (TNF-α) or TNF-related apoptosis-inducing ligand (TRAIL). Conversely, the intrinsic pathway triggers upon internal stimulation, resulting in the opening of the mitochondrial permeability transition pore and loss of the mitochondrial transmembrane potential. As an outcome, several pro-apoptotic proteins such as cytochrome *c* are released into the cytoplasm, eventually leading to caspase activation. The perforin/granzyme B pathway is triggered by cytotoxic T-cells (CTLs) in virus-infected cells or tumor cells. Initially, the pore-forming protein perforin is secreted by CTLs and forms a transmembrane pore in the affected cell. This is followed by the release of cytoplasmic granules from CTLs, which enter the target cell through the pore. The granules contain the protease granzyme B which cleaves various target molecules, such as BID, ICAD and caspase-3. It should be noted that the granules contain other granzymes in addition to granzyme B, although only the latter induces apoptotic cell death (Elmore, 2007, Trapani and Smyth, 2002, Lieberman and Fan, 2003).

Autophagy

Another form of controlled cell death is the "self-eating" process called autophagy. Several forms of autophagy exist, albeit the form most often referred to is macroautophagy. This modality can either promote survival or death of a cell by degrading the bulk, organelles and/or proteins. During periods of moderate stress, such as starvation, it may be sufficient to reduce some of the cellular content in order to generate the nutrients required for extended survival. However, following severe stress, extensive removal of organelles and proteins may lead to cell death (Yonekawa and Thorburn, 2013, Yin et al, 2016). Central for the autophagic machinery is a group of proteins denoted ATG (autophagy-related). These proteins are involved in the formation of a double layered membranous structure called the autophagosome, which engulfs the cytoplasmic contents designated for destruction. Following a series of events, during which it maturates, the autophagosome eventually fuses with a lysosome and, by doing so, exposes the contents to lysosomal proteases for degradation (Mizushima et al, 2002, 2011).

Necrosis

Necrosis is a type of cell death which does not display the features of apoptosis or autophagy. In contrast to apoptotic cells, necrotic cells swell and suffer early ruptures in the plasma membrane. This leads to a release of cellular contents, which in turn trigger the innate immune response, thereby causing localized inflammation (Golstein and Kroemer, 2006, Vanden Berghe et al, 2014). Cells subjected to extremely harsh conditions, *e.g.* during exposure to detergents, typically die in a non-regulated necrotic fashion (Golstein and Kroemer, 2006). As a result, necrosis has long been regarded as an uncontrolled cell death process, occurring when cells take non-physiological damage. This view has partially

changed due to the fact that necrotic death can be induced upon inhibition of caspases (thus steering away from apoptosis). Similar to PCD, necrosis has also been observed during development as well as in the tissue homeostasis of adults (Roach and Clarke, 2000, Barkla and Gibson, 1999). Furthermore, TNF-α-mediated signaling can also bring about necrosis in addition to apoptosis, thus regulating two seemingly different cell death modalities. Taken together it becomes apparent that programmed necrosis (necroptosis) too is a controlled process, and not only a random, unchecked outcome caused by overwhelming cellular damage (Festjens et al, 2006).

p53

Multicellular organisms require healthy cells in order to avoid succumbing to pathologies. Cells have therefore developed various processes to ensure that only healthy and viable cells are allowed to propagate. While cell death is a means to eliminate cells that are beyond "saving", other mechanisms exist which recognize potentially harmful damage and attempts to fix it. Central in many of these processes is the tumor suppressor p53. Due to the role in maintaining genomically healthy cells, p53 has thus been dubbed the "guardian of the genome" (Lane, 1992). Following DNA damage, p53 is responsible for the activation of several DNA-repair factors, as wells as halting the cell cycle to allow time for repair (Brady and Attardi, 2010). Furthermore, p53 is a master regulator of both the intrinsic and extrinsic apoptotic pathways, where it can drive the expression of pro-apoptotic members of the BCL-2 family, *e.g.* BAX and NOXA, in addition to regulating the surface expression of death receptors (Vogelstein et al, 2000). It should also be noted that p53 can regulate other cell death modalities, such as necroptosis and ferroptosis. Being essential for proper surveillance and maintenance of the genome, it is not surprising that loss of p53 function may lead to tumor formation. In fact, mutations in the p53 gene (*TP53*) or p53-regulated pathways are found in the majority of all human cancers (Zilfou and Lowe, 2009, Joerger and Fersht, 2016).

Non-apoptotic functions of caspases

As mentioned previously, not all caspases function in a pro-apoptotic manner. Some caspases mediate other cell death mechanisms in addition to apoptosis, such as autophagy, necroptosis and pyroptosis (Shalini et al, 2015a). Other caspases perform functions that are not directly linked with cell death, at least not during the initial steps. An example is caspase-1, which is important both for the inflammatory and innate immune responses, where it processes interleukins (IL) -18 and -33, and promotes IL-1β secretion (Kuranaga and Miura, 2007, Nadiri et al, 2006). Moreover, even well-established apoptotic caspases have been shown to perform functions which are not necessarily tied with cell death execution. These functions include roles in regulation of cell migration, proliferation and differentiation. Caspase-8 is seemingly involved in calpain-activation and cell migration of mouse embryonic fibroblasts (MEFs) (Helfer et al, 2006). Furthermore, caspase-8 appears to regulate the proliferation of

lymphocytes. Patients carrying mutations in *caspase-8* often display defects in the activation of T-cells, B-cells and natural killer (NK) cells (Chun et al, 2002). Caspase-3, on the other hand, can cleave the cell cycle regulators p27 and p21, thereby promoting or inhibiting proliferation, respectively (Frost et al, 2001, Woo et al, 2003). Both caspase-3 and -8 appear to be required for differentiation of certain cells. While loss of *caspase-8* prevents the differentiation of monocytes into macrophages (Kang et al, 2004), *caspase-3*-deficiency results in decreased osteogenic differentiation of bone marrow stromal cells (Miura et al, 2004). Caspase-3 furthermore appears to be important for erythropoiesis and maturation of erythroid cells (Carlile et al, 2004), although caspase-3-mediated cleavage of GATA-1 has been shown to negatively regulate erythroblast differentiation (Zermati et al, 2001, Solier et al, 2017). In addition, as was previously mentioned, caspase-14 is important for keratinocyte differentiation and skin barrier formation (Rendl et al, 2002).

Caspases are also known to be important during tissue regeneration, neural development, as well as in the pathophysiology of many neurodegenerative diseases (Shalini et al, 2015a, Mukherjee and Williams, 2017, Miura, 2012). Liver regeneration in mice seems to be dependent on the cleavage of iPLA2 by caspase-3 and -7, bringing about the secretion of PGE2 and subsequent tissue restoration (Li et al, 2010). Similarly, the two *Drosophila melanogaster* caspases, DrICE and Dcp-1, induces compensatory proliferation in eye tissues, following cell loss due to injuries (Fan and Bergmann, 2008). The *D. melanogaster* caspase Dronc, on the other hand, is pivotal for dendrite pruning and the development of the adult nervous system (Kuo et al, 2006). Human caspases have also been reported to cleave huntingtin and β-amyloid precursor protein (APP), which is believed to contribute towards developing Huntington's disease and Alzheimer's disease, respectively (Wellington and Hayden, 2000). Together, these findings show that caspases are highly versatile in nature, and may be important for many regulatory processes.

2 INTRODUCTION TO THE STUDY

Caspase-2

In 1992, Kumar and co-workers first described a set of genes which were expressed during early embryonic mouse brain development, yet downregulated in adult mice. The team denoted these as *nedd* genes, based on the neural precursor cells from which they originated, as well as their expressional status. Amongst these, the *nedd-2*, *-9*, and *-10* mRNA transcripts were undetectable in the adult mouse brain (Kumar et al, 1992). A year later, Horvitz and colleagues reported that the *Caenorhabditis elegans* gene, *ced-3*, encodes a protein bearing strong resemblance to ICE (later denoted as caspase-1) and the product of *nedd-2*. Based on the importance of *ced-3* for programmed cell death in *C. elegans*, the group predicted that potential protein members belonging to the CED-3/ICE family might function as apoptotic regulators (Yuan et al, 1993). Later, it was shown that *nedd-2* overexpression in fibroblasts and neuroblastoma cells resulted in an induction of apoptosis, which could be prevented when co-expressing human *bcl-2* (Kumar et al, 1994). Furthermore, both *nedd-2* and its human homologue *ich-1*, encoded proteins with sequences similar to ICE and CED-3 (Wang et al, 1994). Eventually, following the introduction of the term "caspase", NEDD-2/ICH-1 was renamed caspase-2 (Alnemri et al, 1996).

Amongst the caspase protein family, caspase-2 is the most conserved member throughout evolution (Yuan et al, 1993, Kumar et al, 1994). This suggests that the role of the protein provide organisms with an advantage, thereby making the gene beneficial to harbor. Despite this, the main function of caspase-2 is still a subject of debate, as many different roles have been proposed. On top of it all, knock-out mice are viable and do not display any clearly overt phenotype, further questioning the degree of importance of caspase-2. It might, therefore, be possible that the protein functions as a fine-tuning factor in given modalities, potentially by increasing and augmenting the effectiveness of the cellular processes.

Structure and activation

The full structure of caspase-2 consists of a large subunit (p19) containing the active site, and a small subunit (p12). In addition, caspase-2 contains a caspase activation and recruitment domain (CARD) located towards the N-terminus. The identification of a classical nuclear localizing signal (NLS) at the C-terminus of the caspase-2 pro-domain explained why this particular caspase is able to localize to cell nuclei (Baliga et al, 2003). When comparing the structure with its other protein family members, caspase-2 is most similar to caspase-9 (Fava et al, 2012). These features classify the protease as belonging to the group of initiator caspases. However, unlike "typical" initiators, caspase-2 does not appear to cleave effector caspases, which is the case for caspase-8 and -9 (Fava et al, 2012, Guo et al, 2002).

As with other initiator caspases, caspase-2 molecules can undergo autocatalytic cleavage when brought into close proximity of each other, therefore, making ectopic expression of the protein difficult as it might trigger the apoptotic machinery (Butt et al, 1998). Under natural

conditions, caspase-2 has been reported to undergo activation through a complex called the PIDDosome, which is typically formed as a result of DNA damage (Tinel and Tschopp, 2004). This activation platform contains p53-induced death domain protein (PIDD) and RIP-associated ICH-1/CED-3-homologous protein with death domain (RAIDD) in addition to pro-caspase-2. While PIDD and RAIDD interact through their death domains (DDs), RAIDD furthermore possesses a CARD, to which procaspase-2 binds using its own prodomain CARD. It has been shown that a layer of five PIDD death domains can interact strongly with five RAIDD molecules and up to two additional weakly bound RAIDD monomers (Nematollahi et al, 2015). Aside from the involvement in caspase-2 activation, PIDD also plays a role in the NF-κB pathway, where it interacts with receptor interacting protein 1 (RIP1) and NF-κB essential modulator (NEMO) (Janssens et al, 2005). PIDD can thus be regarded as a bifurcated regulator of either pro-death or pro-survival signaling. What determines which of the two possible outcomes that will be "selected", is the level of PIDD processing. Initially the leucine-rich repeats are removed through auto-cleavage, generating a DD-containing fragment denoted PIDD-C. This fragment can then either become further processed to generate PIDD-CC, thereby leading to caspase-2 activation, or form a complex with RIP1 and NEMO, resulting in the recruitment of NF-κB (Tinel et al, 2007).

