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Proteolytic regulation of PGAM5 in non-apoptotic cell death

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A List of Abbreviations:

Abbreviation:	Explanation:
ADCD	autophagy-dependent cell death
AIFM1	apoptosis-inducing factor, mitochondrial
ARH3	ADP-ribosyl hydrolase 3
ASK1	apoptosis signal-regulating kinase 1 = MAP3K5
ATP	adenosine triphosphate
BAX	Bcl-2-associated X protein
Bcl-xL	B-cell lymphoma-extra large
BIR2-domain	baculovirus IAP repeat domain
BPGM	2,3-bisphosphoglycerate mutase
Caspase	cysteinyI-aspartate-specific protease
CD	cell death
cFLIP _L	cellular FADD-like interleukin-1 β converting enzyme-like inhibitory protein (long form)
CHX	cycloheximide, agent commonly used to sensitize cells to induction of cell death
cIAP1/2	cellular inhibitor of apoptosis protein 1/2
CLPB	caseinolytic peptidase B
CRISPR/CAS	clustered regularly interspaced short palindromic repeats/CRISPR-associated protein
CYLD	cylindromatosis
DAI	DNA-dependent activator of IFN regulatory factors
DAMPs	danger-associated molecular patterns
DDR system	DNA damage repair system
DNA/cDNA	(complementary) deoxyribonucleic acid
DR3/6	death receptor 3/6
Drp1	dynammin-related Protein 1
EDTA	ethylenediaminetetraacetic acid
FADD	Fas-associated protein with death domain
FAS	FS-7-associated surface antigen
FLAG-tag	amino acid tag of artificial origin commonly used to mark or capture proteins
GSK'872	N-(6-(Isopropylsulfonyl)quinolin-4-yl)benzo[d]thiazol-5-amine, RIPK3-inhibitor
GST	glutathione S-transferase
HSV1/2	herpes simplex virus 1/2
HT-29	human colorectal adenocarcinoma cell line
HtrA2	high temperature requirement protein A2
IBM	IAP-binding motive
ICD	immunogenic cell death
I-Clips	intermembrane cleaving proteases
IFN	interferon
IMM	inner mitochondrial membrane
IMS	intermembrane space (of mitochondrion)
IRI	ischemia-reperfusion injury
JNK	Jun N-terminal kinase
Jurkat 142	immortalized line of human T lymphocyte cells, from a patient with T-ALL leukemia

kb	kilobase
K48(-linked)-ubiquitin	ubiquitin chain, linked via lysin in AS-position 48, resulting in proteasomal degradation
KEAP1	Kelch-like ECH-associated protein 1
L929Ts	an adherent type of mouse fibroblast cell line, Ts: TRAIL-sensitive subline
LDGD	lysosome-dependent cell death
LPS	lipopolysaccharide
LUBAC	linear ubiquitin chain assembly complex
MAPK	mitogen-activated protein kinase
MEF	mouse embryonic fibroblast
MIF	macrophage migration inhibitory factor
MLKL/pMLKL	(phosphorylated) mixed lineage kinase domain-like
MNNG	N-methyl-N'-nitro-N-nitrosoguanidine, chemical compound leading to strong DNA alkylation
MOMP	mitochondrial outer membrane permeabilization
MPT	mitochondrial permeability transition
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
NAD ⁺	nicotinamide adenine dinucleotide (oxidated)
Nec-1s	necrostatin-1s
NETosis	cell death with formation of neutrophil extracellular traps
NF-κB	nuclear factor 'kappa-light-chain-enhancer' of activated B-cells
Nrf2	NF-E2-related factor 2
OMM	outer mitochondrial membrane
OPA1	optic atrophy 1
PAR	poly(ADP-ribose)
PARG	poly(ADP-ribose) glycohydrolase
PARK genes	known gene loci associated with hereditary forms of Parkinson's disease
PARL	presenilin-associated rhomboid-like
PARP1	poly(ADP-Ribose)-polymerase 1
PBM	PAR-binding motive
PBS	phosphate-buffered saline
PDZ	postsynaptic density 95-discs large-zonula occludens-1
PFKFB	6-phosphofructo-2-kinase/fructose-2,6-biphosphatase
PGAM5 _{L/S}	phosphoglycerate mutase family member 5 (long/short form)
PI	propidium-iodide
PINK1	PTEN-induced kinase 1
PIP	phosphatidylinositol phosphate
PSMC4	gene encoding a subunit of the 19S regulatory subcomplex of the 26S proteasome
PTP	permeability transition pore
Pyr-41	4[4-(5-Nitro-furan-2-ylmethylene)-3,5-dioxo-pyrazolidin-1-yl]-benzoic acid ethyl ester, inhibitor of ubiquitin activating enzyme E1
RCD	regulated cell death
RIG-I	retinoic acid-inducible gene I
RIHM	RIP homotypic interaction motif
RIPK1	receptor-interacting serine-threonine kinase 1
RIPK3	receptor-interacting serine-threonine kinase 3
RNA/siRNA	(small interfering) ribonucleic acid
ROS	reactive oxygen species
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis

SMAC/DIABLO	second mitochondria-derived activator of caspase/direct inhibitor of apoptosis-binding protein with low pI
STARD7	START domain-containing protein 7
STING	(cyclic GMP-AMP synthase)-stimulator of IFN genes
TBO	tert-Butyl hydroperoxide
TLR3/4	toll-like receptor 3/4
TNF	tumour necrosis factor
TNF-R1	tumour necrosis factor receptor superfamily member 1
TRADD	TNF-R1-associated death domain protein
TRAF2	TNF-R-associated factor 2
TRAIL-R1/2	TNF-related apoptosis-inducing ligand receptor 1/2
Tris	Tris(hydroxymethyl)aminomethane
TTC19	tetratricopeptide Repeat Domain 19
UPS	ubiquitin proteasome system
ZnF1/2	zinc-finger domains 1/2
zVAD-fmk	carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]-fluoromethylketone

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1 Introduction

1.1 Regulated Cell Death

In the life of any multicellular organism, RCD (regulated cell death) is indispensable. The individual development of those organisms often relies on remodelling and replacement of tissues or whole organs on their way to their adult form. Furthermore, also the maintenance and homeostasis of adult multicellular organisms requires a way to dispose of cells that are dysfunctional or redundant. Historically, the idea of a physiological form of cell death was first brought up during the investigations on metamorphosis in amphibia in the first half of the 19th century. In the 1839 formulated cell theory, that is usually credited to Matthias Schleiden and Theodor Schwann, it was suggested that structural regression could be a result of the disappearance of individual cells (Schleiden, 1842; Schwann, 1939) and in 1842 Carl Vogt provided a detailed description of the disappearance of cells of the notochord and their replacement by cells of the vertebra in his work on the metamorphosis and development of the common midwife toad (*Alytes obstetricans*) (Vogt, 1842). Vogt's descriptions are viewed as the first mentioning of the process that is today known as apoptosis. The first detailed description of that process followed in 1964 by Lockshin and Williams (Lockshin and WILLIAMS 1965) The term apoptosis was first used in the collaborative work of Kerr, Currie and Wyllie in 1972 (Kerr et al. 1972). In 2002, Howard Robert Horvitz, Sydney Brenner and John Edward Sulston were awarded with the Nobel prize in Physiology or Medicine for their discoveries concerning genetic regulation of organ development and programmed cell death. This award further underscores the importance of investigations on cell death, since they lay the foundation on which the understanding of many, both physiological as well as pathophysiological mechanisms, critically relies. Until the late 1990's, cell death was usually divided into two categories: apoptotic cell death and necrosis. This was due to the morphological differences that dying cells display. While apoptotic cells show condensation of chromatin, membrane blebbing and subsequent fragmentation of the cell which then can be disposed of by phagocytosis, the necrotic morphotype is dominated by the swelling of cell organelles and cytoplasm, followed by the bursting of the cells. Necrosis usually occurs when the cell is confronted with extreme microenvironmental conditions such as elevated pressures, shear forces or high temperatures which provoke the physical disassembly of cellular constituents (Galluzzi et al. 2017). This lead to the assumption that apoptosis is the regulated way for cells to die and that necrosis is rather an accidental form of cell death which results from damage to the structural integrity of a cell (Wyllie et al. 1980).

The investigation of alternative cell death pathways began with the finding that the activity of caspases (cysteinyI-aspartate specific proteases) determine if cells undergo apoptosis or (what then was thought to be) necrosis (Hirsch et al. 1997). Based on that, it was speculated that necrosis (at least in some cases) may rather follow distinct pathways that appear randomly after irreparable damage to the cell

(Kolenko et al. 1999). Since then, intensified investigations revealed a whole set of cell death pathways, condensed and visualized in Fig. 1 (Galluzzi et al. 2018). Though those cell death pathways still need to be further investigated to fully understand them, it is broadly accepted that they represent promising targets for pharmaceutical interventions in many pathophysiological settings. This dissertation focusses on two forms of non-apoptotic RCD, called necroptosis and parthanatos, which will be described in more detail hereinafter.

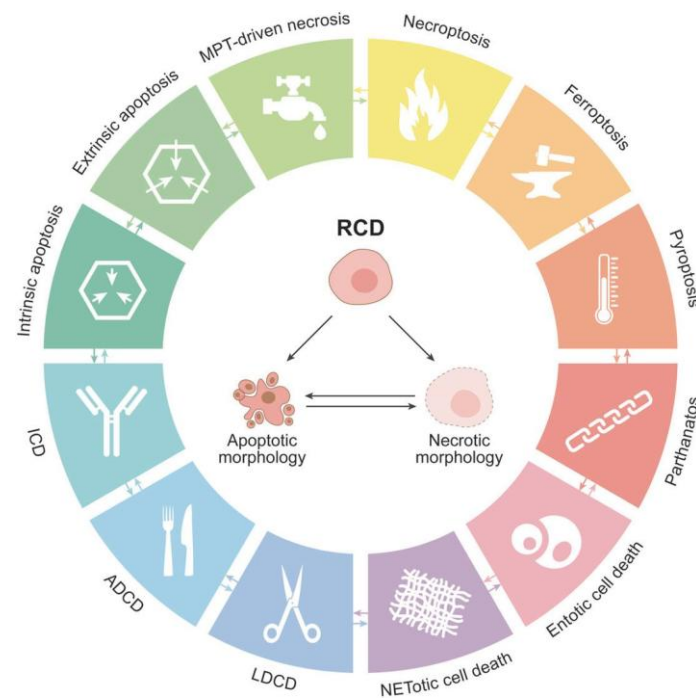


Figure 1: overview of regulated cell death

Depicted are the hitherto described forms of regulated cell death alongside with the possible morphotypes, i.e. necroptosis, ferroptosis, pyroptosis, parthanatos, entotic cell death, NETotic cell death (cell death associated with the release of neutrophil extracellular traps, LDCD: lysosome-dependent cell death, ADCD: autophagy-dependent cell death, ICD: immunogenic cell death, MPT: mitochondrial permeability transition.

Image from: (Galluzzi et al. 2018)

1.2 Necroptosis

In 2005, the term “necroptosis” was first introduced by Degterev and Huang in their investigation of Nec-1 (necrostatin-1), an agent that can prevent necroptosis induced by TNF (tumour necrosis factor) due to its capability to inhibit RIPK1 (receptor-interacting serine-threonine kinase 1) (Degterev et al. 2005).

Necroptosis is today defined as a modality of regulated cell death (RCD) triggered by perturbations of extracellular or intracellular homeostasis (Galluzzi et al. 2018), that critically relies on the phosphorylation of the pseudokinase MLKL (mixed lineage kinase domain-like) by RIPK3 (receptor-interacting serine-threonine kinase 3) (Galluzzi et al. 2017). *In vivo*, the initiation of necroptosis mostly relies on the ligation of specific death receptors. Though this dissertation focusses on the necroptotic pathway initiated by the TNF-R1 (tumour necrosis factor receptor superfamily member 1), it is worth noting that several other death receptors, as well as signalling pathways, are reported to induce necroptosis. Those include FAS (FS-7-associated surface antigen), TRAIL-R1/2 (TNF-related apoptosis-inducing ligand receptor) (Holler et al. 2000), DAI (DNA-dependent activator of IFN regulatory factors) (Upton et al. 2012), DR3 (death receptor 3) (Bittner et al. 2017) and DR6 (Strilic et al. 2016) as well as TLR3 (toll-like receptor 3) and TLR4 (Kaiser et al. 2013), IFN (interferon) signalling (He et al. 2011; Kalai et al. 2002; McComb et al. 2014; Robinson et al. 2012; Thapa et al. 2013), RIG-I (retinoic acid inducible gene I) (Schock et al. 2017) and STING (cyclic GMP-AMP synthase (cGAS)-stimulator of IFN genes) (Brault et al. 2018). Aside from the above mentioned, necroptosis can be the result of severe perturbations of the intracellular homeostasis such as calcium overload (Nomura et al. 2014), excessive ROS (reactive oxygen species) formation (Zhang et al. 2017), inhibition of the proteasome (Moriwaki and Chan 2016), ischemia reperfusion injury (Wen et al. 2017) photodynamic therapy (Coupienne et al. 2011) and last but not least by ionizing radiation (Schildkopf et al. 2010).

Common endpoint of these different receptor pathways in necroptosis is the phosphorylation of RIPK3 which in turn enables it to phosphorylate MLKL on S345 (in mice, T357 and S358 in humans) (Rodriguez et al. 2016). Once MLKL is phosphorylated, it is able to form homo-oligomers which then can bind to PIP (phosphatidylinositol phosphate) located on the inner layer of the cells membrane (Dondelinger et al. 2014). Membrane-bound pMLKL oligomers then mediate the permeabilization of the plasma membrane, though neither the exact stoichiometry of those homo-oligomers nor the exact mechanism that leads to the permeabilization of the cell membrane is sufficiently understood. The permeabilization of the membrane has its visible correlate in the swelling and rupture of the cell, that can be observed in the microscope.

1.2.1 TNF-R1-driven Necroptosis

The events that can follow after ligand binding to TNF-R1 are a good example for the complexity of the regulating network which the different types of RCD seem to constitute. Binding of TNF to the TNF-R1 recruits several proteins to the receptor that together form the so-called complex I. Complex I consists of TNF-R1, TRADD (TNF-R1-associated death domain protein), TRAF2 (TNF-R-associated factor 2) (Hsu et al. 1995), LUBAC (linear ubiquitin chain assembly complex) (Haas et al. 2009) and cIAP1/2

(cellular inhibitor of apoptosis protein 1/2) (Varfolomeev et al. 2008) as well as RIPK1 (Receptor-interacting serine/threonine-protein kinase 1) (Ermolaeva et al. 2008).

From here, the further events depend on the ubiquitination status of RIPK1. By default, cIAP1 in collaboration with the ubiquitin conjugating (E2) enzyme UbcH5a substitute RIPK1 with K63-linked polyubiquitin (Varfolomeev et al. 2008), thus pushing the cell towards survival and synthesis of proinflammatory proteins via the NF- κ B pathway. Alternatively, the activity of cIAP1/2 can be compromised by the mitochondrial protein SMAC (second mitochondria-derived activator of caspase) or the serine protease HtrA2 (high temperature requirement protein A2), which bind to the cIAPs and promote their degradation (Du et al. 2000). The deubiquitinase CYLD (cylindromatosis) can reverse the polyubiquitination of RIPK1 (Moquin et al. 2013; Wertz et al. 2004). This allows RIPK1 to dissociate from complex I, joining RIPK3, FADD (Fas-associated protein with death domain), pro-caspase-8 and cFLIP_L (cellular FADD-like interleukin-1 β -converting enzyme-like inhibitory protein long form) in complex II which constitutes the divergence point between apoptosis and necroptosis in TNF-R1 signalling. The decision which RCD is preferred by the cell now depends on the activity of caspase-8. While high activity of caspase-8 proteolytically inactivates RIPK1 and RIPK3, thus setting the apoptotic machinery in motion, low or no activity of caspase-8 allows RIPK1 and RIPK3 to interact with their RIHM (RIP homotypic interaction motif) domains, resulting in their phosphorylation. This enables MLKL to associate, forming the so-called necrosome where RIPK3 is phosphorylated at Ser232 (in mice, Ser227 in humans) (Chen et al. 2013). As mentioned above, the formation of the necrosome represents the convergence point of the different necroptotic induction pathways.