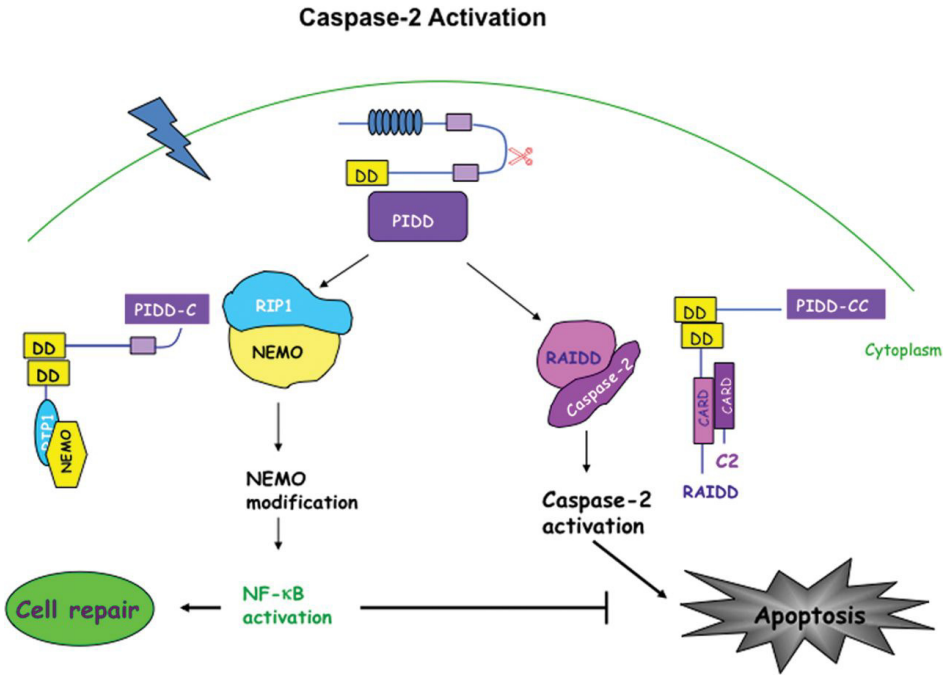

Figure 2. PIDDosome-mediated activation of caspase-2. Image reused with permission from Portland Press (Ribe et al, 2008).

Although PIDD and RAIDD are involved in the activation of caspase-2, *in vivo* studies using *pidd*$^{-/-}$ and *raidd*$^{-/-}$ mice have shown that caspase-2 can nevertheless become activated (Manzl et al, 2009, Kim et al, 2009). Based on these findings it became evident that although the PIDDosome have the ability to promote caspase-2 activation, it is not indispensable for this

task. As it turns out, caspase-2 can also utilize the death-inducing signaling complex (DISC) as an activation platform (Olsson et al, 2009, Lavrik et al, 2006). The DISC is comprised of proteins belonging to the death receptor family, as well as caspase-8 and the protein Fas-associated protein with death domain (FADD), where the latter functions as an adapter between the death effector domain (DED) and the death domain (DD) in the two former factors (Guicciardi and Gores, 2009). In order for caspase-2 to become activated through DISC, it appears that functional caspase-8 needs to be present, as inhibition of the latter abolishes the activation of the former (Olsson et al, 2009). Surprisingly this type of receptor-stimulated processing of caspase-2 does not necessarily couple with apoptosis (Lavrik et al, 2006).

Two known isoforms of caspase-2 exists due to messenger RNA (mRNA) splicing. The first isoform encodes a 435 amino acid protein denoted caspase-2L, which induces cell death upon overexpression. Conversely, the second isoform encodes the anti-apoptotic 312 amino acid protein, caspase-2S. Although many tissues express both variants, there seem to be some degree of tissue-specificity as well. For instance, only caspase-2L was expressed in the murine thymus, whereas caspase-2S was the highest in the embryonic brain (Wang et al, 1994). Interestingly, leukemic cells treated with the anti-cancer agent etoposide seemingly suppress caspase-2L levels, while increasing caspase-2S (Wotawa et al, 2002). Similarly, the anti-apoptotic form was expressed in human macrophages following etoposide and camptothecin treatment, and was observed to be highly expressed in macrophage-derived foam cells found around the core of atherosclerotic plaques (Martinet et al, 2003). These findings may suggest that caspase-2S is initially expressed after cells are exposed to agents causing DNA strand breaks, in order to delay apoptosis and allow DNA repair to commence.

Substrates

Despite being a highly conserved protein, there are few known substrates that are specific for caspase-2. Golgin-160, however, has been demonstrated to become cleaved by caspase-2 at a site unique for the protease, following apoptotic induction. Other caspases, recognizing similar peptide sequences as caspase-2, did not cleave golgin-160 at the same location albeit at other sites (Mancini et al, 2000). Another reported substrate of caspase-2 is the pro-apoptotic protein BID. Although BID is also processed by caspase-8 at higher efficiencies, caspase-2 can nevertheless cleave the protein at Asp^{59}, leading to cytochrome *c* release and apoptosis (Guo et al, 2002). In addition, following DNA damage caused by treatment with doxorubicin, caspase-2 has been reported to cleave mouse double minute 2 (MDM2) at Asp^{367}, thereby protecting p53 from being targeted for proteasomal degradation. The truncated MDM2 can additionally bind to p53 to stabilize the tumor suppressor. Activation of caspase-2 in this context is PIDDosome-mediated, thus creating a positive loop where p53 can promote its own activity. It is worth noting that caspase-3 was also able to process MDM2, although less efficient (Oliver et al, 2011). Taken together, these findings have revealed several settings in which caspase-2 can promote an apoptotic response. Since the substrates described are rarely unique for caspase-2, but can become equally processed by

other caspases, it may imply that caspase-2 mainly supports an already ongoing process and that the primary function of the protein is something else.

Functions of caspase-2

As previously mentioned, many different functions of caspase-2 have been proposed. Due to these cellular events often being closely related, while sharing many of the proteins involved, it is difficult to determine what the primary function of caspase-2 might be (Olsson et al, 2015). The following section reviews some cellular processes to which caspase-2 has been associated.

Cell death

The hallmark function of initiator caspases is to trigger a cellular process, which culminates in the death of the cell. Caspase-2 has indeed been demonstrated to partake in several cases of induced cell death, which strongly supports the notion of it being a pro-cell death protein. For instance, caspase-2 is essential for triggering cell death in macrophages, which have been infected with strains of the intracellular parasite *Brucella abortus* (Chen and He, 2009, Bronner et al, 2013). Similarly, *Salmonella*-induced apoptosis in murine macrophages also appears to be dependent on the protein (Jesenberger et al, 2000). In a separate study, where epithelial cells were subjected to the pore-forming α-toxin (PFT) from *Staphylococcus aureus*, it was concluded that caspase-2 indeed functions as an initiator caspase. Cells with attenuated levels of caspase-2 display a significantly impaired PFT-mediated apoptotic response in this system (Imre et al, 2012).

Infection with the Maraba rhabdovirus is known to elicit a strong endoplasmic reticulum (ER) stress response (Mahoney et al, 2011). ER stress is a condition in which unfolded proteins accumulate in the ER, to which cells respond by activating the unfolded protein response (UPR) in order to restore the protein folding homeostasis (Chen and Brandizzi, 2013, Xu et al, 2005). Failure to do so will cause the UPR to trigger apoptosis as a means to eradicate and prevent the spread of the virus. During Maraba viral infections, caspase-2 activation and subsequent cell death can be stimulated by downregulating inositol-requiring enzyme 1 alpha (IRE1α), an ER stress sensor responsible for activation of the UPR (Mahoney et al, 2011), which potentially reduce the time span that otherwise would be required to initiate apoptosis. The apoptotic modality following ER stress appears to involve BID cleavage by caspase-2. When this cleavage is blocked, by either downregulation or inhibition of caspase-2, it is sufficient to protect cells from apoptosis by the ER stress-inducing agents thapsigargin and brefeldin A (Upton et al, 2008). Interestingly, one group of researchers reported that IRE1α was responsible for cleaving certain microRNA (miRNA) to allow the translation of caspase-2 during ER stress (Upton et al, 2012). However, these findings partially contradict results by Mahoney and colleagues (2011). Moreover, in a separate study using several cell lines, no significant impact was observed on ER stress-induced apoptosis, following loss of caspase-2 (Sandow et al, 2014).

Aside from regulating cell death induced by microbial infections, caspase-2 also seems to control DNA-damage-induced apoptosis. Experiments made in *p53* mutant zebrafish embryos, subjected to γ-irradiation, revealed a different apoptotic machinery which does not include activation of caspase-3 prior to DNA-fragmentation, nor is it affected by loss of *p53* or overexpression of the anti-apoptotic *bcl-2/xl*. Instead the process relies on ataxia telangiectasia mutated (*atm*), ATM and Rad3-related (*atr*) and caspase-2, following removal or inhibition of checkpoint kinase 1 (CHK1) (Sidi et al, 2008). The PIDDosome plays a role in this modality; ATM phosphorylates PIDD at Thr^{788}, thereby allowing RAIDD to bind to PIDD. This is followed by the activation of caspase-2 (Ando et al, 2012). Nucleophosmin (NPM1) has similarly been shown to be important for DNA damage-induced PIDDosome assembly (Sidi and Bouchier-Hayes, 2017), further supporting the view of caspase-2 mediating apoptosis during such conditions. Furthermore, PIDDosome-mediated caspase-2 activation is also a prerequisite for cell death caused by treatment with cytoskeletal disrupting drugs. In MEFs lacking caspase-2, BID and BAX activation, as well as cytochrome *c* release, are delayed following treatment with vincristine, cytochalasin D, and paclitaxel (Ho et al, 2008). These findings might suggest that caspase-2 is vital to prevent genomically abnormal cells from replicating.

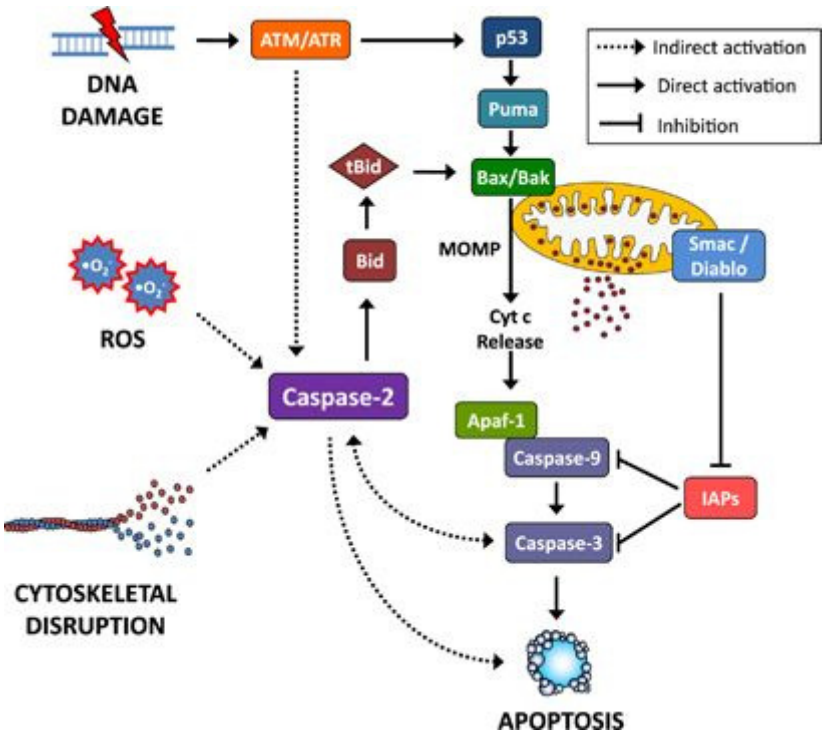

Figure 3. The relationship between caspase-2 and cell death. Image reused with permission from Springer Nature (Puccini et al, 2013b).

Lipoapoptosis is a form of cell death emerging as a consequence of metabolic imbalance. When levels of unoxidized long-chain fatty acids (LCFAs) exceed the storage capabilities of

adipose tissues, the lipids start to accumulate in non-adipose tissues. This build-up can become toxic, causing an apoptotic cell death referred to as lipoapoptosis (Unger and Orci, 2002, Johnson et al, 2013). When mammalian cells were treated with the LCFA palmitate, caspase-2 became activated, while down-regulation of the protease significantly diminished the apoptotic induction (Johnson et al, 2013). These findings strongly suggest that LCFAs are yet another trigger that can cause caspase-2 to initiate cell death.

Oxidative stress and aging

When a shift arises in the balance between oxidants and antioxidants, cells enter a state of so-called oxidative stress. The resulting build-up of reactive oxygen species (ROS) can have harmful effects on cellular structures and processes, as well as on proteins, lipids and DNA (Birben et al, 2012, Betteridge, 2000). It is believed that aging is linked with cumulative damage to DNA and mitochondrial DNA, inflicted by ROS (Cui et al, 2012). Interestingly, caspase-2 has been associated with combating oxidative stress, as well as delaying the aging process in mice. These functions are thus separated from the well-established role in cell death, although one process cannot completely exclude the other. *Caspase-2*$^{-/-}$ mice suffer from hair loss and early hair graying, increased bone loss, reduced body weight and higher levels of oxidized proteins, compared to their *wt* controls (Zhang et al, 2007, Shalini et al, 2012). Furthermore, hepatocytes from young or middle-aged *caspase-2*$^{-/-}$ mice appear to neutralize mitochondrial ROS at rates similar to those of old *wt* mice (Lopez-Cruzan and Herman, 2013). Comparably, *caspase-2*$^{-/-}$ MEFs have higher basal levels of peroxide and superoxide, as well as carbonylated proteins, compared with *wt* cells (Tiwari et al, 2014). Upon treatment with the oxidizing agent paraquat, caspase-2-deficient mice have been shown to display higher serum levels of the inflammatory cytokines IL-6 and IL-1β compared with controls (Shalini et al, 2015b). Taken together these findings suggest that caspase-2 regulates processes in order to mount a response towards increasing oxidative stress. Upon oxidative stress, caspase-2 appears to promote age-related muscle apoptosis in mice, with higher levels of processed caspase-2 found in older animals (Braga et al, 2008). In addition, old caspase-2-deficient mice have been shown to exhibit increased damage caused by free-radicals and decreased activities of glutathione peroxidase (GSH-Px) and superoxide dismutase (SOD), compared with young mice of the same genotype. These findings are believed to be a consequence of reduced expression of the *FoxO1* and *FoxO3a* genes, which are otherwise active during conditions of stress (Shalini et al, 2012). It may, therefore, be possible that caspase-2 functions as a transcriptional regulator in this context, perhaps in an age-depending manner. FoxO3a is known to regulate transcription of SOD during oxidative stress. Yet, the protein is also a known to inhibit proliferation and promote apoptosis, which contradicts its involvement in combating oxidative stress (Nho and Hergert, 2014). Moreover, it makes the role caspase-2 plays in this system much more questionable in the context of protecting the cell rather than promoting cell death during oxidative stress. This does, however, not rule out the possibility that caspase-2 responds differently towards oxidative damage, depending on the underlying cause. For instance, the protease has also been suggested to inhibit autophagy

following oxidative stress. Caspase-2-deficient neurons treated with rotenone, a complex I inhibitor of the mitochondrial respiratory chain, had enhanced levels of autophagy, eventually leading to necrosis due to overwhelming stress (Tiwari et al, 2011). On the other hand, *wt* neurons underwent apoptosis, indicating that the pro-apoptotic function may yet be the mechanism by which caspase-2 resolves the stressful conditions. Nevertheless, the protein does appear to harbor autophagy-suppressing properties, as *caspase-2*$^{-/-}$ cells displayed elevated levels of autophagic markers even under normal growth conditions, without any external stressors (Tiwari et al, 2014). Similar to this function, findings implicate caspase-2 as a suppressor of ROS-driven differentiation of macrophages into osteoclasts, upon treatment with the cytokine receptor activator of NF-κB ligand (RANKL) (Callaway et al, 2015).

Metabolism

Complementary to regulating the energy-providing mechanisms essential for life, metabolic processes are also able to control cell death (Green et al, 2014). If conditions favor growth, the cell death machinery is generally suppressed. Conversely, upon nutrient starvation several processes activate in response, such as a halt in proliferation and, as a last resort, apoptosis (Vakifahmetoglu-Norberg et al, 2017). Caspase-2 has been reported to be regulated by this fashion in *Xenopus laevis* oocytes (Nutt et al, 2005). When nutrient abundance was simulated by the addition of glucose-6-phosphate (G6P), a concomitant inhibition of apoptosis was observed. Further studies revealed that this was due to an inhibitory phosphorylation of caspase-2 at Ser^{135} (figure 4). As it turns out, addition of G6P increases levels of free coenzyme A (CoA), which in turn can directly bind to calcium/calmodulin-dependent kinase II (CaMKII). This interaction facilitates the binding of calmodulin (CAM) to CaMKII, leading to the activation of the latter. Subsequently, CaMKII catalyzes the inhibitory phosphorylation of caspase-2, thereby promoting oocyte survival (McCoy et al, 2013a, b). As a means to maintain the inhibition, the regulatory protein 14-3-3ζ binds to the phosphorylated caspase-2 and, by doing so, prevents dephosphorylation by protein phosphatase-1 (PP1) (Nutt et al, 2009). Interestingly, neither the binding of PP1 to caspase-2 nor the activity of the phosphatase appears to be metabolically regulated. In contrast, the metabolic processes control the interaction between 14-3-3ζ and caspase-2, causing either a lock-down or release of the protease.

Other findings have emerged, hinting towards sex-specific metabolic functions of caspase-2. Reportedly, aged caspase-2-deficient male mice displayed decreased liver mass upon fasting compared to *wt* controls, although the total body weight remained unchanged. Contrary to this, female *caspase-2*$^{-/-}$ mice had reduced total body weight, but not liver mass (Wilson et al, 2016). It thus becomes difficult to predict whether or not the presence of caspase-2 becomes an asset or liability when taken into a metabolic context. For instance, the protease has also been implied to promote obesity and non-alcoholic steatohepatitis (Machado et al, 2015, 2016). When mice lacking caspase-2 were fed a Western diet (rich in fat), they did not notably develop any diabetes mellitus or non-alcoholic fatty liver disease, nor did they suffer from dyslipidemia, hepatic steatosis or abdominal fat deposition, strongly contrasting that of

wt mice (Machado et al, 2016). These reports would indicate that an overload in the lipid metabolism might become severe due to caspase-2. Perplexingly however, the protease might also be important during impaired fatty acid biosynthesis. Treatment of ovarian cancer cells with Orlistat, a fatty acid synthase inhibitor, was highly depending on caspase-2 for cell death induction (Yang et al, 2015). Taken together these findings show that metabolic processes can indeed regulate caspase-2, although it is still not determined if the function of the protease is solely related to cell death in this context. It is possible that caspase-2 exerts different activities, altogether dependent on the metabolic state of the cells.

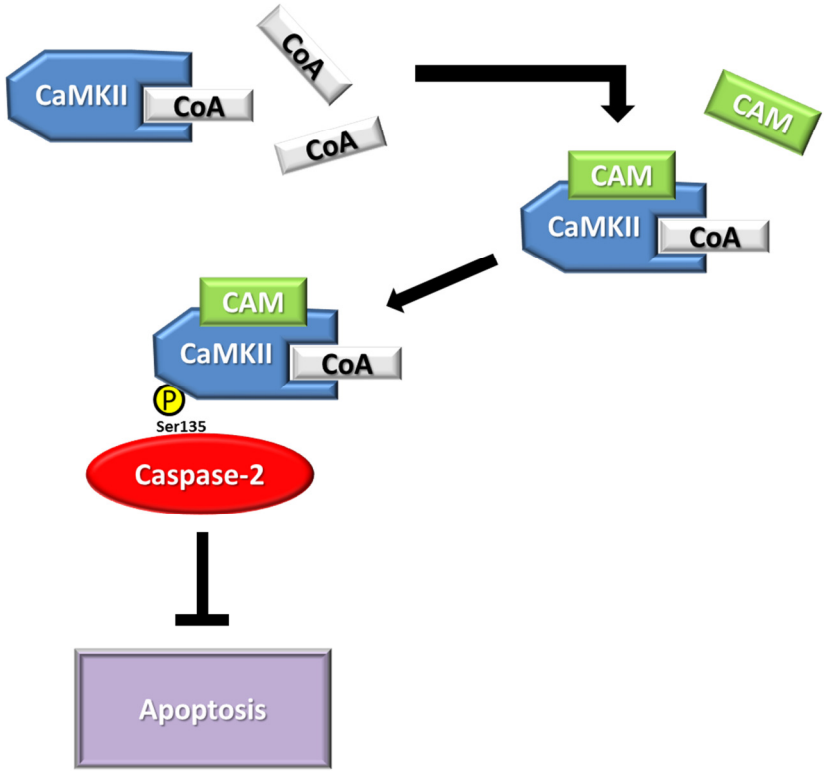

Figure 4. Overview of how nutrient abundance can cause an inhibition of apoptosis by modulating caspase-2. Image reused with permission from Springer Nature (Forsberg et al, 2017).

Cancer and tumor suppression

The *caspase-2* locus is found in the long arm of human chromosome 7 (Kumar et al, 1995). This region is frequently deleted in tumors, especially in acute myeloid leukemias (Johansson et al, 1993, Holleman et al, 2005, Mrózek et al 2008). Although these findings would suggest that loss of *caspase-2* would favor tumorigenesis, very few mutations in the coding region of the protease have been documented in tumors (Kim et al, 2011a, b). Still, reduced levels of caspase-2 mRNA (and protein) have been observed in cancer and tumor material, compared to normal tissues (Ren et al, 2012, Yoo et al, 2004). It might, therefore, be possible that the underlying cause leading to loss of the protein is not determined at the gene level. In the case of leukemia, loss of active caspase-2 correlates negatively in the context of therapeutic response, as well as survival outcome (Holleman et al, 2005, Estrov et al, 1998, Faderl et al, 1999). Despite these findings, indicating a tumor suppressor function of caspase-2, the

opposite has been seen in neuroblastoma (Dorstyn et al, 2014). Based on data from an expression array on human neuroblastoma samples, it became evident that low levels of caspase-2 correlated with increased patient survival. This was however only true for *MYCN*-non-amplified neuroblastomas, strongly suggesting that a potential tumor suppressing function may be context specific.