1.3 Parthanatos

The name parthanatos was introduced in 2006 by Yu *et al.*, in order to delimit it from necrosis and apoptosis (Yu *et al.* 2006). “Parthanatos” is a portmanteau (i.e., a linguistic blend of two words) where “Thanatos” as the name of the greek god of death is combined with the prefix “PAR” referring to poly(ADP-ribose) due to its massive accumulation in cells, that die in a parthanatic manner (Andrabi et al. 2008; David et al. 2009). Parthanatos seems to resemble an emergency reaction upon irreparable damage to the cellular genome. It is, in contrast to most necroptotic signalling, not initiated by extracellular signals but by recognition of severe or prolonged DNA damage in the nucleus. In experimental settings, parthanatos is usually triggered by DNA-alkylating agents such as MNNG (N-methyl-N'-nitro-N-nitrosoguanidine), but the DNA damage can also be the result of oxidative stress, hypoxia, hypoglycaemia or inflammatory cues (David et al. 2009; Virág et al. 2013; Fatokun et al. 2014). Cells dying by Parthanatos display a necrotic morphotype, though some authors mention that the swelling that is observed in necroptosis, is missing in Parthanatos (Yu et al. 2002).

Parthanatos is driven by hyperactivation of PARP1 (poly(ADP-Ribose)-polymerase 1) following its detection of DNA nicks and breaks. The hyperactivation of PARP1 leads to a massive production of PAR-polymers and in the end results in large-scale DNA fragmentation, mediated by AIFM1 (apoptosis-inducing factor 1, mitochondrial) in collaboration with MIF (macrophage migration inhibitory factor) and bioenergetic catastrophe, characterised by the depletion of ATP and NAD⁺ as a result of the inhibition of glycolysis driven by PAR-binding to hexokinase 1 (Andrabi et al. 2014; Fouquerel et al. 2014).

PARP1 is a 116 kDa-protein resident in the cell nucleus and is generally implicated in gene expression and amplification, cell differentiation, cell division, malignant transformation, DNA replication and mitochondrial function (D'Amours et al. 1999; Chiarugi 2002; Kraus and Lis 2003; Hong et al. 2004; Gagné et al. 2008). The PARP protein family consists of 17 isoforms in humans, which all catalyse the attachment of ADP to target proteins, a process that is called ADP-ribosylation (Smith 2001; Hong et al. 2004; Krietsch et al. 2013). Of those PARPs, especially the isoforms 1, 2 and 3 have been shown to be important members of the DDR (DNA damage repair) system (Gibson and Kraus 2012; Vos et al. 2012). The DDR system enables a cell to detect and consequently react to DNA damage. Especially concerning PARP1, those reactions reach from facilitating the repair of the detected damage, to the initiation of the death of the cell by induction of parthanatos. In this dissertation, the other isoforms of PARP are neglected, since PARP1 is responsible for 95 % of PAR synthesized during parthanatos (Dawson and Dawson 2004).

The exact molecular mechanisms under which PARP1 relocates to, recognizes and is activated by the DNA damage site remain not fully understood. Hitherto, at least two ways of PARP1 activation are described in the literature, those are activation by interaction of PARP1 with histones or with DNA.

In the seemingly more common and better understood initiation of PARP1 by DNA interaction, the damage site is recognized and subsequently bound by the two zinc-finger domains of PARP1 (ZnF1/ZnF2). At this point, the course of the following events depends on the severity of the DNA damage to which PARP1 is recruited. Here, PARP1 can operate in two modes in order to fulfil two opposite functions. The detection of mild DNA damage results in the covalent attachment of PAR polymers to PARP1 itself as well as other nuclear protein such as histones, DNA polymerases, topoisomerases and transcription factors (Shall and Murcia 2000; Smulson et al. 2000). This ADP ribosylation is recruiting and regulating these proteins at the binding site of PARP1 in order to restore the integrity of the genome of a cell (Ali et al. 2012).

Mild or normal activation of PARP1 is a transient process that is restricted to the site of damage. This is due to the tight regulation of PAR attachment by PARP1 and its counterplayers Poly(ADP-ribose)

glycohydrolase (PARG) and ADP-ribosyl hydrolase 3 (ARH3). PARG and ARH3 can hydrolyse, thus degrading protein-attached PAR, resulting in free ADP-ribose (Min and Wang 2009; Oka et al. 2006).

Extreme DNA-damage however, hyperactivates PARP1, resulting in accumulation of PAR (Andrabi et al. 2008; David et al. 2009). The accumulating PAR polymers have been shown to function as a signal for cell death (Andrabi et al. 2006). The difference between PAR synthesized by PARP1 in order to repair and PAR synthesized in order to initiate cell death lies not only in the quantity but also in the complexity of the PAR polymers. Both, increasing PAR levels, as well as the amount of branching resulting in higher molecular weight of PAR polymers, have been shown to increase the number of cells dying of parthanatos (Andrabi et al. 2006). It was initially thought, that the massive production of PAR by PARP1 leads to the depletion of the cellular NAD⁺ and ATP pool observed in parthanatos, as NAD⁺ is the substrate of PARP1, while 4 ATP are in turn, required to regenerate NAD⁺ (D'Amours et al. 1999). In 2014, however, it was demonstrated by Andrabi *et al.* and Fouquerel *et al.* that the depletion of the cellular energy pool in parthanatos is a direct consequence of the inhibition of glycolysis by the inhibitory binding of PAR to hexokinase 1. (Andrabi et al. 2014; Fouquerel et al. 2014).

When PARP1 is hyperactivated, the PAR polymers accumulate in the nucleus but also appear in the cytosol and at the mitochondria, where they facilitate the release of AIFM1 (Yu et al. 2006; Yu et al. 2002).

AIFM1 is a flavoprotein primarily located at the inner membrane of mitochondria where it is implicated in the mitochondrial energy generation, functioning as a oxidoreductase (Pospisilik et al. 2007; Susin et al. 1999). It is synthesized as a 67-kDa precursor, transported to the mitochondria, where it is processed to the mature 62-kDa protein (Cao et al. 2007; Otera et al. 2005). AIFM1 was originally described to be a proapoptotic protein, hence its name, with functional similarity to cytochrome c (Susin et al. 1999). Interestingly, the group of Guido Kroemer noticed that in contrast to cytochrome c, AIFM1-mediated chromatin-condensation was not blocked by the pan-caspase-inhibitor zVAD-fmk (that is known to efficiently block apoptosis) (Susin et al. 1999). In 2014, Fatokun *et al.* reviewed mitochondrial-liked mechanisms in parthanatos, calling the initial naming of AIFM1 as “apoptosis-inducing factor” “a misnomer” due to its primary implication in parthanatos (Fatokun et al. 2014).

The question of how PAR polymers are able to bind AIFM1 when considering its residency at the inner mitochondrial membrane was resolved by the finding that 30 % of mitochondrial AIFM1 is bound on the outer side of the mitochondria in neuronal cells (Yu et al. 2009). It could be shown that AIFM1 binds PAR via a PBM (PAR-binding motive) that is separate from the DNA-binding domain (Wang et al. 2011). Wang *et al.* could further show that the binding of PAR to AIFM1 is required for the translocation of AIFM1 to the nucleus during parthanatos.

Once released from mitochondria, PAR-attached AIFM1 can then further bind to cytosolic MIF. MIF is, in addition to its known function as proinflammatory cytokine, a DNA nuclease. Together, AIFM1 and MIF are relocating to the nucleus where they mediate large-scale DNA fragmentation, resulting in fragments of approximately 50 kb (Susin et al. 1999; Daugas et al. 2000; Yu et al. 2002; Wang et al. 2004; Yu et al. 2006; Wang et al. 2011).

In summary, parthanatos is an independent cell death entity, triggered by severe DNA damage and relying on the activity of PARP1, its product PAR, AIFM1 and MIF and is leading to large scale DNA fragmentation and the death of the cell. It is independent of caspases and does not depend on the expression of key proteins of apoptosis. It can be prevented by PARP1 inhibitors or by countering the PAR attachment by activation of the hydrolases PARG or ARH3, preventing the translocation of AIFM1/MIF to the nucleus (Mashimo et al. 2013; Yu et al. 2006).

1.4 Mitochondrial proteins regulating cell death

Mitochondria play a central role in delivering the required metabolites as well as energy equivalents for the cell's survival and proper functioning. In addition, mitochondrial proteins are increasingly implicated in the regulation of cell death pathways, with cytochrome c, SMAC or HtrA2 in apoptosis or AIFM1 in parthanatos being the best-known representatives. The focus of this dissertation lies on three proteins resident to mitochondria, i.e., PGAM5 (phosphoglycerate mutase family member 5), PARL (presenilin-associated rhomboid-like protein) and HtrA2 and their function in necroptosis or parthanatos. In the following, those proteins will be introduced regarding their functions in the regulation of cell death and beyond.

1.4.1 Phosphoglycerate mutase family member 5 (PGAM5)

PGAM5 is a mitochondria-resident protein that occurs in a long (PGAM5_L) and a short (PGAM5_S) form, respectively. This is reported to be the result of alternative splicing events in human cells, resulting in a 32 kDa and a 28 kDa protein (Lo and Hannink 2006). In murine cells, PGAM5 appears similarly in a long and a short form, although there is no description of alternative splicing in murine PGAM5. For murine PGAM5, the appearance of both forms is reported to be mediated by cleavage of full-length PGAM5 by the rhomboid protease PARL (Sekine et al. 2012).

The exact mitochondrial localization of PGAM5 is described inconsistently. In 2008, it was described that PGAM5 was located at the OMM (outer mitochondrial membrane) where PGAM5 can bind KEAP1 (Kelch-like ECH-associated protein 1) and the transcription factor Nrf2 (NF-E2-related factor 2), thus tethering the ternary complex to the outer side of the mitochondria (Lo and Hannink 2008). It was later

proposed by Sekine *et al.* that PGAM5 is located at the IMM (inner mitochondrial membrane), protruding into the intermembrane space (Sekine *et al.* 2012). Additionally, it was demonstrated that PGAM5 is a substrate of the PARL protease (Sekine *et al.* 2012) which was known to be located in the IMM (Cipolat *et al.* 2006; Pellegrini and Scorrano 2007; McQuibban *et al.* 2003). However, it cannot be ruled out that PGAM5_L or PGAM5_S could somehow relocate to the OMM to act in the way reported by Lo and Hannink.

PGAM5 is part of a larger family of phosphoglycerate mutases, consisting of at least 10 distinct proteins, which all contain a structurally conserved PGAM domain (Jedrzejewski 2000). Most of the proteins in the PGAM-family (such as PGAM1, PGAM2, BPGM (2,3-bisphosphoglycerate mutase) and all four members of the PFKFB (6-phosphofructo-2-kinase/fructose-2,6-biphosphatase) (Lo and Hannink 2006) are reported to have phosphoglycerate mutase activity in catalyzing (de-) phosphorylation of small metabolites (Wilkins *et al.* 2014). PGAM5 however lacks the mutase activity and instead catalyzes protein (de-) phosphorylation at serine or threonine residues (Mikhailik *et al.* 2007; Takeda *et al.* 2009; Sadatomi *et al.* 2013). This suggests a function of PGAM5 rather in regulatory signalling than metabolic reactions. As PGAM5 is implemented in a variety of biological processes and signalling cascades, such as mitochondrial homeostasis and ROS-response (Lo and Hannink 2008), in inflammation and differentiation (Takeda *et al.* 2009), cell death (e.g., autophagy (Lu *et al.* 2014), apoptosis (Xu *et al.* 2015) and necroptosis (Wang *et al.* 2012)) mitochondrial dynamics, mitophagic quality control (Lu *et al.* 2014).

Out of these PGAM5-mediated responses, this dissertation focusses on PGAM5's role in response to stress and its role in the field of regulated cell death.

1.4.1.1 PGAM5 in regulated cell death

PGAM5 can function in a pro-apoptotic manner in intrinsic apoptosis. PGAM5_L seems to promote the translocation of BAX (Bcl-2-associated X protein) to the mitochondria leading to increased apoptotic rates, as Xu *et al.* could measure decreased apoptosis rates in experiments featuring a knockdown of PGAM5_L (Xu *et al.* 2015). This implication in intrinsic apoptosis is further corroborated by the finding of Zhuang *et al.*, who showed that truncated PGAM5 can trigger apoptosis through caspase activation (Zhuang *et al.* 2013). This pro-apoptotic role of PGAM5 is opposed by findings that ascribe PGAM5 anti-apoptotic functions mediated by Bcl-xL (B-cell lymphoma-extra large) protein (Yang *et al.* 2017; Ishida *et al.* 2012). This could indicate that PGAM5 exerts context-dependent pro- and anti-apoptotic functions in apoptosis.

In 2012, the group of Xiaodong Wang proclaimed an essential role of both PGAM5_L and PGAM5_S in necroptosis, where PGAM5_S was reported to act downstream of a RIP1/RIP3/MLKL/PGAM5_L complex. This finding initially seemed to fit with previous observations of the group of Prof. Dr. Dieter Adam

(Kiel University, Kiel, Germany), who found that PGAM5 protein-levels accumulate in cells that become necroptotic (Voigt 2014). Notably, the claim of Wang *et al.* that PGAM5 plays an essential role in necroptosis was refuted due to the findings of several groups who could demonstrate that PGAM5 is not required for necroptosis *in vitro*, using MEF (mouse embryonic fibroblast) cells from a PGAM5^{-/-} mice model (Lu et al. 2016; Moriwaki et al. 2016) or by downregulation of PGAM5 using siRNA (Murphy et al. 2013; Remijns et al. 2014).

1.4.1.2 PGAM5 in ROS-response

Nevertheless, PGAM5 is reported to be implicated in a variety of other processes. As mentioned above, the group of Lo and Hannink could demonstrate the interaction of PGAM5 with KEAP1 and Nrf2, arguing that PGAM5 is implicated in the response to ROS (Lo and Hannink 2008). The transcription factor Nrf2 is known to be a major regulator of cytoprotective genes which encode enzymes neutralizing reactive molecules in the cell, such as GST (glutathione S-transferase) and NAD(P)H-quinone oxidoreductase 1. Nrf2 activity is controlled by a ubiquitin ligase complex containing KEAP1, that, in the absence of ROS, targets Nrf2 for proteasomal degradation (Wakabayashi et al. 2003; Itoh et al. 1999; Dhakshinamoorthy and Jaiswal 2001). If, however ROS are present, they prevent the degradation of Nrf2 by perturbing the assembly of the ubiquitin ligase complex due to modifications on cysteine-residues on KEAP1 (Zhang and Hannink 2003). In this context, downregulation of PGAM5 was shown to increase the expression of genes controlled by Nrf2 whereas upregulation of PGAM5 attenuates their expression (Lo and Hannink 2008).