Many experiments have been carried out in order to investigate how loss of caspase-2 might facilitate tumorigenesis. Somewhat surprisingly, *caspase-2*$^{-/-}$ mice do not display any increase in tumor incidence when compared to *wt* counterparts (Shalini et al, 2012, Zhang et al, 2007). In addition, loss of caspase-2 does not sensitize mice to develop cancers more readily than *wt* mice, following γ-irradiation or treatment with the DNA-damaging compound 3-methylcholanthrene (Manzl et al, 2013, Manzl et al, 2012). What appears to be vital, however, is the p53 status, as loss of this protein accelerates tumorigenesis. For instance, although p53 levels was shown to be largely the same in *caspase-2*$^{-/-}$ and *wt* MEFs, caspase-2 deficient cells had lower amounts of *p21* transcripts (Ho et al, 2009). Since p53 is a known regulator of p21, these findings indicate that the function of p53 is impaired in *caspase-2*$^{-/-}$ cells. So far, only one study has shown that loss of caspase-2 can promote tumorigenesis following treatment with a DNA-damaging reagent (Shalini et al, 2016). When *caspase-2*$^{-/-}$ mice were injected with diethylnitrosamine (DEN), they consequently developed hepatocellular carcinoma at an increased rate compared to controls. DEN is a known inducer of ROS production, aside from being an agent that can alkylate DNA. This may imply that caspase-2 only limits tumor formation caused by a specific type of external agents.

Interestingly, caspase-2 seems to act as a tumor suppressor in established tumor cells, driven by oncogenes (Ho et al, 2009, Manzl et al, 2012, Parsons et al, 2013, Puccini et al, 2013a). *Caspase-2*$^{-/-}$ MEFs, transformed with E1A/Ras, proliferated faster *in vitro* than transformed *wt* controls. Furthermore, the caspase-2-deficient MEFs quickly formed large colonies when grown in soft agar, compared to the slow growth of the transformed *caspase-2*$^{+/+}$ cells. Similar results were observed when the MEFs were injected into nude mice, where *caspase-2*$^{-/-}$cells rapidly formed large and aggressive tumors, in stark contrast to the controls, which formed small or undetectable tumors (Ho et al, 2009). Eμ-*Myc* mice, a transgenic model, which readily develops lymphomas and leukemia, combined with knockout of *caspase-2*, resulted in accelerated tumorigenesis compared to Eμ-*Myc* mice alone (Ho et al, 2009). Although the underlying mechanism behind the potential tumor suppressive function of the protease still remains to be described, it has been concluded that it is PIDDosome-independent (Manzl et al, 2012). *Caspase-2* deficiency has also been reported to boost the formation of lymphomas caused by a loss of ATM (Puccini et al, 2013a). While *atm*$^{-/-}$ mice spontaneously acquire thymic lymphomas, *atm*$^{-/-}$*caspase-2*$^{-/-}$ mice were shown to have a strong increase in tumor incidence. Moreover, offspring of the double-knockout animals died shortly after birth. The few that survived, displayed growth retardation compared to controls. There was no significant difference in the amount of apoptotic cells in the lymphomas, regardless of genotype. Yet *atm*$^{-/-}$*caspase-2*$^{-/-}$ tumor cells proliferated faster than those from

atm$^{-/-}$-mice, indicating that the tumor suppressive function mediated by caspase-2 is not due to the apoptotic role of the protease (Puccini et al, 2013a).

Aside from oncogene-driven lymphomagenesis, caspase-2 reportedly plays a role in the prevention and limitation of other types of cancer, such as murine breast cancer (Parsons et al, 2013) and lung cancer (Terry et al, 2015). Mice expressing the oncogene *c-neu*, under the transcriptional control of the mouse mammary tumor virus (MMTV) promoter, readily develop tumors in the mammary epithelium of multiparous mice. Consistent with observations made in the lymphoma models, *caspase-2*$^{-/-}$*MMTV* animals displayed an acceleration in the tumor formation rate, when compared to *caspase-2*$^{+/+}$*MMTV* mice (Parsons et al, 2013). Enhanced *Kras*-driven lung tumorigenesis has also been demonstrated following loss of *caspase-2*. The knockout-mice developed larger numbers of tumors, which were greater in size, than mice harboring one or both of the *caspase-2* alleles. Although *caspase-2*-null tumors were more sensitive towards cisplatin compared to controls, they swiftly rebounded following a break in drug administration (Terry et al, 2015). These results are partly contrasting observations made in Eμ-*Myc* mice, where *caspase-2*-deficiency made cells resistant to chemotherapy-induced apoptosis (Ho et al, 2009).

Taken together these findings indicate that loss of caspase-2 does not by itself increase the susceptibility of organisms to develop cancer, but rather makes it harder to halt the rapid growth of the established tumor(s). So far, the proposed tumor suppressive function appears to become a necessity for cells following oncogenic pressure, due to loss of other important proteins, or exposure to certain DNA-damaging agents. Cells that just lack caspase-2, without being subjected to additional genetic alterations, do not automatically turn tumorigenic.

Preventing genomic instability

Genomic instability is a term, which refers to changes in nucleic acid sequences (*e.g.* mutations) as well as alterations to the karyotype (*e.g.* aneuploidy). The former case is primarily caused by defects or inefficiencies in DNA-repair mechanisms, while the latter case can involve nearly all parts of chromosomal regulation (Heng et al, 2013). Both types of genomic alterations are hallmarks of cancer (Hanahan and Weinberg, 2011). If cells are unable to correct the damage, they typically undergo cell death in order to prevent the accumulation of defective cells (Roos and Kaina, 2006, Vitale et al, 2017). Whether genomic instability becomes a means for tumorigenic cells to shape their genomes in order to survive, or a "fail-safe" mechanism to bring about the destruction of cancer cells, still remains to be discussed (Forsberg et al, 2017).

Several studies have shown that caspase-2 ostensibly promotes genomic stability. Whether or not this is directly linked to the proposed tumor suppressor function of the protease is still unclear, although the two modalities are intricately connected. Blood cells lacking *caspase-2* seemingly have deficient systems for maintaining a correct genome. Alas, T-cells from premalignant *atm*$^{-/-}$*caspase-2*$^{-/-}$mice displayed higher levels of aneuploidy (Puccini et al, 2013a). Similar to this, bone marrow cells from old knockout mice suffered from increased

rates of aneuploidy and DNA-damage, while hematopoietic stem cell differentiation was impaired (Dawar et al, 2016). Other characteristics of genomic instability, observed upon loss of caspase-2, include micronuclei formation, abnormal mitoses, karyomegaly and multinucleation (Dorstyn et al, 2012, Parsons et al, 2013). *Caspase-2*-deficient MEFs treated with the compounds vincristine and paclitaxel, which target microtubule functions, were resistant to the apoptotic induction normally elicited by the two drugs (Ho et al, 2008). In the same line, splenocytes appear to require catalytically active caspase-2 to be able to get rid of aneuploid cells following treatment with mitotic poisons (Dawar et al, 2017). The ability for caspase-2 to process specific proteins does thus appear to be a requirement for maintaining genomic stability in certain biological contexts. For instance, colorectal cancers, caused by *B-cell lymphoma 9-like protein* (*bcl9l*) dysfunction, display reduced basal levels of caspase-2 mRNA and protein, which contributes to aneuploidy. Reportedly, upon faulty chromosomal segregation, functional BCL9L permits transcription of caspase-2, which can then either cleave MDM2 (thereby stabilizing p53) or BID (López-García et al, 2017). Both cases ultimately lead to apoptosis.

A common trait pertaining to a role of caspase-2 in maintaining genomic integrity is the intricate connection the protease has with the cell cycle. For example, cyclin D3 expression has been shown to bring upon the activation of caspase-2, possibly by stabilizing the latter (Mendelsohn et al, 2002). Conversely, cyclin-dependent kinase 1 (CDK1) – cyclin B1 could suppress mitotic apoptosis by the inhibitory phosphorylation of caspase-2 at Ser^{340} (Andersen et al, 2009). Above all, caspase-2 is implied to partake in processes, which are active during malfunctioning mitosis. It was reported that an abnormal increase in centrosomes led to PIDDosome-mediated activation of caspase-2, resulting in cell cycle arrest (Fava et al, 2017). Similar to previous studies, this involved the cleavage of MDM2, stabilization of p53, and a p21-mediated halt in proliferation. Deficiencies in the checkpoints governing the DNA structure and spindle assembly can trigger a cell death response in mitosis, known as “mitotic catastrophe” (Castedo et al, 2004a). Although this was initially believed to be dependent on caspase-2 acting upstream of mitochondria, triggering activation of effector caspases and apoptotic cell death (Castedo et al, 2004b), it has also been shown to occur independently of caspase-2 in a necrotic manner (Vakifahmetoglu et al, 2008, Vitale et al, 2011).

Neuronal maintenance

Caspase-2 was originally identified as being a down-regulated protein during murine brain development (Kumar, 1992). Since then, a number of reports have implicated a possible function of caspase-2 in regulating neuronal cell death or survival. These findings are however elusive and seem to be highly context dependent. During the development of caspase-2-deficient mice, cell death of motor neurons was accelerated. Furthermore, sympathetic neurons deficient in caspase-2, isolated from the superior cervical ganglia, were more sensitive to cell death caused by nerve growth factor (NGF) deprivation than their *wt* counterparts (Bergeron et al, 1998). While these observations would indicate that the protease

acts in an anti-apoptotic manner, conflicting results have been obtained from other neurons. For instance, cells from the dorsal root ganglion of 2-day-old *caspase-2*-deficient mice were equally sensitive towards NGF withdrawal as controls (O'Reilly et al, 2002). Moreover, inhibition of caspase-2 protected adult glia and dorsal root ganglion neurons from cell death following serum deprivation (Vigneswara et al, 2013). Similarly, targeting *caspase-2* with siRNA largely decreased the loss of retinal ganglion cells following axon clamping (Ahmed et al, 2011).

Caspase-2 is believed to be involved in various neuropathological conditions, in addition to roles related with normal brain development. Loss of *caspase-2* has been observed to reduce excitotoxicity-induced brain damage in neonatal mice (Carlsson et al, 2011). Furthermore, sympathetic and hippocampal neurons were greatly protected from cell death induced by β-amyloid (which are peptides believed to cause Alzheimer's disease) when caspase-2 was downregulated with antisense oligonucleotides (Troy et al, 2000). Likewise, *caspase-2*-deficient mice were protected from the neurodegeneration caused by β-amyloid (Jean et al, 2013). Aside from Alzheimer's disease, unregulated caspase-2 does also seem to be part of the mechanisms leading towards developing Huntington's disease. This may be dependent on the catalytic activity of the protease, as expression of an inactive mutant partly protected against Huntingtin-mediated cell death (Hermel et al, 2004). Moreover, *caspase-2*-deficient mice maintained higher degrees of motor functions, as well as cognitive functions, when compared with *wt* mice (Carroll et al, 2011).

Collectively, research has shown an involvement of caspase-2 in the fate of neuronal cells. Due to contrasting findings, it becomes difficult to determine whether or not the protease promotes or inhibits cell death during brain development. The possibility exist that caspase-2 performs various functions throughout the different developmental stages and may, therefore, adopt multiple characteristics depending on the timing. Furthermore, findings indicate that caspase-2 may be a contributing factor towards developing neurodegenerative diseases, although the precise mechanisms remain to be determined.

3 AIM OF THE STUDY

The main aim of the PhD study was to characterize new processes involving caspase-2, as the protein is believed to be highly versatile. Although several, diverse functions have been proposed for caspase-2, few interaction partners of the protease have been described. Therefore, a yeast two-hybrid (Y2H) screen was carried out in order to identify potential interacting proteins, while using the full-length caspase-2 as bait. Based on the results from the screen and the established view/knowledge of caspase-2 acting as an initiator of apoptosis following DNA damage, three individual projects were formed, with the specific aims as follows:

Paper I – To investigate an interaction between caspase-2 and RFXANK, and the extent of which this interaction would affect MHC II expression.

Paper II – To study the involvement of caspase-2 in FAN-mediated processes, as well as confirming the interaction between the two proteins.

Manuscript I – To evaluate the potential role caspase-2 would play in Gemtuzumab ozogamicin-induced cell death.

4 MATERIALS AND METHODS

Cell lines

Cells were grown in either RPMI-1640 or DMEM (supplemented with 10% heat-inactivated fetal bovine serum and 100 U/mL penicillin, 100 µg/mL streptomycin), with the exception of OCI-AML2 cells which were grown in alpha-modified MEM (20% heat-inactivated fetal bovine serum and 1% L-glutamine).
The cell lines used in the papers and manuscript, included in this thesis, are listed in Table I.

Table I. Cell lines employed in the studies.

Cell line	Origin (disease)	Cell type	Culture medium
Daudi *wt*	Burkitt's lymphoma	B lymphoblast	RPMI-1640
HCT116 *wt*	Colorectal carcinoma	Epithelial	DMEM
HEK293T *wt*		Epithelial	DMEM
HEK293T *shCtrl*		Epithelial	DMEM
HEK293T *shCasp2*		Epithelial	DMEM
HeLa *wt*	Cervical carcinoma	Epithelial	DMEM
HeLa *shCtrl*		Epithelial	DMEM
HeLa *shCasp2*		Epithelial	DMEM
HL60 *wt*	Acute promyelocytic leukemia	Myeloid	RPMI-1640
OCI-AML2 *wt*	Acute myeloid leukemia	Myeloid	MEM
OCI-AML2 *shCtrl*		Myeloid	MEM
OCI-AML2 *shCasp2*		Myeloid	MEM
THP1 *wt*	Acute monocytic leukemia	Monocyte	RPMI-1640
THP1 *shCtrl*		Monocyte	RPMI-1640
THP1 *shCasp2*		Monocyte	RPMI-1640

Protein extraction and quantification

Adherent cells were detached from culture vessels by adding trypsin directly to the cells. All cells were centrifuged at 1000 rpm (≈200 rcf) for 5 minutes, and subsequently washed in phosphate-buffered saline (PBS). This was followed by another centrifugation before removing the supernatant. Cells were then lysed for 10 minutes at room temperature in cOmplete™ Lysis-M, supplemented with protease inhibitors (Roche Diagnostics). Subsequently each sample was centrifuged for 5 minutes at 13 000 rcf, before collecting the supernatants. Protein concentrations were measured using the BCA Protein Assay (Thermo Fisher Scientific). The samples were then mixed with 5x Laemmli buffer and stored at -20°C.

Western blot

Western blot is semi-quantitative technique which can be used for comparing protein levels between different samples. For this reason, the technique was used in all projects of this thesis. Briefly, quantified protein samples were denatured at 95-97°C for 10 minutes, before

being separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). The separated proteins were then electroblotted to 0.45 µm nitrocellulose membranes, which were subsequently blocked with 5% (w/v) non-fat dry milk in PBS for 60 minutes at room temperature. Incubation with primary antibodies was carried out overnight at 4°C. Membranes were then washed repeatedly with PBS and PBS-Tween20 (0.1%). Horseradish peroxidase (HRP)-conjugated, or fluorophore-labeled secondary antibodies were subsequently added, and the membranes were left to incubate at room temperature for 60 minutes. This was again followed by repeated washing steps. Finally, proteins were detected by using enhanced chemiluminescence (ECL) (in cases when HRP-conjugated antibodies were utilized), or measuring fluorescence using the 700 nm and 800 nm channels. Densitometric analysis was carried out using the Image J software.

Immunoprecipitation

One method that can be used for studying protein-protein interactions is immunoprecipitation. Typically, the protein of interest can be captured while using immobilized antibodies. If the protein of interest is interacting with other proteins, these could potentially become co-captured. This method was, therefore, selected for studying the interactions between caspase-2 and RFXANK or FAN. In some settings, tagged versions of the three proteins were overexpressed prior to the protein extraction, in order to facilitate capture. The capture antibodies (targeting either the proteins directly, or the tags) were immobilized on magnetic beads. Cells were lysed in NP40-buffer (with added protease and phosphatase inhibitors) and centrifuged at 16000 rcf for 20 minutes. The supernatants, containing proteins, were then collected. Each protein sample was then added to the bead-antibody mixtures, and placed under rotation overnight at 4°C. The following day, the samples were washed repeatedly using the lysis buffer, before being resuspended in Laemmli buffer.

ELISA

IL-6 secretion was measured using the IL-6 Quantikine® ELISA kit (R&D Systems), according to the manufacturer's protocol.

Fluorescent imaging

Fluorescent microscopy was applied in order to study co-localization of proteins, as well as cellular effects. For this purpose, mCherry-, GFP-, or RFP-tagged versions of the proteins were overexpressed in cells. Nuclei were stained using Hoechst 33342. Lysosomes were stained using LysoSensor™ Green DND 189 (Invitrogen), according to the manufacturer's protocol. Images were acquired with a Zeiss LSM 510 META confocal laser scanner microscope (Carl Zeiss MicroImaging).

Cell proliferation assay

An assay using Cell Proliferation Reagent WST-1 (Roche) was performed in order to confirm that shRNA-mediated knockdown of caspase-2 would not affect proliferation rates of the

cells. This was carried out according to the manufacturer's recommendations. Absorbance was measured at 450 nm, with a reference wavelength at 650 nm.

Cellular fractionation

In addition to fluorescent microscopy, the localization of proteins can be determined by first fractionating the cellular compartments, followed by detection with *e.g.* western blot. This method was, therefore, used in combination with immunoprecipitation, in order to further evaluate where the interaction between caspase-2 and RFXANK takes place. Harvested cells were resuspended in a fractionation buffer (150 mM KCl, 1 mM $MgCl_2$, 0.2 mM EGTA, 5 mM Tris, and 0.01% digitonin) and left to incubate for 10 minutes at room temperature. Subsequently, samples where centrifuged and the supernatants (cytoplasmic fractions) were separated from the pellets (nuclear fractions). Immunoprecipitation was then carried out on the different fractions.

Wound-healing assay

The wound-healing assay, also known as a scratch assay, is a technique which can be used for studying cell migration. This method was, therefore, selected when investigating the effect of caspase-2-deficiency, as well as FAN-deficiency on cell motility. Cells with attenuated levels of the two proteins were grown in 6-well plates. When the cells had formed a monolayer, upon complete confluency, a scratch was made in the cell layer and initial (t0) images were obtained, using a Nikon microscope. This was repeated each day until cells had successfully repopulated the scratched areas. Cell-free areas in each image were measured using the wound-healing plugin in the Image J software.

Yeast two-hybrid screen

Yeast two-hybrid screening is a commonly used approach for discovering interacting proteins. For this reason, the technique was applied in order to identify proteins with the potential to interact with caspase-2. The screen was performed by Hybrigenics Services, Paris, France.

Sphingolipid analysis

Quantitative measurements of sphingolipids in caspase-2-deficient and control cells were carried out by the Medical University of South Carolina, Department of Biochemistry and Molecular Biology. This was done using HPLC-MS/MS, as has been described previously by Bielawski and coworkers (2009).

Transfection of cell lines

In some cases, prior to performing immunoprecipitation or fluorescent imaging, constructs encoding tagged RFXANK or NSMAF (FAN) were overexpressed in cells. These constructs were purchased from OriGene. Transfection was carried out using Lipofectamine® LTX (Invitrogen), according to the manufacturer's recommendations. The same procedure was

applied when expressing catalytically inactive (and tagged) caspase-2, control plasmids, and a tagged construct corresponding to the four ankyrin repeats of RXANK.

For the transfection of THP1 cells with siRNA constructs, HiPerFect® Transfection Reagent (Qiagen) was used, according to the manufacturer's protocol.

Transfection of yeast cells

To confirm that the bait fragment (used in the Y2H screen) indeed encoded caspase-2, the construct was expressed in *Saccharomyces cerevisiae* W303a. The cells were vortexed and transfected by heat-shock for 45 minutes at 42°C, before being centrifuged and recovered in YPD (1% yeast extract, 2% peptone, 2% dextrose w/v). Cells were then plated on a selective plate (synthetic defined medium without tryptophan). During the following days, individual colonies were selected, from which overnight cultures were made. Proteins then were obtained from the cells by performing a Rödel extraction. Briefly, harvested cells were resuspended in H_2O, before adding 50 µL Rödel mixture (741 µL H_2O, 185 µL 10 M NaOH, 74 µL 14.3 M β-mercaptoethanol). Subsequently, each sample was left on ice for 10 minutes, before adding 60 µL 72% trichloroacetic acid. This was followed by 20 minutes incubation at -20°C, and thereafter 30 minutes of centrifugation at 28,000 rcf. Finally, pellets were washed with acetone, before being resuspended in Laemmli buffer.

Flow cytometric analysis

Flow cytometry is a technique employed for counting and sorting cells, based on different parameters. This method was, therefore, used in order to estimate the surface distribution of MHC class II proteins on a wide range of murine antigen presenting cells. Murine B-cells were obtained through fluorescent-activated cell sorting (FACS), using a BD Aria III FACS sorter.

Analysis of caspase-3 activation and conformational changes of BAX were carried out on 0.5% PFA-fixed cells, using anti-caspase-3 and anti-BAX antibodies.

Pulsed field gel electrophoresis

In order to quantify DNA double strand breaks caused by GO-treatment, pulsed field gel electrophoresis (PFGE) was carried out according to a protocol presented by Stenerlöw et al, 2003. Prior to the treatment, cells were cultured for 48 hours in 1000 Bq/mL ^{14}C-thymidine-containing medium.

Statistical analysis

Results are presented as means ± standard deviations.