1.4.1.3 PGAM5 in inflammation and differentiation

Additionally, PGAM5 is reported to be an important activator of ASK1 (Takeda et al. 2009), which is known to mediate the JNK- and the p38-pathway (Ichijo 1997). Both the JNK- as well as the p38-pathway are stress response pathways that can result in inflammation as well as differentiation, proliferation or apoptosis (Wagner and Nebreda 2009). Takeda *et al.* could show that PGAM5 is able to activate ASK1 (apoptosis signal-regulating kinase 1) by dephosphorylation, which in turn leads to the activating phosphorylation at Thr-838 (Thr-845 in murine cells) of ASK1. ASK1 is also called MAP3K5 (mitogen-activated protein kinase kinase kinase 5) referring to ASK1's function up-stream of the MAP kinases JNK and p38.

1.4.2 Presenilin-associated rhomboid-like (PARL) protease

PARL is a serine protease that belongs to the family of rhomboid proteases, which are in turn a subgroup of the so-called I-Clips (intermembrane cleaving proteases) (Sekine et al. 2012). PARL was found to be resident to the mitochondria (McQuibban et al. 2003), where its seven transmembrane helices anchor it to the IMM (Sík et al. 2004). The name "Presenilin-associated rhomboid-like" was later challenged by Spinazzi and Strooper *et al.*, who proposed to change the name to "PINK1/PGAM5-

associated rhomboid-like protease”, since they consider the link between PARL and presenilin 2 to be artificial (Spinazzi and Strooper 2016).

1.4.2.1 Substrates of PARL

The two best described substrates of PARL are PINK1 (PTEN-induced kinase 1) and PGAM5. Interestingly, PARL is not cleaving PINK1 and PGAM5 simultaneously but rather “chooses” its preferred substrate depending on the integrity of the mitochondria (Sekine et al. 2012). In healthy mitochondria, PARL cleaves PINK1 thus preventing its accumulation, since PINK1 is usually rapidly degraded by the UPS (ubiquitin proteasome system) after cleavage by PARL (Spinazzi and Strooper 2016). Dysfunctional or damaged mitochondria, however, lose their membrane potential, resulting in a substrate-change of the PARL protease, that now preferentially cleaves PGAM5 (Sekine et al. 2012). This is thought to be at least partially a quality-control mechanism, since the substrate-change of PARL prevents the degradation of PINK1. PINK1 accumulates at the OMM where it interacts with parkin resulting in lysosomal degradation of the dysfunctional mitochondrion (Youle and van der Bliek 2012), a process that often is referred to as mitophagy as specialized subgroup of autophagy. While the research concerning Parkinson’s disease and related neurodegenerative diseases has led to intensive investigation of the PARL-PINK1-pathway (Pickrell and Youle 2015), little is known about the function of PARL in regulating PGAM5.

Besides of PGAM5 and PINK1, there are several other substrates of PARL. In mammals, the serine protease HtrA2 as well as OPA1 (optic atrophy 1) were described to be substrates of PARL (Spinazzi and Strooper 2016; Chao et al. 2008; McQuibban et al. 2003), although other authors presented convincing evidence that PARL is not responsible for the proteolytic processing of OPA1 in mammals (Anand et al. 2014; Duvezin-Caubet et al. 2007; Ishihara et al. 2006). Concerning HtrA2, the group of Thomas Langer from cologne could similarly demonstrate that PARL does not influence the proteolytic maturation of HtrA2 though it contains the required amino acid sequence for cleavage by PARL (Saita et al. 2017). In their work, Saita *et al.* could however identify other substrates of the PARL protease, namely SMAC, STARD7, TTC19 and CLPB. Here, especially the identification of SMAC is interesting because it implies PARL in a pro-apoptotic rather than the hitherto proposed anti-apoptotic function as PARL seems to be employed in maturation of SMAC and its release into the cytosol upon induction of apoptosis (Saita et al. 2017). SMAC then is able to bind and subsequently degrade IAP’s, lifting their inhibitory effect on effector-caspases in the apoptotic machinery.

1.4.2.2 Role of PARL in the organism

Since the biological implications for PARL derive from its substrates, its role is well understood in the context of mitophagy via the PARL-PINK1-parkin-pathway which preserves the homeostasis of the mitochondria as well as disposes of mitochondria that are non-functional. Lesser is the knowledge on

how PARL influences regulated cell death. In mice, a PARL knockout does not seem to affect the embryonal development, but around 4 weeks after birth, the animals suffer from severe progressive cachexia that reduces their lifespan drastically. Furthermore, mice deficient for PARL display atrophic muscular, splenic, and thymic tissue, whereas fibroblasts and myoblasts derived from PARL-deficient mice are reported to be more susceptible to intrinsic apoptotic stimuli (Cipolat et al. 2006). This anti-apoptotic effect of the PARL protease was corroborated by the finding that cytochrome c release occurred earlier in PARL-deficient cells than in wildtype controls (Cipolat et al. 2006). Notably, as outlined above, the group of Thomas Langer has demonstrated that PARL is required for the proper functioning of SMAC, and also that PARL-deficient cells are protected against TNF-induced apoptosis (Saita et al. 2017), thus ascribing PARL a proapoptotic function. In summary, PARL's exact function in apoptosis remains somewhat enigmatic.

1.4.3 High temperature requirement protein A2 (HtrA2)

The high temperature requirement protein A2, also called OMI, is a serine protease resident to the mitochondria where it is thought to be located at the IMM, protruding into the intermembrane space (IMS) (Vande Walle et al. 2008). HtrA2 is transported to the IMS as a 49-kDa proenzyme where the proteolytic maturation is taking place, though it is worth mentioning that there is a subfraction of endogenous HtrA2 protein, which is supposed to be located in the nucleus (Martins et al. 2002; Kuninaka et al. 2007; Gray et al. 2000). The protein contains, besides its serine protease domain and the mitochondrial localisation signal, a C-terminal PDZ (postsynaptic density 95-discs large-zonula occludens-1) domain that is known to arbitrate protein-protein interaction, as well as an N-terminal transmembrane domain that is in proximity with HtrA2's IBM (IAP-binding motif), so that if HtrA2 is cleaved from the membrane, its IBM is freed and can bind to IAP's. Interestingly, the PDZ domain of HtrA2 enables it to bind hydrophobic residues of misfolded proteins in the IMS, an event that has been shown to increase HtrA2's protease activity. It is therefore speculated that HtrA2 plays a role in the recognition and degradation of misfolded mitochondrial proteins (Zhang et al. 2007). In humans, HtrA2 has 3 paralogs (HtrA1/3/4) that, in contrast to HtrA2, are not targeted to the mitochondria but rather are directed into the secretory pathway (Vande Walle et al. 2008) and are therefore not further discussed in the present dissertation.

1.4.3.1 HtrA2 in regulated cell death

HtrA2 is an important part of the apoptotic cascade where it is released into the cytosol in parallel with cytochrome c and SMAC upon MOMP (mitochondrial outer membrane permeabilization) (James et al. 2007). There, HtrA2 can bind IAPs with its N-terminal IAP-binding motif and thereby prevent the binding of IAPs to apoptotic effector caspases, thus amplifying apoptosis (Suzuki et al. 2001; van Loo et al. 2002; Yang et al. 2003).

Notably, the idea of HtrA2 being involved in caspase-independent cell death had already come up in parallel with its implication in apoptosis in the early 2000s (Suzuki et al. 2001; van Loo et al. 2002; Blink et al. 2004; Okada et al. 2004; McCormick et al. 2008; Vande Walle et al. 2010). Accordingly, in recent years, the group of Prof. Dr. Dieter Adam could identify HtrA2 to be a component of the necroptotic machinery in mouse (L929Ts, MEF) as well as in human (HT-29, Jurkat I42) cells, since either pharmacological inhibition or genetic ablation of HtrA2 protected cells from necroptosis induced by TRAIL or TNF (Sosna et al. 2013; Sosna et al. 2016; Fuchslocher Chico et al. 2017). Though it has been initially suggested that HtrA2 does not leave the mitochondrion during necroptosis (van Loo et al. 2002; Blink et al. 2004; Fuchslocher Chico et al. 2017), evidence for a release of HtrA2 in necroptosis was presented recently (Zhang et al. 2019a).

1.4.3.2 Role of HtrA2 in the organism

HtrA2 fulfils several functions in the cell. Belonging to a highly conserved family of chaperone proteins, HtrA2 is a member of the mitochondrial quality control system, degrades misfolded mitochondrial proteins (Goo et al. 2017) and attenuates cellular processes leading to mitophagy (Quirós et al. 2015). Accordingly, HtrA2 has been implicated in neurodegenerative diseases such as Parkinson's disease/syndrome in mice (Martins et al. 2004) and humans (Vande Walle et al. 2008) or Alzheimer's disease. In Alzheimer's disease, the expression of HtrA2 was shown to prevent the oligomerization of amyloid β 1-42 (Kooistra et al. 2009), a peptide typically seen in Alzheimer's disease patients and largely responsible for neurodegeneration.

Furthermore, the protease function of HtrA2 has been connected to hypoxia-reperfusion-injury of neurons and cardiomyocytes, connecting HtrA2 to the pathogenesis of clinical highly important diseases such as stroke (Degterev et al. 2005; Xu et al. 2010; Zhu et al. 2012) or myocardial infarction (Smith et al. 2007; Oerlemans et al. 2012; Luedde et al. 2014).

1.5 RCD in medicine

1.5.1 Necroptosis in medicine

Necroptosis was initially understood as (and reduced to) a backup mechanism for apoptosis that allows for cells with an impaired apoptotic machinery (as frequently found in cancer cells or cells infected by certain viruses) to commit suicide (Thon et al. 2006; Conrad et al. 2016). This still holds true, but is expanded by the modern understanding of this cell death entity. Today, necroptosis is understood as a cell death routine that has evolved to counter malignant and infectious threats (e.g., viral, bacterial, fungal or parasitic infections). Unlike apoptosis, which is usually immunologically silent, the strong proinflammatory nature of necroptosis potently triggers the immune response (Heib et al. 2020),

mainly via the release of DAMPs (danger-associated molecular patterns) from the dying cells (Kaczmarek et al. 2013).

Viral infections were one class of diseases where evidence concerning the pathophysiological relevance of necroptosis was gathered. It was found that some viruses are able to prevent apoptosis in their host cells by expression of proteins that inhibit the activity of caspase 8 (Mocarski et al. 2011). In some of those cases, the necroptotic machinery nevertheless allowed for the host cells to die, and additionally amplified the immune response by supporting an inflammatory reaction. The importance of necroptosis as an antiviral mechanism is additionally corroborated by the evolutionary adaptation of several viruses expressing factors preventing necroptosis in their host cells (e.g., the proteins ICP6 and ICP10 by HSV1/2 (herpes simplex virus 1/2) (Guo et al. 2015), E3L by vaccinia virus (Koehler et al. 2017), or M45-dependent RIPK3 inhibition by the murine cytomegalovirus (Nailwal and Chan 2019). Further evidence for an antiviral role of necroptosis came from a study showing that necroptosis restricts influenza A virus as a stand-alone cell death mechanism (Shubina et al. 2020).

Since its discovery, necroptosis has been implicated in numerous other pathological conditions as summarized in Table 1. Amongst them are diseases of affluence such as atherosclerosis, the underlying cause of about 50 % of all deaths in Western society (Pahwa and Jialal 2020), or cancer, which is listed by the WHO (world health organisation) as the second leading cause of death in the Western world, but also diseases such as chronic liver disease with a lower global incidence of 20.7/100,000 (Moon et al. 2020). All the diseases listed below represent fields with large potential for the development of new drugs or therapeutic strategies that modify the necroptotic cell death response.

Table 1: Implications of necroptosis in diseases

Pathological Setting/Disease(-categories)	Source
Acute and chronic liver injury	(Roychowdhury et al. 2013; Gautheron et al. 2014; Deutsch et al. 2015; Afonso et al. 2015; Wang et al. 2016)
Inflammatory skin diseases	(Bonnet et al. 2011; Dannappel et al. 2014; Takahashi et al. 2014)
Acute pancreatitis	(He et al. 2009; Sun et al. 2023)
Atherosclerosis	(Meng et al. 2015; Lin et al. 2013)
Myocardial infarction	(Smith et al. 2007; Oerlemans et al. 2012; Luedde et al. 2014; Yuan et al. 2022)
Ischemia-reperfusion injury	(Rosenbaum et al. 2010; Zhang et al. 2016a)
Acute kidney injury	(Linkermann et al. 2013; Müller et al. 2017)
Systemic inflammatory syndrome	(Meng et al. 2015)

Sepsis	(Duprez et al. 2011; Linkermann et al. 2012; Chen et al. 2020; Sun et al. 2023)
Stroke	(Degterev et al. 2005; Xu et al. 2010; Zhu et al. 2012; Peng et al. 2023)
Multiple sclerosis	(Ofengeim et al. 2015)
Retinal disorders	(Trichonas et al. 2010; Dong et al. 2012; Murakami et al. 2012; Hanus et al. 2013; Tamura et al. 2014)
Amyotrophic lateral sclerosis	(Re et al. 2014; Greco et al. 2018)
Traumatic brain injuries	(You et al. 2008; King et al. 2014; Wu et al. 2023)
Traumatic spinal cord injuries	(Wang et al. 2014; Liu et al. 2015; Fan et al. 2016)
Morbus Alzheimer/Alzheimer's disease	(Qinli et al. 2013; Caccamo et al. 2017; Huang et al. 2018; Balusu et al. 2023)
Morbus Huntington	(Zhu et al. 2011)
Inflammatory bowel disease	(Caprioli et al. 2008; Günther et al. 2011; Welz et al. 2011; Weinlich et al. 2013; Dannappel et al. 2014; Pierdomenico et al. 2014; Takahashi et al. 2014; Negroni et al. 2017; Matsuzawa-Ishimoto et al. 2017; Aden et al. 2018; Zhang et al. 2019a; Zhang et al. 2019b)
Autoimmune and autoinflammatory diseases	(Alvarez-Diaz et al. 2016; Zhang et al. 2019b)
Lysosomal storage disorders	(Vitner et al. 2014)
Duchenne's muscular dystrophy	(Morgan et al. 2018)

Modified from: (Heib et al. 2020)

In cancer, the most obvious implication of necroptosis is its possible role as additional inducible cell death mechanism, since the resistance or resilience to apoptosis is a hallmark of cancer and is found in abundance throughout most cancer entities (Mohammad et al. 2015). In contrast, many common cancer types (e.g. colorectal cancer, leukemia, multiple myeloma, lung cancer, ovarian cancer, breast cancer, hepatocarcinoma, bladder carcinoma, head and neck carcinoma, glioblastoma, cervical cancer, and neuroblastoma) are reported to be commonly susceptible to the induction of necroptosis (Su et al. 2016). Regarding this common susceptibility, new methods to establish more effective pro-necroptotic therapies are sought after. On the other hand, anti-necroptotic agents could be used in cancer therapy since there are reports of necroptosis favouring cancer progression by generating an inflammatory microenvironment that surrounds the necroptotic cells and which promotes angiogenesis, metastasis, drug resistance of cancer cells and is able to diminish the response of the adaptive immune system at least in some cases (Colotta et al. 2009).

Some of the agents already used to overcome apoptosis resistance achieve that goal by redirecting an initially proapoptotic stimulus to trigger the necroptotic pathway, as done with SMAC-mimetics (second mitochondria-derived activator of caspase, a pro-apoptotic mitochondrial protein) upon TNF-stimulation in the (apoptosis resistant) T-ALL leukemia cell line Jurkat (Laukens et al. 2011). Other drugs already approved or in clinical trials, such as TRAIL, obatoclox, 3-bromopyruvate plus chloroquine and

shikonin and its analogs, have been found to induce necroptosis at least in some cancer types (Su et al. 2016). In table 2, a list of drugs known to modify the intensity of necroptosis is shown (derived and modified from Molnár et al. 2019).