5 RESULTS

Paper I

Forsberg J, Li X, Akpinar B, Salvatori R, Ott M, Zhivotovsky B, Olsson M (2018). A caspase-2-RFXANK interaction and its implication for MHC class II expression. Cell Death and Disease, 9: 80

In this project, we aimed at investigating the potential interaction between caspase-2 and RFXANK. To date, RFXANK is only known for being involved in transcriptional regulation of Major histocompatibility complex II (MHC II) genes. RFXANK forms a complex with RFX5 and RFXAP, which then binds to the X-box motif of MHC II gene promoters. Subsequently, the Class II transactivator (CIITA) interacts with the bound factors, thereby initiating transcription. Based on this, we hypothesized that a caspase-2-RFXANK interaction could either promote or inhibit expression of MHC II. Since MHC II is primarily expressed by antigen-presenting cells (APCs), the majority of the experimental work was performed on cells belonging to this category.

The study started with the confirmation of the construct used in the screen as bait. Indeed, the construct encoded a full-length caspase-2 protein, as was seen when expressed in *S. Cerevisiae*. Next, the interaction between caspase-2 and RFXANK was confirmed by co-expressing tagged versions of the two proteins followed by immunoprecipitation. The caspase-2 construct encoded a catalytically inactive form of the protein, tagged to mCherry. This not only allowed us to overexpress caspase-2 without causing an induction of apoptosis, but also facilitated IP. The RFXANK construct was purchased from OriGene, and contained myc-DDK tags. IP results, both while overexpressing the constructs, as well as on endogenous material from B-cells, showed that the two proteins indeed can interact. This was also suggested when visualizing overlapping localization through fluorescent microscopy. Moreover, when expressing a construct consisting only of the four ankyrin repeats of RFXANK, the interaction with caspase-2 could still be observed, indicating that this region is important for forming a complex. Next, we decided to look at how loss of caspase-2 would affect MHC II expression. Surprisingly, we could not detect any differences in the surface expression of MHC II in APCs originating from thymus, the bone marrow or spleen of *caspase-2*$^{-/-}$ mice. However, when analyzing total lysates of B-cell lines, or B-cells from *caspase-2*-deficient mice, there was a clear induction of MHC II following loss of caspase-2. These findings suggest that caspase-2 exhibits a negative effect on MHC II gene expression, while other mechanisms control the installment of the protein at the cell surface.

In conclusion, the data from this study show that caspase-2 can interact with RFXANK, and might, therefore, influence the regulatory processes of the immune system.

Paper II

Forsberg J, Li X, Zamaraev AV, Panaretakis T, Zhivotovsky B, Olsson M (2018). Caspase-2 associates with FAN through direct interaction and overlapping functionality. Biochemical and Biophysical Research Communications, 499: 822-828

FAN is a protein which has been reported to bind to a specific region on TNF receptor 1 (TNFR1), where it is involved in TNF-α-mediated activation of neutral sphingomyelinase (nSMase), cell migration, IL-6 secretion and vesicular/lysosomal dynamics (Montfort et al, 2010).

Since caspase-2 was suggested to interact with FAN in the yeast-2-hybrid screen, we set out to investigate the impact this interaction would have on the known FAN-mediated processes. Similar to as in Paper 1, the interaction was confirmed by immunoprecipitation while overexpressing tagged versions of the two proteins. Based on the fact that FAN is an important factor in nSMase activation and ceramide production, we decided to evaluate how loss of caspase-2 would affect these processes. Caspase-2 levels were reduced in HEK293T and HeLa cells through shRNA-mediated knockdown, which was followed by measurements of the sphingolipid contents using HPLC-MS. No significant differences were observed in the quantity of the measured ceramide species when comparing control cells with caspase-2-deficient cells. This indicates that caspase-2 is not involved in ceramide production, at least not in unstimulated cells. Next, we looked into how caspase-2 would influence IL-6 secretion, keeping in mind that FAN-deficiency has been reported to attenuate IL-6 levels. Using ELISA, it was concluded that loss of caspase-2 impaired the secretion of IL-6 into the culture media, regardless of whether or not cells had been treated with TNF-α. In addition, confocal microscopy revealed that caspase-2 and FAN-deficient cells contained enlarged endosomal/lysosomal structures, possibly reflecting abnormal vesicular traffic. Wound-healing assays further demonstrated that loss of caspase-2 decreases cells ability to migrate. Thus, although caspase-2-deficient cells were still able repopulate cell-free areas, they did so at a reduced rate compared with controls.

Together these findings show that caspase-2 is able to influence a wide range of cellular processes, and acts in a similar fashion as FAN. Since the two proteins are able to interact, it is highly possible that caspase-2 acts together with FAN to regulate these modalities.

Manuscript I

Hååg P, Lagergren Lindberg M, **Forsberg J**, Olsson M, Zielinska Chomej K, Zong D, Kanter L, Stenerlöw B, Lewensohn R, Viktorsson K, Zhivotovsky B, Stenke L. Caspase-2 is a mediator of apoptotic signaling in response to gemtuzumab ozogamicin in AML (manuscript, in revision).

Acute myeloid leukemia (AML) is the most prevalent acute leukemia in adults. The disease has a poor outcome, where only 25% of patients survive a 5-year period. Treatment of AML typically involves high-dose chemotherapy. Although complete remission is often achieved, most patients eventually relapse with a chemo-resistant form of leukemia. Due to this, different ways for treating AML patients are currently being investigated. One such approach involves targeted delivery of cytotoxic agents to the leukemic cells, where surface proteins on the cells act as beacons. CD33 is transmembrane glycoprotein, which is found in normal myeloid cells, albeit the expression levels are elevated in AML blasts. Utilizing this is the antibody-drug conjugate Gemtuzumab ozogamicin (GO; Mylotarg®). GO consists of a humanized antibody that is directed against the CD33 antigen, and to which a derivative of calicheamicin γ_1 is bound.

In this study we showed that GO-mediated cytotoxicity involves the processing of caspase-2. Inhibition of the proteolytic activity of caspase-2 reduced the GO-induced activation of caspase-3. Similarly, removal of caspase-2 using siRNA caused the same outcome. Contrastingly, caspase-2 inhibition did not block BAX activation following GO-treatment, though it partially prevented BID processing. Expression levels of caspase-2 or caspase-3 did, however, not appear to be linked with the duration of complete remission for AML patients.

Taken together, the findings show that GO-induced cell death in AML cells is to a great extent mediated by caspase-2, although the exact mechanism still remains to be determined. Despite this, GO also appears to be able to act in a caspase-2-independent manner to some degree.

6 DISCUSSION

Cell death is a term encompassing tightly controlled cellular events, with several different modalities, leading to cell elimination. Although many of the proteins involved have defined properties in regulating the mechanisms of cell death, they also perform additional functions. Caspase-2 is a protease which originally was regarded as an initiator of apoptosis following DNA damage. Though this perception of the protease still holds true, it has become apparent that caspase-2 also partakes in processes which are not necessarily tied to cell death. As it appears, caspase-2 may very well function as a multi-regulatory protein to either enhance or suppress cellular processes. However, due to the lack of well-defined caspase-2-specific substrates or interacting partners (aside from during apoptosis), the protein still remains one of the least understood members of the caspase family.

In the present study we aimed at discovering proteins which can interact with caspase-2, potentially in a non-cell death context. To identify these unknown proteins, we performed a yeast-2-hybrid screen, from which the two proteins RFXANK and FAN were suggested.

MHC class II proteins are found on the cell-surface of antigen-presenting cells (Reith et al, 2005), where they present extracellular proteins from pathogens to components of the immune system. Similar to MHC class I, class II proteins are heterodimers consisting of an α- and a β-chain. The expression of MHC II is mainly regulated at a transcriptional level (Reith et al, 2005). Central for this process are the *trans*-acting proteins RFX5, RFXAP, RFXANK and CIITA. While the three RFX-proteins are ubiquitously expressed in cells, CIITA expression is a highly regulated event. Together, RFX5, RFXAP and RFXANK form the so-called RFX complex, which binds the X-box promoter motif of MHC II genes. CIITA can then interact with the bound complex in order to promote gene expression. Mutations in the four transactivators (in particular RFXANK) are known to cause MHC class II deficiency, resulting in a condition known as Bare lymphocyte syndrome (BLS) (Lisowska-Grospierre et al, 1994, Wiszniewski et al, 2003, Reith et al, 2005). In **paper I** we confirmed that caspase-2 was able to interact with RFXANK in the cytoplasm of cells. This interaction appears to take place at the ankyrin repeat region of RFXANK. Ankyrin repeat motifs are known to mediate protein-protein interactions (Li et al, 2006). Moreover, in a report aiming at designing a caspase-2-specific inhibitor, the protease was demonstrated to possess binding properties towards ankyrin repeats (Schweizer et al, 2007). It is, therefore, not surprising that our results point towards an interaction taking place at this region. Furthermore, the ankyrin repeat motifs of RFXANK are responsible for the interaction with CIITA and RFXAP (Wiszniewski et al, 2003). Interestingly, dendritic cells can produce a CARD-containing splice-isoform of CIITA, which has the ability to inhibit caspase-mediated degradation of nitric oxide synthase-2 or, conversely, promote the production of nitric oxide (NO). This, in turn, regulates the capability of dendritic cells to trigger T-cell activation/proliferation (Huang et al, 2010). In our study, while analyzing total protein levels from APCs, we observed a concomitant increase in MHC II following a loss of caspase-2. These findings indicate that caspase-2 can act as a suppressor of MHC II expression. While the mechanism is still unknown, it is

possible that caspase-2 binds to the ankyrin repeats of RFXANK, as well as the CARD on CIITA, thereby disrupting the interaction between the two *trans*-acting proteins. Moreover, cells with CARD-deficient CIITA are able to express functional MHC class II genes, suggesting that CIITA may be less "hampered" when losing the ability to bind caspases (Zinzow-Kramer et al, 2012). Surprisingly, we did not observe any clear differences in surface expression of MHC II on APCs from *caspase-2*$^{-/-}$ mice. While this seemingly contrasts the findings made on total protein levels, it may indicate that caspase-2 also affects mechanisms responsible for delivering MHC II to the plasma membrane. It is also important to note that no pathogenic pressure was applied during the experiments in paper I, which potentially could affect the surface distribution of MHC II.