Table 2: Available drugs to modify necroptosis intensity

Drug/Agent	Effect on necroptosis	Mechanism	Cells tested in necroptosis	General application in the clinic/in clinical trials (not necroptosis)
Anthracycline Mitoxantrone (Yang et al. 2016)	↑	Induces MLKL phosphorylation	Inhibits TC1 and EL4 cell line-induced tumour growth in mice	Used in chemotherapy for various cancers
Bortezomib/PS-341 (Zhang et al. 2016b)	↓	Disrupts the formation of RIPK1-RIPK3 complex through stabilization of cIAPs	Primary bone marrow-derived macrophages	Bortezomib (PS-341) is used in Multiple Myeloma treatment
Carfilzomib (Ali and Mocarski 2018)	↓	Inhibits induction of pRIPK3 and pMLKL	HT-29 cells	Approved for Multiple Myeloma
Cisplatin (Xu et al. 2017; Choi et al. 2019)	↑	Induces necrosome formation	Various cell lines (Jing et al. 2018; Xu et al. 2017) and rats (Choi et al. 2019)	Used in the treatment of numerous human cancers (Dasari and Tchounwou 2014; Sun et al. 2018)
Cyclosporine A (Fakharnia et al. 2017)	↓	Reduction of the necroptosis markers RIPK1 and RIPK3	Rat models of cerebral ischemia-reperfusion injury	Widely used immunosuppressive drug
Dabrafenib (Li et al. 2014; Cruz et al. 2018)	↓	RIPK3 inhibition by competing with ATP binding	Normal human hepatocytes and mouse models of ischemic injury	Approved in BRAF-mutant melanoma
Dasatinib (Xu et al. 2018)	↑	Plays a role in HMGB1-induced necroptosis.	CCC-HEH-2 human embryonic cardiac tissue-derived cell lines	Used as an anticancer drug in CML patients
Dexmedetomidine (Chen et al. 2019)	↓	Inhibition of HMGB1 expression	H9C2 embryonic rat heart-derived cells	Used in the intensive care setting for light to moderate sedation

Diacerein (Abd-Ellatif et al. 2019)	↓	Decreased renal expression of RIPK3 and MLKL; prevents necroptosis	Acute kidney injury in rats	Registered in some European Union and Asian countries to treat joint diseases
Dimethyl Fumarate (Xie et al. 2015; Schroeder et al. 2017)	↑	Depletion of GSH; increases MAPK and ROS activation, inhibits the Trx1/NF-κB axis	Gastrointestinal CT26 and lymphoid cancer cell lines Se-Ax, HH and CEM cells	Used in relapsing-remitting Multiple Sclerosis
Fluorouracil (Oliver Metzger et al. 2016)	↑	Reduces cIAP1 protein level; stabilizes binding between RIP1 and RIP3; blocks tumour growth	Xenograft experiments with HT29 cells	Used in chemotherapy in for various cancers
Hypomethylating agents (decitabine, 5-azacytidine and RG108) (Koo et al. 2015)	↑	Restores RIPK3 in cancer cells where RIPK3 had previously been silenced	Human breast tumour and AML (acute myeloid leukemia) samples	Decitabine and Azacytidine are used in Myelodysplastic Syndrome and AML
Interferons, Type III (Thapa et al. 2013; Günther et al. 2016; McComb et al. 2014)	↑	Increases expression of RIPK3 and/or MLKL	Mouse studies in a sepsis model	Used in treatment of viral infections, autoimmune diseases and cancer
Lithium (Wang et al. 2017)	↑	Induces AKT- and mTOR-mediated necroptosis	RT4 cells and human primary schwannoma cells	Treatment of bipolar disorders
Melatonin (Zhou et al. 2018)	↓	Represses the RIPK3-PGAM5-CypDmPTP pathway	Mouse studies in cardiac ischemia-reperfusion models	Used for jetlag sleep disorder
Miconazole (Chengzhu et al. 2019)	↑	Upregulates RIPK3 and MLKL	MDA-MB-231 cells	Anti-fungal medication
Oxaliplatin (Yang et al. 2016)	↑	Induces ATP release in RIPK3- and MLKL-expressing cells; inhibits tumour growth	TC1 and EL4 cell line-induced tumour growth in mice	Used in colorectal cancer
Phenhydan (Moerke et al. 2019)	↓	Suppresses phosphorylation and activation of RIPK1, RIPK3 and MLKL	MEF, L929, NIH3T3, HT-29, U937, and Jurkat mouse and human cell lines	Used as an anti-convulsive drug
Phenytoin (Mässenhausen et al. 2018)	↓	Partial inhibition of RIPK1	HT29 cells and RAW 264 cells, human colon cancer cell lines	Used as anti-arrhythmic class Ib and as anticonvulsant

Pazopanib (Fauster et al. 2015)	↓	Inhibits RIPK1	FADD-deficient Jurkat cells	Approved for renal cell carcinoma and soft tissue sarcoma
Ponatinib (Fauster et al. 2015)	↓	Inhibits both RIPK1 and RIPK3	FADD-deficient Jurkat cells	Approved in some chronic myeloid leukemia and some acute lymphoblastic leukemia
Rapamycin (Ding et al. 2019)	↓	Inhibits RIPK1 expression	Experimental retinal detachment in rats	Approved for prevention of transplant rejection in Lymphangioleiomyomatosis, and to prevent restenosis in coronary arteries following balloon angioplasty
SAHA/Vorinostat (Di Wang et al. 2013)	↓	Activates NF-κB and p38 MAPK; inactivates JNK and Akt kinase; enhances cFLIPL expression	L929 cells and human neuroblastoma SH-SY5Y cells	Approved for the treatment of Cutaneous T cell lymphoma
Sorafenib (Martens et al. 2017; Feldmann et al. 2017)	↓	Reduces interaction of RIPK1 with RIPK3, inhibits RIPK1 and RIPK3 kinase activity protects against TNF-induced SIRS	TNF-induced SIRS and renal ischemia reperfusion Injury models	Approved for advanced thyroid and renal cell cancer, hepatocellular carcinoma
Valproic acid (Bollino et al. 2015)	↑	Induces JNK1 activation and RIPK1 expression	Rat PC12 cells	Used in epilepsy and mood disorders (Stephen 2003)

Modified from: (Molnár et al. 2019); ↑ indicating increase, ↓ indicating decrease

1.5.2 Parthanatos in medicine

Many of the *in-vivo* experiments on parthanatos have focussed on the field of IRI (ischaemia reperfusion injury), where it could be shown that a knock-out of PARP1 is able to reduce the damage following a focal ischaemic occlusion (Goto et al. 2002; Shimizu et al. 2013). Especially for PARP1-inhibitors, a protective function in neuronal IRI-models has been shown by diminishing the infarct size and oedema volume, as well as attenuating neurovascular damage after acute ischemic stroke (Matsuura et al. 2011). The PARP1 inhibitor JPI-289 (also called Amelparib) proved effective in an animal stroke model (Kim et al. 2017) and is therefore currently investigated in a multi-center, randomized, double-blind, placebo-controlled, phase IIa clinical trial and tested in patients with acute ischemic stroke (Jeil Pharmaceutical Co. 2017).

Another important role of parthanatos may be in the pathogenesis of Parkinson's disease. A large part of the experiments investigating the pathogenesis as well as potential treatments of Parkinson's disease critically rely on the use of MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine), and MPTP-

toxicity in mice is the most commonly studied animal model of Parkinson's disease (Dauer und Przedborski 2003). Since 1996, evidence accumulated that PARP1-inhibitors offer protection against the neurotoxic effect of MPTP/MPP+-treatment (Cosi et al. 1996; Outeiro et al. 2007; Yokoyama et al. 2010). Additionally, it was shown that there is a nuclear translocation of AIFM1 (as observed during parthanatos) exclusively in dopaminergic neurons of deceased Parkinson's disease patients and it could further be shown that a similar nuclear translocation of AIFM1 could be prevented by blocking PARP1 in an LPS-injection model of Parkinson's disease (Burguillos et al. 2011). This indicates a participation of PARP1 (Outeiro et al. 2007; Burguillos et al. 2011) in the pathogenesis of Parkinson's disease. This was further corroborated in 2018 when parthanatos (and PAR) was identified as the key mediator of alpha-synuclein-toxicity and related neurodegeneration (Kam et al. 2018). Additionally, parthanatos is reported to be implicated in other neurodegenerative diseases such as Alzheimer's disease (Love et al. 1999) Huntington's disease, spinal cord injury (Maier et al. 2007) and retinal disease (Greenwald and Pierce 2019). It must be mentioned that PARP1 also has become a promising target in cancer therapy with more than 70 clinical trials testing PARP1-inhibitors in therapy of Ovarian, Breast, Prostate, Head and Neck, Lung, Gastric, Pancreatic, Endometrial, Colon, Rectal and Germ cell cancer as well as Glioblastoma, Ewing's sarcoma and Leukemia (Lin and Kraus 2017). However, the therapeutic approach with PARP1-inhibitors rather targets PARP1's function as member of the DDR system, allowing cancer cells to repair DNA damage to survive, than its role in parthanatos.

2 Aims

2.1 Mechanism behind the accumulation of PGAM5 in necroptosis

The observation of the group of Prof. Dr. Dieter Adam that PGAM5 levels increase in necroptotic cells suggests a function of PGAM5 in necroptosis, although this role may be accessory rather than essential (Voigt 2014). In addition, a similar accumulation of PGAM5 in apoptosis is reported, where it is thought to sensitize cells to death signals (Zhuang et al. 2013). This raised the question if there could be a similar sensitizing function to the accumulation of PGAM5 in necroptosis. Thus, this was addressed in the present dissertation by (a) further investigating the mechanism behind the accumulation of PGAM5 upon induction of necroptosis, and (b) by re-evaluation of a possible participation of PGAM5 in the necroptotic signalling cascade.

2.2 Influence of PARL on necroptosis

The initial claim by Wang and colleagues that both the long (PGAM5_L) and the short form (PGAM5_S) of PGAM5 are employed in necroptotic cell death (Wang et al. 2012) gave rise to the question whether the rhomboid protease PARL also plays a role in necroptosis, especially since PARL-mediated cleavage

of PGAM5 is responsible for the appearance of PGAM5_L and PGAM5_S in murine cells (Sekine et al. 2012). Given that, in contrast to the findings of Wang and colleagues, PGAM5 was shown not to be an essential member of the necroptotic cascade by other investigators (Lu et al. 2016; Moriwaki et al. 2016), another aim of this dissertation was to clarify whether PGAM5 could play a PARL-dependent, accessory role in necroptosis, supporting necroptotic cell death (CD), though not as an essential member of the necroptotic signalling cascade. It should further be clarified whether PARL could mediate the pro-necroptotic effect of PGAM5_{L/S}, measured by Wang and colleagues by releasing PGAM5_S upon loss of the mitochondrial membrane potential. Independent from its function in cleaving PGAM5, there have been contradictory reports whether PARL is also responsible for the proteolytic maturation of HtrA2 (Spinazzi and Strooper 2016; Chao et al. 2008; Saita et al. 2017). Since HtrA2 is known to participate in necroptosis, another aim of this dissertation was to investigate a participation of the PARL protease in necroptosis.

2.3 Role of PGAM5 in MNNG-induced parthanatos

Since parthanatos is known to be elicited by ROS-induced damage and PGAM5 is, via the KEAP1 Nrf2-axis employed in the regulation of oxidative stress, a possible connection between PGAM5 and parthanatos should be investigated in this dissertation. Additionally, this aim was further corroborated by experimental results obtained by Wang and colleagues, indicating that PGAM5 is important for cell death following H₂O₂- as well as tert-butyl hydroperoxide (TBO) treatment, both being strong oxidants which could induce parthanatos. Moreover, Wang *et al.* did not specify the exact cell death entity in their study, but merely reported “necrosis” following H₂O₂ or TBO-treatment (Wang et al. 2012).

2.4 Role of HtrA2 in MNNG-induced parthanatos

A possible role of HtrA2 in parthanatos was postulated for this dissertation based on a literature research concerning their parallel implication in diseases such as Parkinson’s disease or Stroke/IRI. Parthanatos has repeatedly been implicated in the pathogenesis of Parkinson’s disease (Cosi et al. 1996; Dauer and Przedborski 2003; Outeiro et al. 2007; Yokoyama et al. 2010; Burguillos et al. 2011; Kam et al. 2018) and Stroke/IRI (Goto et al. 2002; Matsuura et al. 2011; Shimizu et al. 2013; Kim et al. 2017), whereas HtrA2 has also been connected to Parkinson’s disease (Martins et al. 2004; Vande Walle et al. 2008), has been identified as a disease-related gene, called PARK13 (Strauss et al. 2005), and is reported to fulfil important functions in the pathogenesis of Stroke/IRI (Degterev et al. 2005; Xu et al. 2010; Zhu et al. 2012). Therefore, HtrA2 might itself be connected to or employed in the cascade that elicits parthanatos. Given that all required cells and methods to analyse a potential role of HtrA2 in parthanatos were already available in the group of Prof. Dr. Dieter Adam, the corresponding analyses were included as an additional aim of this dissertation.