TNFR1 is a surface-bound receptor which is central in many signaling processes, such as cell survival, cell death induction and regulation of immune responses (Naudé et al, 2011). Upon ligand stimulation, the adaptor protein TRADD becomes recruited to the death domain of TNFR1 (Hsu et al, 1995). At this point the cell is presented with two possible pathways to follow, leading either to cell death or survival. If TRADD interacts with RIP1 and TRAF2, it will bring about the activation of NF-κB, which in turn leads to the expression of proteins involved in survival and/or inflammation (Hsu et al, 1996a, b). Conversely, if TRADD interacts with FADD and procaspase-8, thereby forming the DISC, it causes the activation of the caspase cascade and subsequent cell death (Muzio et al, 1996). TNFR1 can also induce caspase-3 activation and cell death via neutral sphingomyelinase (nSMase), in addition to acting through the DISC (Neumeyer et al, 2006). This alternative pathway results in the production of ceramides through the hydrolysis of sphingomyelin, a reaction catalyzed by nSMase. The adaptor protein FAN is essential for this TNF-α-induced activation of nSMase (Montfort et al, 2010). FAN binds to a specific domain of TNFR1, known as the NSD domain. This interaction is mediated through the PH and BEACH domains of FAN (Adam et al, 1996). While bound to TNFR1, the WD repeats of FAN allows nSMase to dock (Adam-Klages et al, 1996). Aside from the critical role during nSMase activation, FAN is seemingly involved in other cellular processes as well. For instance, FAN is essential for full expression of the pro-inflammatory genes encoding IL-6 and CXCL2 (Montfort et al, 2009). Furthermore, FAN-deficient fibroblasts have been demonstrated to contain larger lysosomes compared with wild-type counterparts, possibly indicating a role in regulation of vesicular traffic (Möhlig et al, 2007, Montfort et al, 2010). In addition, FAN appears to be required for normal filopodia formation following TNF-α-stimulation (Haubert et al, 2007), and may, therefore, affect cell motility. While caspase-2 has been linked to death receptor biology (Espín et al, 2013, Olsson et al, 2009), no report had previously connected it to FAN. In **paper II** we showed that caspase-2 and FAN can interact when exogenously expressed. Moreover, when we systematically investigated the effect that caspase-2-depletion would have on cells, we noticed that the results greatly resembled those reported for FAN-deficiency. IL-6 secretion greatly diminished when caspase-2 levels were reduced using shRNA-mediated knock-down. Cells deficient in caspase-2 also showed signs of reduced motility, as was demonstrated using wound-healing assays. Furthermore, caspase-2 deficient

cells contained large, spherical structures, indicating impaired vesicular/lysosomal homeostasis. Remarkably we did not observe any differences in the production of ceramide species, when comparing caspase-2-deficient cells with controls. This may indicate that a caspase-2-FAN interaction does not act to promote ceramide synthesis. However, it should be taken into account that we did not stimulate cells with TNF-α prior to the analysis, which may be required for triggering this specific pathway. Nevertheless, we showed that loss of caspase-2 causes the same outcomes as FAN-deficiency does, thus, supporting the hypothesis that the two proteins function together to regulate various processes.

At a first glance, it would appear like the interactions we reported in **papers I** & **II** are connected to two different and unrelated functions. Interestingly, however, TNF-α has been demonstrated to influence MHC class II expression. Under normal conditions, MHC II expression is regulated to a great extent by the cytokine interferon gamma (IFN-γ). TNF-α has been shown to act synergistically with IFN-γ to induce expression of MHC II (Chang and Lee, 1986, Pfizenmaier et al, 1987, Arenzana-Seisdedos et al, 1988, Kim et al, 2002, Keller et al, 2011). On the other hand, in several other cases, TNF-α was found to antagonize the effect of IFN-γ on MHC II, thus, the link between TNF receptor-functions and MHC II appears to be very context specific (Makhoul et al, 2012, Melhus et al, 1991, Han et al, 1999). Factors determining the fate of MHC II expression seem to involve differentiation and maturation. For instance, TNF-α augments IFN-γ-mediated MHC II expression in the immature B-cell lines THP-1 and HL-60. However, when the cells were made to differentiate, the synergistic effect of TNF-α was lost (Watanabe and Jacob, 1991). Collectively, these findings highlight tightly regulated pathways, which to date are not fully understood. The precise role of caspase-2 in these processes, therefore, becomes a subject of speculation. Although a well-described mechanism remains to be explained, it is highly possible that the protease partakes in the interconnection between TNFR1-functions and MHC class II regulation. The interaction with FAN could serve as a way to anchor caspase-2 to TNFR1. Upon stimulation, caspase-2 (and possibly also FAN) may dissociate from the receptor to allow interaction with RFXANK or other factors. This in turn prevents CIITA from interacting with the RFX complex, thereby reducing the expression of MHC class II genes. In this scenario, caspase-2 acts as a second messenger, conveying the signal from the exterior of the cell to the nucleus. Since we were able to detect the caspase-2-RFXANK interaction in the cytosolic compartment, it is possible that caspase-2 shuttles RFXANK out from the nucleus, thus further preventing MHC II expression (figure 5).

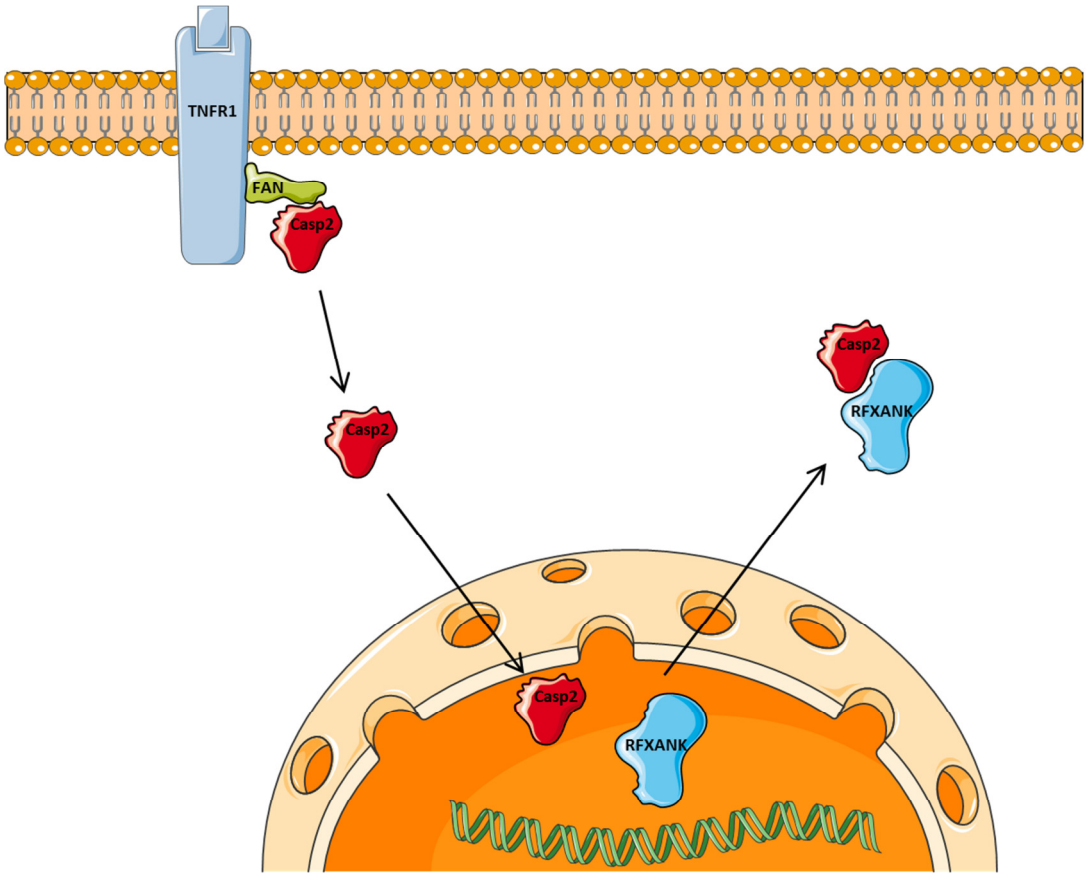

Figure 5. A proposed explanation to how caspase-2 may suppress MHC class II gene expression by inhibiting the function of RFXANK. The image was made using templates from Laboratoires Servier.

We have seen that, like FAN-deficiency, loss of caspase-2 seemingly disrupts normal vesicular traffic. This could potentially explain why we detected an increase in the total levels of MHC II when analyzing cell lysates, yet no clear difference in the surface expression of MHC II. Could it be that caspase-2-deficiency promotes the expression of MHC class II genes, yet prevents the encoded protein complex from being transported to the surface of the cell (figure 6)? Several reports have demonstrated how B-cells can secrete MHC II-containing exosomes with the ability to elicit T-cell responses (Raposo et al, 1996, Buschow et al, 2010). Moreover, neutral sphingomyelinases are known to regulate the release of exosomes from the plasma membrane (Trajkovic et al, 2008, Menck et al, 2017). Although we did not observe any effects of caspase-2-deficiency on ceramide production, it is possible that a FAN-caspase-2 interaction mediates exosomal transport of proteins via nSMase. Loss of either caspase-2 or FAN could, by this mechanism, prevent the transport of MHC II to the surface of the cell (or release into the extracellular space). This could in turn connect to the observed impairment in cell migration (**paper II**), following ablation of FAN or caspase-2. As it turns out, MHC II is an important regulator of cell motility in both B- and T-cells (Partida-Sánchez et al, 2000, Fischer et al, 2007). It is, therefore, possible that loss of caspase-2 or FAN subsequently traps MHC II in the cell interior, thereby preventing MHC II from promoting cell migration. Since FAN- and/or caspase-2-deficiencies are sufficient to impair

cell migration, a complimentary reduction in the surface expression of MHC II would possibly render APCs immobile. Another way in which caspase-2 could potentially regulate MHC II expression is by modulating IL-6. The cytokine IL-6 is known to inhibit expression of MHC II in a range of cells by various mechanisms (Kitamura et al, 2005, Abós et al, 2016). In our study we observed that caspase-2-deficiency led to decreased secretion of IL-6. Although, the levels of MHC II were not investigated in the study (**paper II**)**,** it is tempting to speculate that the observed increase in MHC II (presented in **paper I**) may be caused by attenuated secretion of IL-6, following a loss of caspase-2.

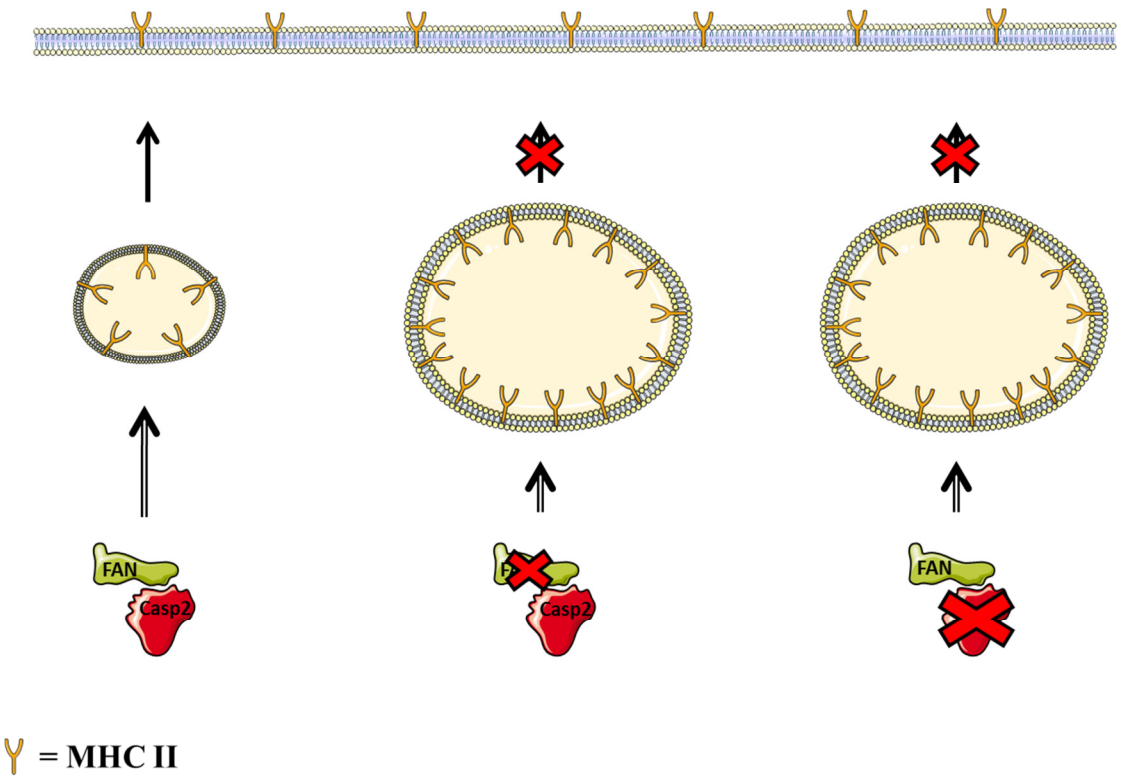

Figure 6. A proposed explanation to how caspase-2 may be important for MHC II surface expression, by modulating vesicular traffic. Both caspase-2 and FAN-deficiency causes an accumulation of large vesicular/lysosomal structures. The image was made using templates from Laboratoires Servier.