3 Materials and Methods

3.1 Materials

3.1.1 Technical equipment

Table 3: Technical equipment

Company	Equipment
BD Biosciences, Heidelberg, Germany	FACSCalibur flow cytometer Software: BD CellQuest™ Pro V.4.0.2 5 mL polystyrene round-bottom tubes
Bender & Hobein, Zurich, Switzerland	Vortex Genie 2™ vortexer (a mixer)
Biometra, Göttingen, Germany	Rocking platform WT12 (automatic tilting table)
Bio-Rad, Munich, Germany	Glass Plates SmartSpec™ 3000 protein blotting cell PowerPac 300 spectrometer
BRAND, Wertheim, Germany	Neubauer counting chamber Pipette boy Accu-jet® pro
Corning, Corning, NY, USA	Tissue culture dish 100 x 20 mm
Eppendorf, Hamburg, Germany	Refrigerated Centrifuge 5417R Centrifuge 5415D Thermomixer compact Pipettes (2, 10, 20, 100, 200 and 1000 µl)
GE Healthcare, Munich, Germany	HiTrap™ Protein GHP 1mL columns Hyperfilm™ Nitrocellulose blotting membrane 0.2 µm Whatman™ filtering paper Grade 3 CHR
GFL, Wunstorf, Germany	Water bath 1003
Gilson, Middleton, WI, USA	Pipettes (10, 20, 100, 200 and 1000 µl)
Greiner Bio-One, Frickenhausen, Germany	15 and 50 mL Tubes Serological pipettes (1, 5, 10, 25 and 50 mL)

	6- and 12-well plates Cell culture flasks (50, 250 and 650 mL)
Heraeus, Osterode, Germany	LaminAir® HB2472GS (ventilated work bench) Megafuge 1.0 Minifuge RF
Hirschmann Laborgeräte, Eberstadt, Germany	Pipetus®
Kern & Sohn, Balingen, Germany	KERN 440-47N precision scales
Gerlinde Kisker, Mühlhausen, Germany	X-Ray cassette 24 x 30 cm
Lonza Group, Basel, Switzerland	Amaya Biosystems Nucleofector™ 2b Device
Mitenyi Biotec, Bergisch Gladbach, Germany	μ Columns OctoMACS™ Separator
Petra electric	Water boiler
Qualitron Incorporation, Korea	DW-41 Microcentrifuge
Sartorius Group, Göttingen, Germany	Precision scales A120S-D1
Stuart®, Stone, Staffordshire, UK	Roller mixer SRT9
Tecan Austria, Grödis, Austria	Infinite M200 ELISA Microplate Reader, Software: i-Control 1.3 (used for readout of ATP assays)
Thermo Fischer Scientific, Waltham, MA, USA	HERAcell 150i CO ₂ Incubator 96 well plate
Zeiss, Göttingen, Germany	Axiovert 135 microscope

3.1.2 Chemicals and reagents

Table 4: Chemicals and reagents

Company	Chemical
Bachem AG, Bubendorf, Switzerland	zVAD-fmk
BASF Bioreserach, Ludwigshafen, Germany	TNF
Biochrom, Berlin, Germany	L-Glutamine

	PBS Dulbecco w/o Ca ²⁺ McCoy's modified medium 10 x Trypsin/EDTA penicillin/streptomycin
Biovision Incorporated, Milpitas, CA, USA	RIPK1 inhibitor Nec-1s (Necrostatin 1s)
Carl Roth, Karlsruhe, Germany	Methanol ≥99 % (v/v) Hydrogen Peroxide 30 % (w/v) Ethanol 96 % (v/v) Sodium chloride 99,8 % (w/v) Tris Glycine
Cell Signaling Technology, Danvers, MA, USA	LumiGLO®
GE Healthcare, Munich, Germany	Ammonium persulphate TEMED
Invitrogen, Darmstadt, Germany	Agarose electrophoresis grade pcDNA 3.1 Vector
Heirler Cenovis, Radolfzell, Germany	Skimmed milk powder
Lonza Group, Basel, Switzerland	Amaya™ cell line nucleofector™ kit V, R and C
Merck, Darmstadt, Germany	β-mercaptoethanol Dimethyl sulfoxide RIPK3 Inhibitor GSK'872 Glycerol 85 % (v/v) Paraformaldehyde
Miltenyi Biotec, Bergisch-Gladbach, Germany	μMACS Protein G micro beads
PAN Biotech, Aidenbach, Germany	Click's RPMI 1640
Promega, Madison, WI, USA	Cell Titer-Glo® Luminescent Viability Assay
Roche Diagnostics, Mannheim, Germany	Protease Inhibitor Cocktail Tablets
Serva, Heidelberg, Germany	Acrylamide/bis-solution Dodecylsulfate-Na-salt in pellets Trypan blue solution

Sigma-Aldrich, Munich, Germany	Cycloheximide Tween®20 Propidium Iodide Ponceau S
Thermo Fischer Scientific, Waltham, MA, USA	Coomassie Blue Protein Assay Albumin standard NP-40 detergent BSA standard PageRuler™ Prestained Protein Ladder Accutase (Life Technologies) Fetal bovine serum (Life Technologies) Sodium pyruvate (Life Technologies)

3.1.3 Antibodies

Table 5: Antibodies

Target and clone	Dilution	Article number	Company
Primary antibodies:			
actin	1:10000	A1978	Sigma Aldrich, Heidelberg, Germany
Flag M2	1:500	F3165	Sigma Aldrich, Heidelberg, Germany
HtrA2	1:1000	15775-1-AP	OriGene Europe/Acris Antibodies, Herford, Germany
Anti-Ubiquitin, Lys48-Specific	1:500	05-1307	Sigma Aldrich, Heidelberg, Germany
PARL	1:1000	ab45231	Abcam, Cambridge, UK
PARP	1:2000	#9542	Cell Signaling Technology, Danvers, MA, USA
PARP	1:1000	SA-253	Biomol, Hamburg, Germany
PGAM5	1:3000	ab126534	Abcam, Cambridge, UK

pMLKL (human)	1:1000	ab187091	Abcam, Cambridge, UK
pMLKL (mouse)	1:1000	ab196436	Abcam, Cambridge, UK
PMSC-4	1:1000	PA5-44602	Thermo Fisher Scientific, Waltham, MA, USA
poly-ubiquitin (FL76)	1:500	sc-9133	Santa Cruz Biotechnology, Heidelberg, Germany
RIPK3	1:1000	PRS2283	Sigma Aldrich, Heidelberg, Germany
Secondary antibodies:			
goat	1:10000	Peroxidase-conjugated AffiniPure Anti-goat IgG	Jackson ImmunoResearch Europe, Suffolk, UK
mouse	1:10000	Donkey anti-mouse IgG peroxidase-conjugated	Jackson ImmunoResearch Europe, Suffolk, UK
rabbit	1:10000	Donkey anti-rabbit IgG peroxidase-conjugated	Jackson ImmunoResearch Europe, Suffolk, UK

3.1.4 Plasmids and siRNAs

Table 6: Plasmids and siRNA's

Product (Target)	Catalog number	Company
siRNA 120471 (PSMC4)	AM16708	Thermo Fisher Scientific
PGAM5 cDNA in a pCMV6-entry vector	RC201678	OriGene
pcDNA3.1	V79020	Thermo Fisher Scientific
nontargeting siRNA	D-001206-13-20	Horizon Discovery

3.2 Methods

3.2.1 Cell lines and culture

The murine fibrosarcoma cell line L929Ts was derived from L929 cells originally obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) by prolonged passaging in the laboratory. The L929Ts C47 cells that overexpresses PGAM5 were derived from L929Ts cells which were stably transfected with a PGAM5-Flag construct by Dr. Susann Voigt, a former member of the group of Prof. Dr. Dieter Adam (Voigt 2014).

The PGAM5-deficient L929Ts clones A2, A6, A8, B5, B9, B11, C2 and C3 were created using a CRISPR/CAS knock-out performed by Johaiber Fuchslocher Chico, during his dissertation in the group of Prof. Dr. Dieter Adam (Fuchslocher Chico 2017). This also applies to the HtrA2-deficient L929Ts clone C2 and the HtrA2-reconstituted clone C2E2.

Wildtype, PARL-deficient and a PARL-reconstituted MEF as well as HtrA2-deficient MEF (Martins et al. 2004) were kindly provided by Prof. Dr. Thomas Langer (Cologne University, Cologne, Germany). HtrA2-reconstituted MEF were generated in the group of Prof. Dr. Dieter Adam (Weiß et al. 2023).

3.2.2 Generation of cell lysates and measurement of protein concentration

To generate lysates, the cells were detached using phosphate buffered saline (PBS) containing 0.5 % (v/v) trypsin and 0.2 % (v/v) EDTA. Detached cells were pelleted by centrifugation at 400 x g for 5 min at room temperature and washed twice with cold PBS. Then, TNE lysis buffer (50 mM Tris pH 8.0, 150 mM NaCl, 1 % (v/v) NP-40, 3 mM EDTA) containing 10 µg/mL cOmplete protease inhibitor (Roche, Mannheim, Germany) was added for 10 min. Lysates were centrifuged at 20,000 x g at 4 °C and the supernatants were stored at -20 or -80 °C until further analysis. The protein concentration of the lysates was measured using Coomassie Blue Protein Assay. After dilution of 1-5 µL of the lysate in 1mL Coomassie Blue reagent, the Absorbance was measured at a wavelength of 595 nm with a spectrometer. The protein concentration was then calculated by comparison of the absorbance with a previously prepared protein standard.

3.2.3 SDS-Page and Western blot

Standard protocols (LAEMMLI 1970) were followed to prepare 12 % (w/v) SDS-PAGE gels. The lysate, containing 20 µg of protein (if not indicated differently) was diluted into a volume of 20 µL with TNE lysis buffer (see: 3.2.2) and further mixed with 5 x sample buffer (10 % (v/v) β-mercaptoethanol, 125 mM Tris-HCL pH 6.8, 4 % (w/v) SDS, 20 % (v/v) glycerol, 0.02 % (w/v) bromophenol blue) to a final volume of 25 µL. The required protein denaturation was achieved by boiling the samples at 100°C for 5 min. Afterwards, the samples were loaded onto the gel, which was mounted on the mini PROTEAN® tetra system, using PageRuler™ Prestained Protein Ladder as a standard. For separation, an electrical current of 25 mA (per gel) was applied for 1 hour.

The proteins were then transferred to a nitrocellulose membrane via (wet) Western blotting at 100 V for 1 hour. The membrane was then exposed to a blocking solution (PBS with 0.1 % (v/v) TWEEN 20 and 5 % (w/v) milk powder). For incubation with the primary antibody, the membrane was placed in the blocking solution containing the primary antibody at 4°C overnight. The membrane then was washed under gentle agitation in 3 cycles of 10 min in PBST (PBS with 0.1 % (v/v) Tween 20) to reduce unspecific signals. Afterwards, the membrane was incubated with the secondary antibody, diluted in blocking solution, at room-temperature for one hour. After 3 more cycles of washing, the membrane was developed using LumiGLO® reagent. Signals were detected by exposure to Hyperfilm ECL for 10 seconds up to 1 hour.

If the detection of additional proteins was required, enzymatic activity of the peroxidase-conjugated secondary antibodies were inactivated by washing the membrane in 15 % (v/v) H₂O₂ for 1 hour. After 3 cycles of washing, the new primary and secondary antibody were applied to the membrane in the above described manner. The identification of the molecular mass of the submitted proteins was done by comparing their signal with that of the control-bands derived from the PageRuler™ Prestained Protein Ladder.

3.2.4 Propidium-iodide (PI-) assay

As a marker for cell death, the loss of membrane integrity was measured. Cells were seeded in 6-well plates and left overnight to attach. After treatment, cells were detached using Accutase and then centrifuged for 6 minutes at 860 x g and 4°C. After discarding the medium, the precipitated cells were resuspended in PBS and the centrifugation step was repeated with the same settings one more time. Following the second centrifugation, cells were resuspended in 200 µl PBS/EDTA at 4°C. After that, propidium iodide was added at a concentration of 2 µM/mL. The suspension was then measured with FACSCalibur flow cytometer at red fluorescence to discriminate live (unstained) cells from propidium-iodide-positive (dead) cells that had lost membrane integrity.

3.2.5 Immunoprecipitation

Miltenyi µMACS columns were used for immunoprecipitations. The precipitation was performed by incubating 500 µg cell lysate with 1 or 2 µg antibody and 50 µL Protein G Microbeads for 1 to 12 hours on ice. Subsequently, µMACS columns were placed in the magnetic field of the OctoMACS™ Separator and were rinsed with 200 µL of lysis buffer. Afterwards, the µMACS columns were carefully stocked with the prepared lysates, followed by 3 washing cycles with 200 µL lysis buffer each. Then, the columns were rinsed with 100 µL high-salt wash buffer (50 mM Tris/HCl pH 8.0, 1 M NaCl, 1 % (v/v) NP-40, 2 mM EDTA), followed by a last washing step with 100 µL lysis buffer.

The elution of the enriched proteins was achieved by application of 20 µL of 1 x SDS gel loading buffer at 95°C. After 5 min of incubation at room-temperature, additional 30 µL of preheated 1 x SDS gel

loading buffer was applied onto the μ MACS columns. The eluted immunoprecipitate was collected in a fresh tube and was subsequently analysed by Western blot.

3.2.6 Transfections

All constructs that were used for transfections are listed under “4.1.4 Plasmids and siRNAs”. For the transfection of L929 cells, the Amaxa® Cell line Nucleofector Kit was selected. According to the recommendations of the manufacturer 1×10^6 cells were collected and resuspended in 100 μ L room temperature solution V. Afterwards 5 μ g DNA or 150 pmol siRNA was added to the cell solution and the electroporation was performed using the Nucleofector® Program T-20. The cells were then carefully transferred to cell culture plates containing warm medium and cultured for 24 hours. After that, the cells were detached and submitted to further experiments. The success of the transfection (i.e., protein expression) was investigated via Western blot.

4 Results

4.1 Analysis of the mechanism behind the accumulation of PGAM5 during necroptosis

L929Ts cells are a well-established model to investigate TNF-induced necroptosis (e.g., Thon et al. 2006 and Voigt 2014). The accumulation of PGAM5 during necroptosis was addressed in L929Ts C47 cells which stably overexpress a PGAM5-Flag construct (Voigt 2014). Hence, L929Ts C47 cells display 2 bands for endogenous PGAM5, and 2 bands for the slightly larger FLAG-tagged PGAM5 in Western blot analysis (Fig. 2A).

4.1.1 PGAM5 accumulates during necroptosis

Consistent with previous findings in the group of Prof. Dr. Dieter Adam (Voigt 2014), it could be shown that PGAM5-levels increase in L929Ts C47 cells upon induction of necroptosis with TNF and that this accumulation occurs for both endogenous PGAM5 as well as transfected PGAM5 (Fig. 2B). As the stably transfected PGAM5-FLAG construct is expressed from a different promoter than the endogenous gene, this indicates that the accumulation of PGAM5 is mediated by alterations in the degradation of the PGAM5 protein rather than by changes in transcriptional processes (Voigt 2014).

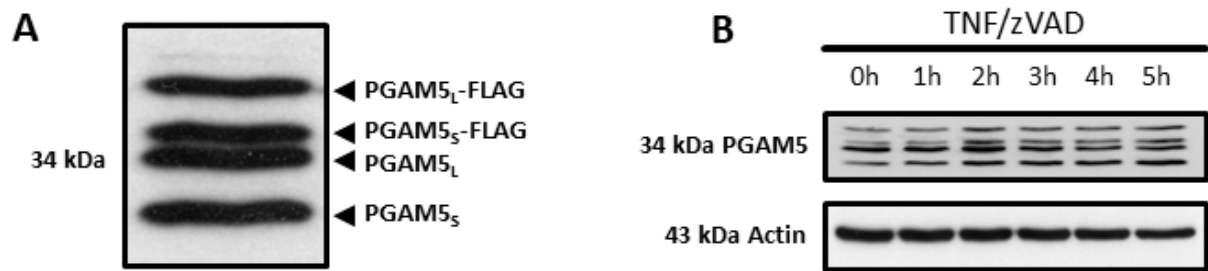


Figure 2: PGAM5 levels in L929Ts C47 cells during necroptosis

(A) Wildtype PGAM5 is detected in a long and a short form in Western blots. L929Ts C47 cells are stably transfected with PGAM5-FLAG and overexpress the protein, thus displaying 4 bands in Western blots .
 (B) Necroptosis was induced in L929Ts C47 cells by addition of 100 ng/mL TNF, 30 min after the pretreatment with 20 μ M zVAD-fmk. Lysates were generated at the indicated time points and analyzed in Western blots with a PGAM5-detecting antibody. Detection of actin served as control for equal loading.

4.1.2 Blockage of the proteasome increases intracellular PGAM5 levels

The above findings suggested that the accumulation of PGAM5 could be result of an attenuated proteolytic degradation during necroptosis. In accordance, previous experiments in the group of Prof. Dr. Dieter Adam had led to a similar increase of PGAM5 when cells had been treated the proteasome inhibitor MG-132 together with the pan-caspase inhibitor zVAD-fmk (Voigt 2014). To further validate the hypothesis of an attenuated proteasomal degradation during necroptosis, the initial experimental setting was expanded in this thesis. In the new setting, Nec-1s, a RIPK1-inhibitor was added in order to

inhibit necroptosis. This was necessary, since cells treated only with MG-132 and zVAD-fmk were still dying with a necrotic morphotype (data not shown), and displayed phosphorylation of MLKL in Western blots (Fig. 3), suggesting that treatment with MG-132 and zVAD-fmk was sufficient to induce necroptosis, even in the absence of TNF, and in agreement with a previous study (Moriwaki and Chan 2016). Since necroptosis increases PGAM5-levels itself, this would confound the effects that the proteasome inhibition with MG-132 had on PGAM5-levels. After the inclusion of Nec-1s (Fig. 3, right

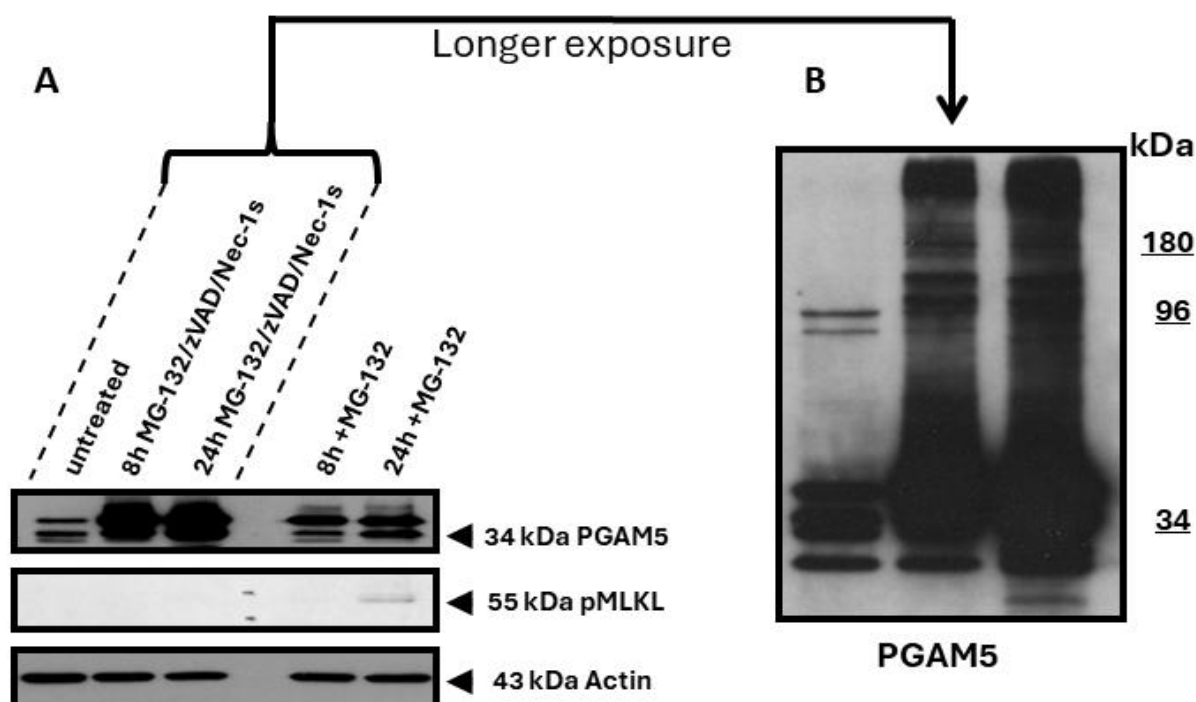


Figure 3: PGAM5 accumulation in L929Ts C47 cells after proteasome inhibition with MG-132
L929Ts C47 cells underwent a treatment with 5 μ M of the proteasome inhibitor MG-132. Additionally, in half of the cells, 20 μ M zVAD-fmk and 50 μ M Nec-1s were added. Lysates were generated 8h and 24h after treatment.
(A) Accumulation of PGAM5 in Western blot when the cells are treated with MG-132 in combination with the indicated inhibitors. Detection of pMLKL indicates necroptotic CD. Detection of actin served as control for equal loading. Exposure time for PGAM5 Western blot: 2 min
(B) long exposure (10 min) of the left upper panel showing high molecular (above 34 kDa) signals for PGAM5.

panel) to the experimental setting, the observed cell death was diminished and there were no more signals detected for pMLKL in Western blot, validating that necroptosis was indeed inhibited under the applied conditions. Interestingly MG-132's ability to induce necroptosis is reported to be mediated by MG-132's effect on RIPK3 (Moriwaki and Chan 2016; Omoto et al. 2015) that acts downstream of RIPK1 in the necroptotic cascade. The question why Nec-1s (RIPK1-inhibitor) was able to prevent necroptosis caused by blockage of the proteasome in the experiments performed for this dissertation remains to be answered and is readdressed in the discussion of this dissertation.

After MG-132-treatment (with or without inhibitors of cell death), the cells showed a strong accumulation of PGAM5 within the first 8 hours which was further increased after 24 hours of treatment (Fig. 3, left panel). Simultaneously, high molecular weight bands occurred in Western blots when the film was exposed for a longer time, possibly representing polyubiquitinated forms of PGAM5 (Fig. 3, right panel).

4.1.3 Immunoprecipitation of PGAM5 with a K48-polyubiquitin-detecting antibody

To validate this assumption, PGAM5 was immunoprecipitated with an antibody that detects K48-linked polyubiquitination. K48-linked polyubiquitin (a chain of polyubiquitin where one monomer is connected to the other only by the lysin in position 48 of ubiquitin) is the form of polyubiquitination that targets proteins to the proteasome to be degraded. If an attenuated proteasomal degradation was the cause of the observed accumulation of PGAM5, a decrease in the amount of PGAM5 immunoprecipitated with the K48-detecting antibody during a necroptotic time-kinetic was to be expected.

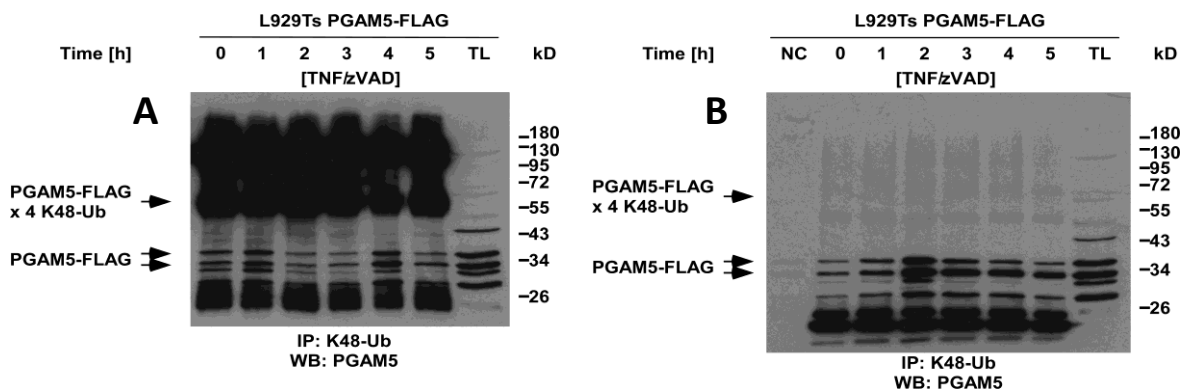


Figure 4: Immunoprecipitation of PGAM5 with two different lots of K48-ubiquitin-detecting antibody

(A) Immunoprecipitation of FLAG-tagged PGAM5 previously performed in the group of Prof. Dr. Dieter Adam. (B) Immunoprecipitation under equal conditions in the course of the present dissertation, except that a new lot of the anti-K48-linked-ubiquitin antibody was employed.

In both experiments, necroptosis was induced by 100 ng/mL TNF following a 30 min preincubation with 20 μ M zVAD-fmk and lysates were taken at the indicated timepoints. The lysates were then immunoprecipitated with the K48-linked-ubiquitin-detecting antibody and subsequently analyzed in Western blots with a PGAM5 antibody.

Therefore, necroptosis was induced in L929Ts C47 cells for 5 hours. Lysates were prepared every hour and immunoprecipitated with an antibody against K48-linked polyubiquitin followed by Western blot with an anti-PGAM5 antibody.

In contrast to a previous, albeit preliminary experiment that had been performed in the group of Prof. Dr. Dieter Adam (Fig. 4A) it was not possible to immunoprecipitate PGAM5 in the above described

manner, and therefore not possible to replicate the result of the preliminary experiment. After multiple repetitions of this experiment, which consistently did not detect K48-polyubiquitinated PGAM5 (one example is shown in Fig. 4B) the approach was abandoned due to the finding that the anti-K48-linked-ubiquitin antibody used in the previous experiment was from a different, no longer available lot than the antibody lot used in this thesis.

4.1.4 Inhibition of the ubiquitin-activating enzyme E1 increases PGAM5 levels

Because of the above results, an alternative approach was employed, using the drug Pyr-41 (4[4-(5-Nitro-furan-2-ylmethylene)-3,5-dioxo-pyrazolidin-1-yl]-benzoic acid ethyl ester). Pyr-41 inhibits the activity of the ubiquitin-activating enzyme E1, which in turn facilitates the very first step in the process of ubiquitin-attachment to target proteins (Haas et al. 1982). Pyr-41 was the first cell permeable inhibitor of the ubiquitin-activating enzyme E1 and was suggested as potential cancer therapeutic by its developers (Yang et al. 2007). Yang *et al.* could demonstrate that the ability of Pyr-41 to inhibit the

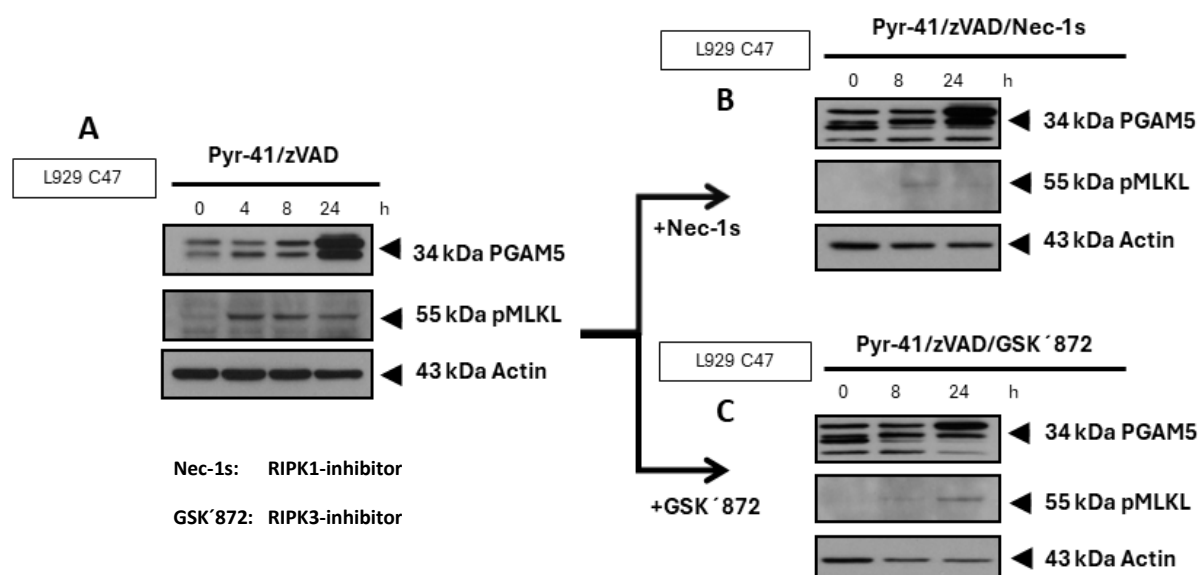


Figure 5: Treatment of L929Ts cells with Pyr-41 increases PGAM5-levels

(A) Cells were preincubated with 20 μ M zVAD-fmk for 30 min to inhibit apoptosis. After treatment with 100 μ M Pyr-41 for the indicated times, cell lysates showed an increase in PGAM5 levels as well as the appearance of phosphorylated MLKL (pMLKL) in Western blots.

(B) To exclude a possible confounding effect of necroptosis on PGAM5 levels in this experimental setting,

the cells were additionally preincubated with either 50 μ M of the RIPK1 inhibitor Nec-1s or

(C) 10 μ M of the RIPK3 inhibitor GSK'872 in order to prevent necroptosis and apoptosis. Detection of pMLKL indicates necroptotic CD. Detection of actin served as control for equal loading.

proteasomal degradation of proteins results from a blockade of protein ubiquitination (including, but not limited to K48 ubiquitination). In consequence, the accumulating proteins in cells that are treated with Pyr-41 lack a polyubiquitination and are not degraded despite a functional proteasome. This is in contrast to cells treated with MG-132, where inhibition of the proteasome causes an accumulation of

proteins that are polyubiquitinated (Yang et al. 2007). In a first experiment with Pyr-41, its optimal working concentration in L929Ts C47 cells was found to be 100 μ M (data not shown). In the subsequent analyses, apoptosis had to be blocked by inhibitors, since Pyr-41 was known to be able to induce apoptosis (Yang et al. 2007) which in turn may alter PGAM5 protein levels. Cells treated with Pyr-41 in combination with zVAD-fmk to block apoptosis did show elevated PGAM5-levels. They also displayed a necrotic morphotype (data not shown) as well as the appearance of phosphorylated MLKL in subsequent Western blot analysis of the lysates (Fig 5A).

In the next step, two different inhibitors of necroptosis were additionally employed, targeting either RIPK1 (Nec-1s) or RIPK3 (GSK'872) to prevent an accumulation of PGAM5 by necroptosis and apoptosis. As shown in Fig. 5B, C, the treatment with the ubiquitin-activating enzyme E1 inhibitor still resulted in elevated PGAM5 levels, though the increase in PGAM5 protein levels was observed at later timepoints when compared with the experiments utilizing MG-132 (Fig. 3). Both inhibitors of necroptosis were able to mostly prevent the phosphorylation of MLKL (Fig. 5B, C) indicating necroptosis as the underlying mechanism.

4.1.5 Knockdown of PSMC4 elevates PGAM5 levels

In a third line of experiments, it was tested whether the knockdown of the gene PSMC4 would likewise result in increased PGAM5 protein levels in L929Ts C47 cells. PSMC4 encodes for the regulatory subunit 6B (called subunit 6B afterwards) of the 19S regulatory subcomplex of the 26S proteasome, that has been shown to be essential for the assembly of the 26S-proteasome, and a knockdown of PSMC4 results in attenuated proteasomal activity (Kumar et al. 2010).

In order to investigate the effect of a PSMC4 knockdown, L929Ts C47 cells were transfected with either a siRNA targeting PSMC-4, or with a non-targeting control siRNA, resulting in two subfractions of C47 cells. Afterwards, the transfected cells were again subdivided in two fractions, and one fraction was left untreated, while in the other fraction, necroptosis was induced by treatment with zVAD-fmk and TNF to test the hypothesis that elevated PGAM5 levels sensitize for necroptosis. After 4 hours, the cells were detached from their wells followed by lysis and subsequent subjugation to Western blot analysis (Fig. 6A) or flow cytometric analysis to determine the percentage of dead cells (Fig 6B). The knockdown of PSMC4 in cells without further treatment as well as in cells treated with TNF/zVAD resulted in elevated PGAM5 levels (Fig 6A). Hence, PGAM5 levels also do increase in cells with a defect proteasome assembly, providing further evidence for a proteasomal degradation of PGAM5 in healthy cells under physiologic conditions. Notably, the knockdown of PSMC4 also increased the basal cell

death in the untreated fractions (Fig 6B). This elevated basal cell death was not necroptosis as indicated by the lack of phosphorylated MLKL in Western blots (Fig 6A).

In fractions treated with TNF/zVAD, the knockdown of PSMC4 did likewise increase the cell death (Fig 6B) which was identified as necroptosis, since phosphorylated MLKL appeared in Western blot analysis (Fig. 6A). Furthermore, the increased rate of necroptotic cell death in the cells with PSMC4 knockdown does correspond to a stronger signal for phosphorylated MLKL detected in that fraction (Fig. 6A).