Caspase-2 is known to become activated when a cell sustains DNA damage. Depending on the situation, the protease is believed to regulate the cell cycle and maintenance of the genome. However, severe damage can lead to the activation of the pro-apoptotic function of caspase-2, as is often the case following chemotherapeutic treatment (Schmitt et al, 1999, Robertson et al, 2002, Olsson et al, 2009, Heikaus et al, 2010). One of the challenges with conventional chemotherapy is that the drugs generally affect both normal and tumorigenic cells. In addition, patients often relapse with chemo-resistant tumors that are increasingly aggressive. Due to this, there is a growing need for treatment strategies that specifically target tumor cells or, at least, completely eradicates them, thus ensuring complete remission.

Gemtuzumab ozogamicin is an antibody-drug conjugate (ADC) designed to specifically target AML cells. The drug binds to the CD33 antigen, leading to the internalization of the complex. Upon joining with lysosomes, the intercalating agent calicheamicin is released from the antibody conjugate, thereby causing DNA double strand breaks (Godwin et al, 2017). In **manuscript I** we showed that caspase-2 is involved in the cytotoxic response towards GO. HL60 cells treated with GO displayed clear apoptotic features. Furthermore, there was an increase in the cleavage of caspase-2, indicating the activation of the protease. Similar findings were seen in patient-derived cells, where full-length caspase-2 levels decreased after GO-treatment. Inhibition of the proteolytic activity of caspase-2, using z-VDVAD-fmk prior to GO-treatment, resulted in decreased caspase-3 activity. Likewise, siRNA-mediated knockdown of caspase-2 prevented GO-mediated activation of caspase-3. Together these findings indicate that caspase-2 acts upstream of caspase-3 during GO-induced cell death. Surprisingly, however, inhibition of caspase-2 did not appear to block GO-mediated BAX activation, although it slightly prevented BID processing. This may indicate that the involvement of caspase-2 in GO-mediated apoptosis is not necessarily tied to the main mechanism of action induced by the ADC. It is thus possible that the protease functions in a parallel pathway, following the sustained DNA damage caused by GO, where it acts like an augmenting factor in the cell death execution. Removal of caspase-2, or inhibition of the proteolytic activity, would therefore reduce the velocity of the apoptotic machinery but not completely block it. Another potential manner in which inhibition or removal of caspase-2 could suppress the apoptotic response is if the protease is important for the uptake and processing of the drug itself. GO enters the cell through endosomes, which are eventually required to fuse with lysosomes in order to release the active compound calicheamicin. As described in **paper II**, loss of caspase-2 appears to disrupt the normal vesicular/lysosomal traffic. This raises the question whether or not caspase-2 mainly is important for the drug delivery process, rather than the actual cytotoxic mechanism. Partially supporting this hypothesis, are the findings that caspase-2 and caspase-3 expression levels, prior to treatment with GO, do not influence the duration of complete remission in AML patients (**manuscript I**). It is, therefore, possible that caspase-2 is of less importance for the drug effect, but rather pivotal for the uptake of the ADC. A third fashion, in which loss of caspase-2 could reduce the effectiveness of GO, would be if caspase-2 is an important regulator of cellular mechanisms by which GO exerts its function. For instance, DNA damage generally leads to a halt in the cell cycle in order to allow time for repair to commence. However, when the damage turns out to be irreparable, cell death is triggered as a final outcome. The purpose of this is to prevent faulty cells from replicating. Caspase-2 has repeatedly been implied to partake in the cell cycle, and is important for maintaining genetic stability (Oliver et al, 2011, Dorstyn et al, 2012, Fava et al, 2017). Impaired cell cycle arrest due to a loss of caspase-2, could thus result in that the GO-induced damage becomes “overlooked”, thereby reducing the potency of the drug.

Taken together it is gradually becoming more apparent that caspase-2 possesses a variety of functions. What determines if and when the protease engages in a given modality, still

remains to be described in detail. So far caspase-2 has been reported to regulate cell death, maintaining genomic stability, suppressing tumorigenesis, combating oxidative stress and ageing, *etc*. Here we additionally report the ability of caspase-2 to regulate MHC class II expression, as well as being involved in TNF-receptor biology. The possibility exists that many of these functions are connected to each other, where the factors determining the outcome are highly context specific. Future studies should thus aim to cross-link the reported pathways in which caspase-2 partakes.

7 CONCLUSIONS AND OUTLOOK

Caspase-2 is a protein which appears to be able to influence or regulate a wide range of cellular processes. In the presented studies, we show that caspase-2 is able to interact with the two proteins RFXANK and FAN. Loss of caspase-2 results in increased total levels of MHC class II proteins. Moreover, caspase-2 and RFXANK co-localize in the cytoplasm, but not in the nucleus. Collectively these findings indicate that caspase-2 acts as a suppressor of MHC II in antigen presenting cells. As this study did not involve any pathogenic pressure on the cells or animals, future studies should address this matter.

While a lack of FAN has been reported to affect a multitude of processes, such as IL-6 secretion, vesicular/lysosomal dynamics, cell migration and ceramide production, we show that loss of caspase-2 largely resembles that of FAN-deficiency. Attenuated caspase-2 levels impaired the ability of cells to repopulate cell-free areas. Likewise, IL-6 secretion was hampered upon loss of the protease. Presence or absence of caspase-2 does not appear to affect ceramide production under the experimental settings used here. However, large, abnormal vesicular structures were observed in caspase-2-deficient cells, indicating disrupted vesicular/lysosomal traffic.

Since many FAN-mediated processes are important for a fully functioning immune system, it would be interesting to determine if caspase-2 plays a role in regulating the immune response as well. Stimulating the TNF-α pathways and subsequently evaluating the effect it would have on MHC II expression, could provide insights regarding whether or not caspase-2 connects these two modalities. Strong hints would be if the co-localization between caspase-2 and RFXANK or FAN would change following stimulation. Hypothetically, caspase-2 may be anchored to TNFR1 (through FAN), and upon stimulation with TNF-α, dissociates and forms a complex with RFXANK. This should be possible to confirm with immunoprecipitation following TNF-α-stimulation.

The antibody-drug conjugate Gemtuzumab ozogamicin (Mylotarg®) induces apoptosis in AML cells. This seems to, at least in part, be acting through caspase-2, as inhibition or removal of the protease reduces caspase-3 activation. Although caspase-2 promoted BID processing, it did not affect BAX activation following treatment with GO. Whether or not caspase-2 is directly involved in the mechanism of action of GO, or rather acts in parallel, remains to be determined. Since the possibility exists that caspase-2-deficiency prevents proper delivery of GO, it would be interesting to evaluate if removal of the protease would also reduce GO-induced DNA damage. This could, in theory, be tested using comet assays on GO-treated caspase-2-deficient cells and controls. If the degree of DNA damage decreases following the removal of caspase-2, it could indicate a failure in calicheamicin (the active compound of GO) reaching the DNA.

To date, several roles in different processes have been proposed for caspase-2, although direct, mechanistic explanations still remain largely unknown. One of the challenges in pinpointing a precise function of the protease arises due to the fact that the suggested

processes often are tightly related (figure 7). The work presented in this thesis demonstrates, for the first time, yet another involvement of caspase-2 in two additional areas of cell biology, namely immunologic regulation and TNF-mediated biology. These findings could potentially provide the research field with clues to what the true function of this enigmatic protein may be.

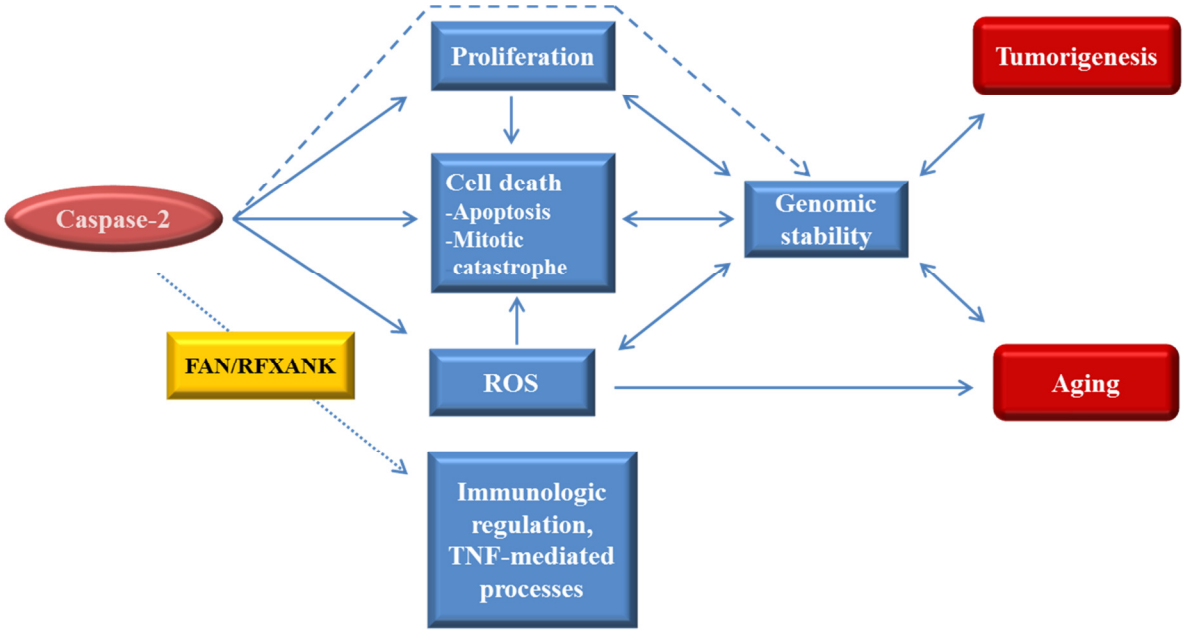

Figure 7. The complexity of caspase-2 and the many processes in which it has been suggested to partake. Adapted from a figure by Olsson et al, 2015.

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