In summary, the knockdown of PSMC4 does increase PGAM5 levels, basal cell death and possibly the susceptibility to induction of necroptosis by TNF/zVAD.

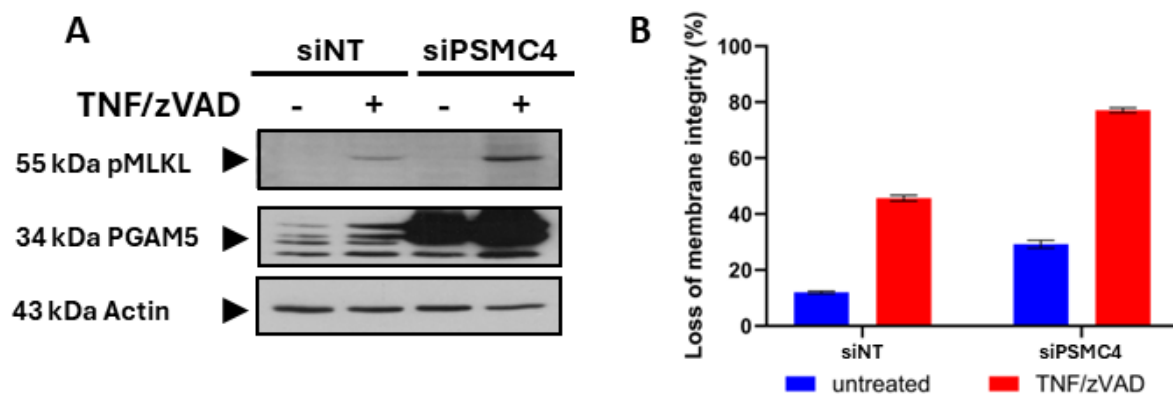


Figure 6: Knockdown of PSMC4 increases PGAM5 levels and susceptibility to necroptosis

L929Ts C47 cells were treated with either an siRNA targeting PSMC4 (siPSMC4) or with a non-targeting siRNA (siNT), then necroptosis was induced by addition of 100 ng/mL TNF after the cells were preincubated 30 min with 20 μ M zVAD-fmk.

(A) Lysates were taken, followed by Western blot analysis of the indicated proteins. Detection of pMLKL indicated necroptotic CD. Detection of actin served as control for equal loading.

(B) Additionally, the loss of membrane integrity (as a marker for cell death) was measured in PI assays using flow cytometry. NT = not transfected. Each bar represents the mean of at least 3 individual measurements, error bars indicate the corresponding standard deviations.

4.1.6 Influence of PGAM5 levels on the susceptibility to necroptosis induced by TNF/zVAD

To test whether the accumulation of polyubiquitinated PGAM5 is involved in the observed increase in necroptotic cell death, PGAM5-deficient L929Ts B11 cells (Fuchslocher Chico 2017) were introduced to the experimental setting. This allowed it to compare the reaction upon induction of necroptosis with regard to both PGAM5- and proteasome subunit 6B-expression. PSMC4 was knocked down in the above described manner in L929Ts C47 and B11 cells and lysates were taken to investigate them with regard to proteasome subunit 6B- and PGAM5-expression (Fig. 7A). PSMC4 could be knocked down in both cell lines, though the appearance of a weak signal for the proteasome subunit 6B in the L929Ts C47 siPSMC4 fraction indicates that the knockdown was less efficient than in the L929Ts B11 clone (Fig.

7A). PGAM5 increased strongly upon knockdown of PSMC4 in L929Ts C47 cells and was not detected in both fractions of the L929Ts B11 clone, validating their PGAM5-deficiency (Fig. 7A).

In parallel, necroptosis was induced with TNF/zVAD and cell death was analyzed using flow cytometry. As shown in Fig. 7B, there was no difference in cell death rates between TNF/zVAD-treated C47 and B11 cells that had been previously subjected to PSMC4 knockdown. This indicates that the increased cell death after knockdown of PSMC4 is unlikely to be mediated by PGAM5. Only in the untreated fractions of these cells, there was higher cell death measured in B11 than in C47 cells, which can most likely be attributed to the difference in the efficiency of the PSMC4 knockdown. When comparing the fractions transfected with a non-targeting control siRNA (NT), PGAM5 seems to offer protection against induction of necroptosis since the PGAM5-expressing C47 clone reacted with lesser cell death to the TNF/zVAD-treatment than the PGAM5-deficient clone B11. However, this seemingly protective effect of PGAM5 is most likely an artifact since no other experiment in the group of Prof. Dr. Dieter Adam that investigated the influence of PGAM5 on necroptosis indicated a protective role (Fuchslocher Chico 2017; Voigt 2014). It must be mentioned in addition, that, due to time constraints, this experiment could only be performed once.

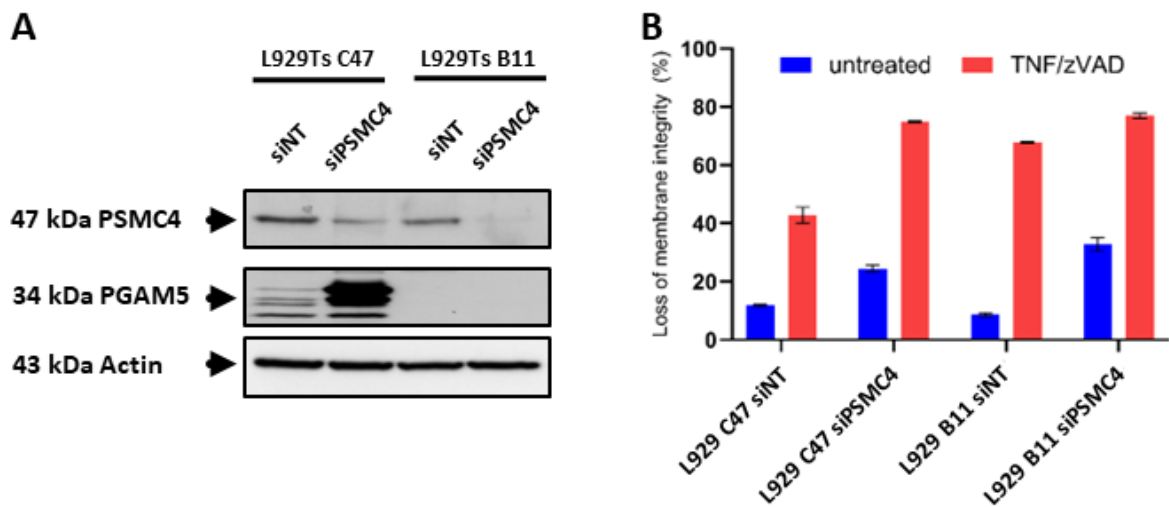


Figure 7: Susceptibility to necroptosis in PGAM5-deficient and -overexpressing clones after knockdown of PSMC4

(A) PGAM-overexpressing L929Ts C47 and PGAM5-deficient L929Ts B11 cells were treated with siRNA targeting PSMC-4 (siPSMC4) or a non-targeting siRNA (siNT) and lysates were taken and submitted to Western blot analysis with regard to PSMC4 and PGAM5 expression. Detection of actin served as control for equal loading.

(B) In parallel, necroptosis was induced by treatment with 100 ng/mL TNF for 4h following a preincubation with 20 μ M zVAD-fmk for 30 min. Loss of membrane integrity was measured as indicator of cell death. Each bar represents the mean of at least 3 individual measurements, error bars indicate the corresponding

4.1.7 Conclusions

The obtained experimental findings strongly suggest that the accumulation of PGAM5 during necroptosis is mediated by the impairment of a constitutive degradation of PGAM5 by the UPS, since all three approaches, the block of the proteasome as well as the inhibition of ubiquitin-attachment by Pyr-41 and the knockdown of the 26S proteasome subunit 6B led to increased levels of PGAM5. It could be shown further, that the accumulation of PGAM5 during necroptosis does not seem to change the number of cells dying by TNF/zVAD-induced necroptosis, which means that the exact function of the PGAM5 accumulation during necroptosis, driven by the arrest of its continuous degradation, needs to be further investigated.

4.2 PARL does not influence the outcome of necroptosis

Since both the long and the short form of PGAM5 had been implicated in necroptosis (Wang et al. 2012) and since the long and short form of PGAM5 occur due to a proteolytic cleavage by the rhomboid-protease PARL (Sekine et al. 2012), the impact of PARL for necroptosis was investigated using a MEF (murine embryonic fibroblast) cell line deficient for PARL (PARL^{-/-}) as well as control cells reconstituted with a PARL-FLAG construct (PARL^{-/-}+PARL). The absence or presence of PARL in these cells was validated via an immunoprecipitation of the FLAG-tagged PARL protein (Fig. 8A). Since a lack of available antibodies prevented the detection endogenous PARL, the cleavage of PGAM5 by PARL was used to validate the knock-out of endogenous PARL in PARL^{-/-} MEF. As shown in Fig. 8B, PARL^{-/-} MEF did not display the lower band of PGAM5 that is present in PARL^{-/-}+PARL MEF as the result of cleavage by PARL.

To investigate the influence of PARL on necroptosis, PARL^{-/-}+PARL and PARL^{-/-} MEF were pre-treated with either zVAD-fmk alone or zVAD-fmk and cycloheximide (CHX) followed by treatment with TNF. CHX is a eukaryotic protein synthesis inhibitor that can be used to sensitize cells to TNF-induced necroptosis (Sosna et al. 2014). After 20 h, the loss of membrane integrity was measured with PI assay using flow cytometry. As shown in Fig. 8C, neither treatment revealed a significant difference in cell death between PARL-reconstituted and PARL-deficient MEF. However, these results are still preliminary and should be validated in future experiments.

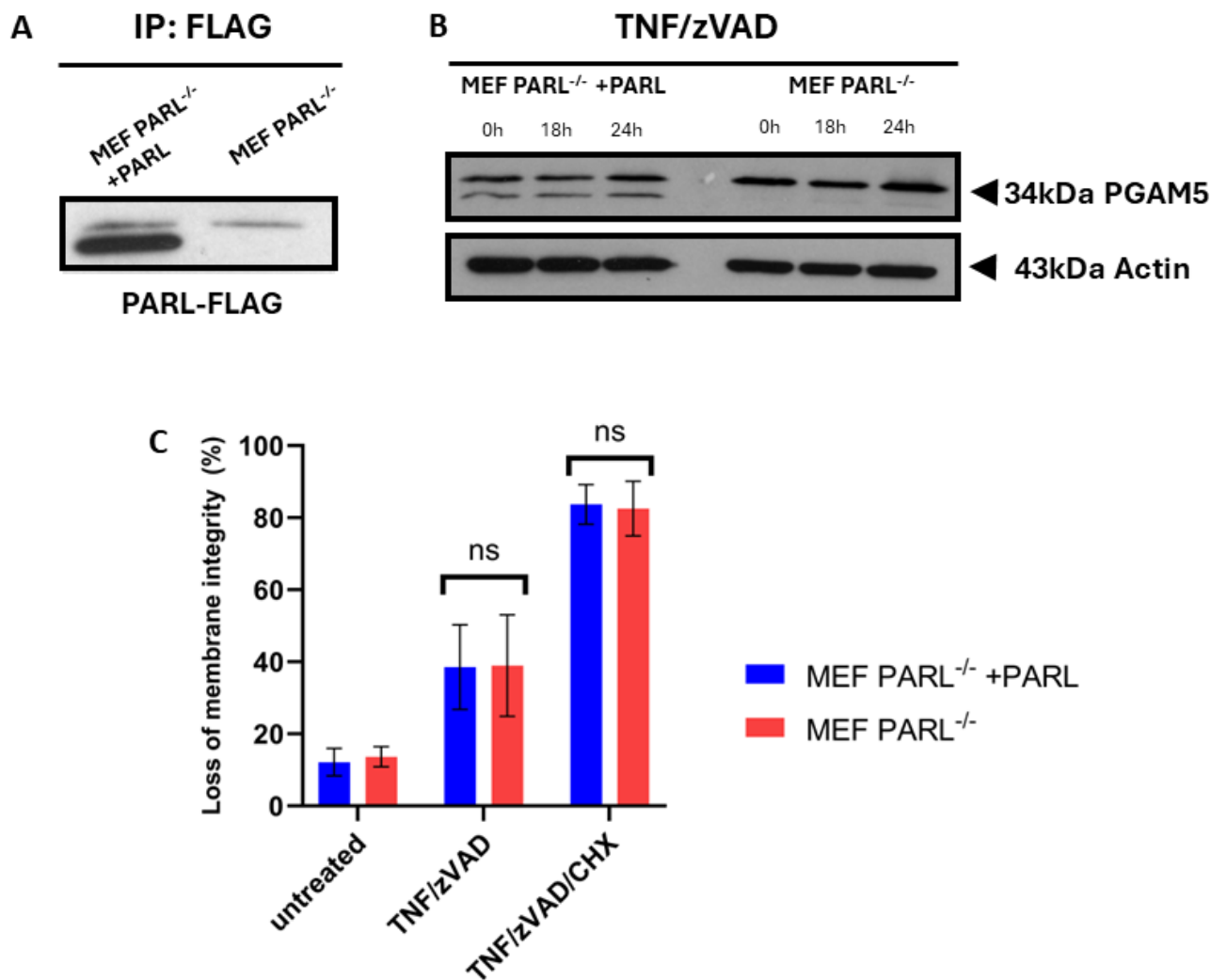


Figure 8: PARL does not influence the outcome of necroptosis in MEF

(A) PARL^{-/-} MEF and PARL^{-/-}+PARL MEF were validated by immunoprecipitation with an antibody against FLAG-tagged PARL.

(B) To test for endogenous PARL activity in PARL^{-/-} MEF, necroptosis was induced in both cell lines by addition of 100 ng/mL following 30 min of preincubation with either with 20 μ M zVAD-fmk or 5 μ g/ml CHX and 20 μ M zVAD-fmk. Lysates were taken, followed by Western blot analysis for cleavage of PGAM5 (lower band). Detection of actin served as control for equal loading.

(C) To investigate whether PARL is implicated in the process of necroptosis, the cells were treated with 100 ng/mL TNF for 20h after 30 min of preincubation with either 20 μ M zVAD-fmk or 20 μ M zVAD-fmk and 1 μ g/mL CHX. Loss of membrane integrity was then measured in PI assays using flow cytometry. Each bar represents the mean of at least 4 individual experiments (*n*=4 also mit 12 Werten), error bars indicate the corresponding standard deviations. Statistical significance was calculated using a two-tailed Student's t-test (ns, not significant; *p*= 0.9255 (TNF/zVAD) and 0.6684 (TNF/zVAD/CHX)).

4.3 Role of PGAM5 in MNNG-induced parthanatos:

In the third part of this dissertation, it was investigated whether PGAM5 influences the outcome of the PARP1-dependent, AIFM1-mediated cell death entity called parthanatos. To address this issue, a set of PGAM5-deficient L929Ts clones were used alongside with the PGAM5-overexpressing L929Ts clone C47 that served as control. The cells were treated with MNNG, since this is the gold standard for induction of parthanatos (Fatokun et al. 2014). At the beginning, the PGAM5-deficient clones were validated in Western blots for PGAM5 (Fig. 9A). After that, a subset of clones (A2, A6, A8, B11, C2) was used for further experiments and clones B5, B9 and C3 were excluded due to their slow growth rates. The PGAM5-deficient clones as well as the PGAM5-overexpressing clone C47 were treated with MNNG and cell death was measured by PI assay using flow cytometry. As shown in Fig. 9B, there was no significant difference in the reaction to MNNG-treatment between the C47-control and the PGAM5-deficient clones A2 and A6. The clone A8 did show increased sensitivity to MNNG-treatment whereas the clones B11 and C2 seem to be protected (Fig. 9B). As a caveat, this potential protection of B11 and C2 was seen only in one of two experiments and therefore could be of artificial origin.

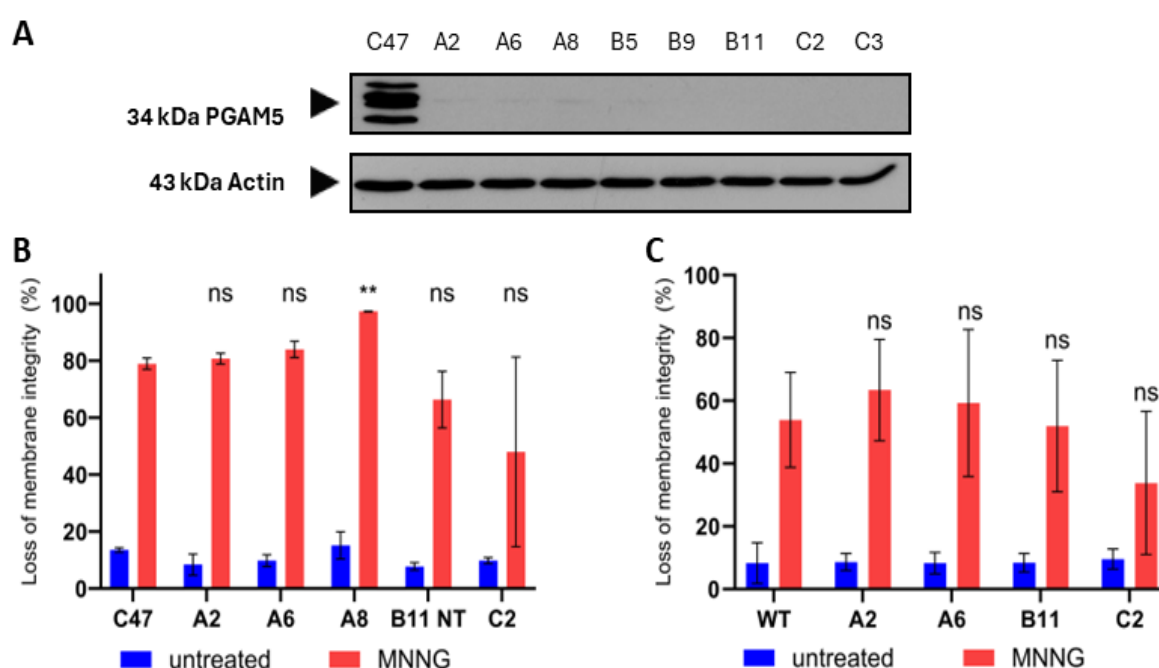


Figure 9: Role of PGAM5 in MNNG-induced parthanatos

(A) The PGAM5 expression status of the employed L929Ts clones was analysed by Western blot using a PGAM5 antibody, detection of actin served as control for equal loading.

(B) The PGAM5-overexpressing clone C47 or (C) wildtype L929 (WT) were compared to the PGAM5-deficient clones A2, A6, A8, B11 and C2 in response to the treatment with 0.5 mM MNNG. MNNG was applied for 15 min, then the medium was changed, followed by 10 hours of incubation at 37°C. Cell death was measured with PI assay using flow cytometry. Each bar represents the mean of 2 independent experiments. Statistical significance was calculated using a two-tailed Student's t-test (ns, not significant for C47 or WT vs A2, A6, B11, C2; ** indicating statistical significance ($p=0.0084$) for the later excluded clone A8).

In a second set of experiments, L929Ts wildtype (WT) cells were tested against the clones A2, A6, B11 and C2 (Fig 9C). The previously used clone A8 displayed increasing rates of cell death both in the treated as well as the untreated fraction (data not shown) and was therefore excluded. In these experiments, the measured cell death of the clones A2 and A6 was higher compared to the wildtype, while B11 showed a comparable response and C2 had diminished cell death rates after treatment with MNNG (Fig. 9C). The large error bars indicate the strong difference between the three independent experiments. Accordingly, statistical analysis revealed no significant differences in the reaction of the individual clones to the treatment with TNF/zVAD (Fig. 9C). The problem of strong differences in the reaction to MNNG was persistent though all experiments with MNNG and has multiple reasons which are addressed in the discussion part of the present dissertation. In summary, the majority of PGAM5-deficient clones didn't seem to be protected from parthanatos induced by MNNG.

Clones that did show signs of protection were however selected for a subsequent retransfection experiment. The clones B11 and C2 were either transfected transiently with a control vector (pcDNA3.1) or with PGAM5 cDNA. An fraction of the transfected cells was analysed by Western blot validating the success of the PGAM5 transfection (Fig 10A) and the remaining cells were treated with

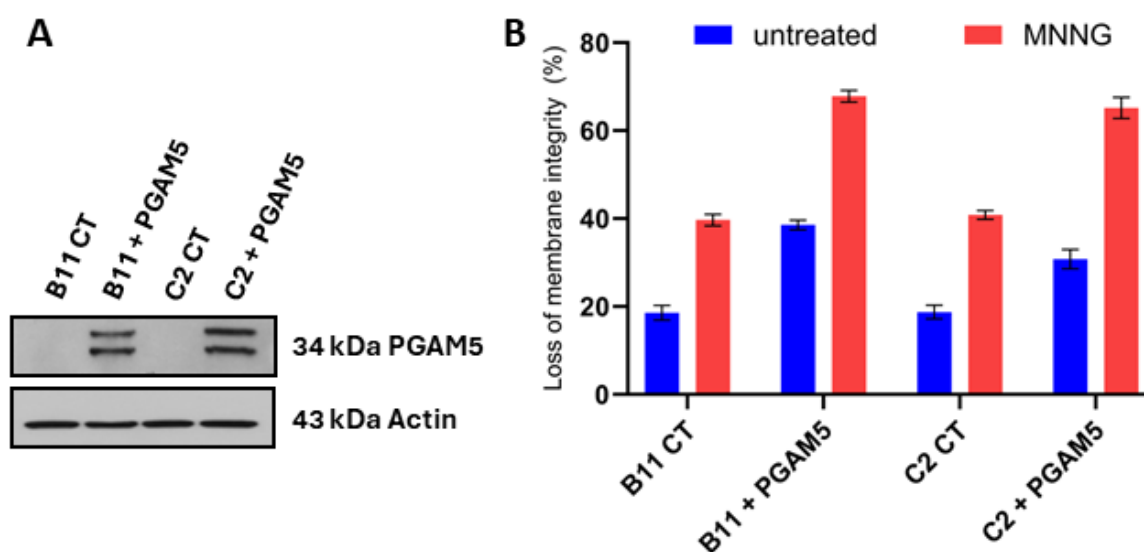


Figure 10: PGAM5-reconstitution in MNNG-induced Parthanatos

In PGAM5-deficient L929 B11 and C2 cells, a transfection of PGAM5 cDNA (+PGAM5) or pcDNA3.1 as a control (CT = control-transfected) was performed. (A) Lysates were taken and subjected to Western blot analysis to validate the success of the reconstitution of PGAM5. Detection of actin served as control for equal loading.

(B) After transfection, cells were either treated with 0.5 mM MNNG for 5 min followed by 14 h of incubation or left untreated for 14 h to serve as a control. After that, cell death was measured using flow cytometry. Each bar represents the mean of at least 3 individual measurements, error bars indicate the corresponding standard deviations.

MNNG followed by flow cytometric analysis (Fig 10B). Notably, the PGAM5-transfected cells, but not the control-transfected B11 and C2 clones, showed increased rates of basal cell death in regular microscopical controls (data not shown). This increase in basal CD was still apparent when cell death was measured by flow cytometry after induction of parthanatos by MNNG for 14 hours (see Fig 10B). This is important for the interpretation of the results of the PGAM5-reconstituted cells in response to MNNG-treatment and is re-addressed in the discussion part of this dissertation. In both PGAM5-transfected clones, the cell death exceeded that of the control-transfected cells after treatment with MNNG (Fig 10B). However, a similar increase in cell death was observed when comparing the cell death in the untreated fractions of control-transfected with the PGAM5-re-transfected cells (Fig 10B). This indicates that the PGAM5 re-transfection (at least in large parts) is the cause of the observed increased cell death, and not a different sensitivity of the cells to MNNG treatment. When the cell death mediated by PGAM5 transfection alone (difference between control-transfected and PGAM5-transfected death of untreated cells) is subtracted from the cell death excess in the treated fractions only 5 % (B11) or 13 % (C2) of cell death are left as potentially caused by participation of PGAM5 in MNNG-induced parthanatos. Since B11 and C2 were the only two out of five PGAM5-deficient clones that showed a certain protection from parthanatos (Fig. 9), their enhanced sensitivity of 5 % and 13 % upon retransfection with PGAM5 is most likely caused by an unspecific variation between the clones rather than mediated by PGAM5-expression. Therefore, it can be concluded that PGAM5-deficiency of murine L929Ts cells does not alter their sensitivity for MNNG-induced parthanatos.

4.4 Relevance of HtrA2 in MNNG-induced parthanatos

To investigate whether the serine protease HtrA2 plays a role in the parthanatic cascade, HtrA2-deficient as well as HtrA2-reconstituted L929Ts cells (Fuchslocher Chico 2017) were tested with regard to their viability after MNNG treatment. As a control, the appearance of phosphorylated MLKL (marker of necroptosis) was investigated in Western blots, in addition to verifying the presence or absence of HtrA2 (Fig. 11A). The lack of pMLKL indicated that the cells died indeed by parthanatos and not by necroptosis. In the initial experiments, HtrA2-deficient L929Ts cells seemed to be completely protected from the treatment with MNNG, since the cell death rates between untreated and treated samples differed only by 2 %. In contrast, in HtrA2-reconstituted L929Ts cells, the sensitivity to parthanatos induced by MNNG was clearly restored (Fig. 11B). This difference was statistically significant in an unpaired t-test ($p=0,0067$).

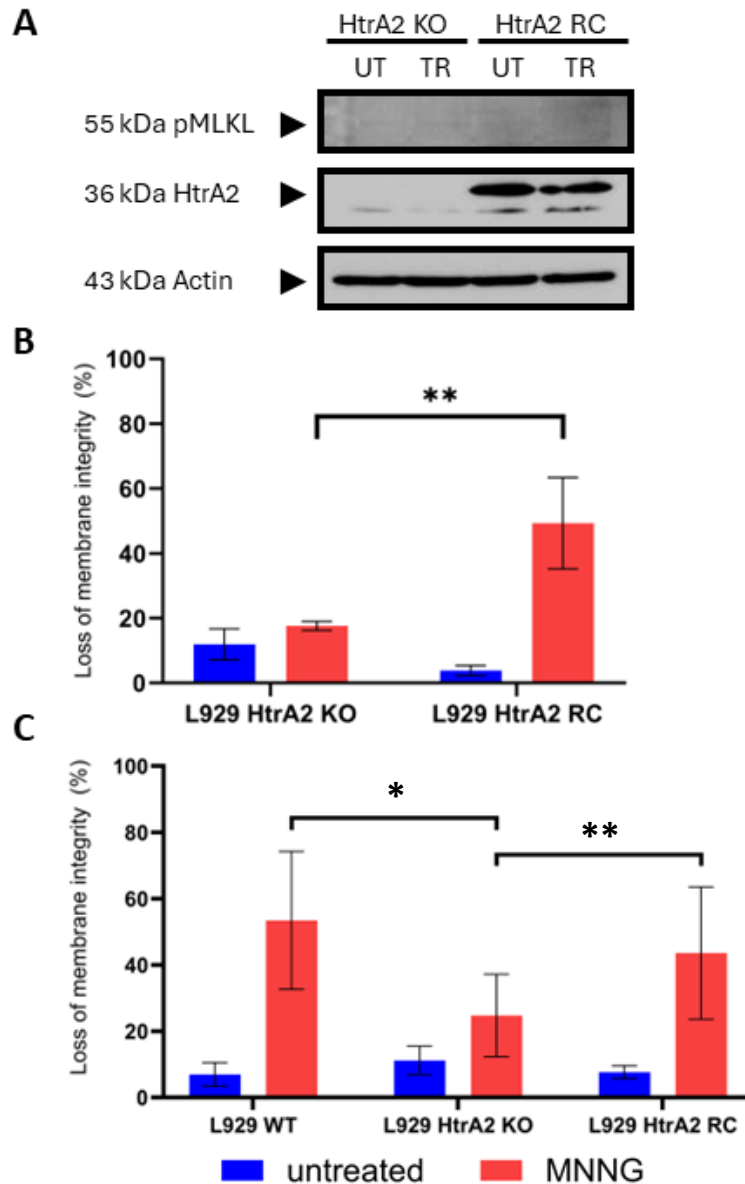


Figure 11: Relevance of HtrA2 in MNNG-induced Parthanatos

HtrA2-deficient (KO) and -reconstituted (RC) L929Ts-cells were either treated with 0.5 mM MNNG for 5 minutes followed by 14h of incubation (TR) or left untreated (UT) for 14 hours.

(A) The cell lysates were analysed in Western blot with regard to their HtrA2 expression as well as appearance of pMLKL to exclude necroptosis. Detection of actin served as control for equal loading.

(B) In parallel, cells were collected and analysed with PI assay using flow cytometry. Each bar represents the mean of 4 independent experiments, error bars indicate the corresponding standard deviations. Asterisks indicate a statistical significant difference between treated fractions in an unpaired Student's t-test ($p=0.0067$).

(C) In a new set of experiments, wildtype L929 cells were additionally analysed. Each bar represents the mean of 4 independent experiments, error bars indicate the corresponding standard deviations. Asterisks indicate a statistical significant difference between treated fractions in a ratio paired Student's t-test (WT vs KO: $p=0.0273$; KO vs RC: $p=0.0036$).

Since the initial experiments suggested an important role for HtrA2 in parthanatos, wildtype L929Ts cells were included in the following experiments. Again, the protection of the HtrA2-deficient L929Ts cells was persistent while HtrA2-reconstituted L929Ts cells were sensitive to MNNG-induced parthanatos, although not to the extent of wildtype L929Ts cells (Fig. 11C). However, it must be mentioned that the new set of experiments didn't indicate a complete protection of the HtrA2-deficient cells, since treatment with MNNG increased their death rates by approximately 10 %. Nevertheless, the above results justify the interpretation that HtrA2 is important for the cytotoxic effects of parthanatos induced by MNNG. Due to strong fluctuations in response to MNNG treatment between the individual experiments in the second part of the investigation of HtrA2 (Fig. 11C), a ratio paired t-test was performed to evaluate statistical significance. Here, the protection of the HtrA2 deficient clone again proved significant (WT vs KO: $p=0.0273$; KO vs RC: $p=0.0036$) providing strong evidence for an important, if not essential role of HtrA2 in parthanatos.

5 Discussion

5.1 Mechanism behind the accumulation of PGAM5 during necroptosis

5.1.1 Blockage of the proteasome increases PGAM5 levels

In the experiments with MG-132, it could be shown that the inhibition of the proteasome does lead to an increase in PGAM5-levels and that this effect is independent from apoptosis or necroptosis, which both can also increase PGAM5 levels (see Fig. 2 and (Zhuang et al. 2013)). The results are in accordance with the hypothesis of a constitutive proteasomal degradation of PGAM5 by the UPS, further strengthening previous results obtained in the group of Prof. Dr. Dieter Adam (Voigt 2014). The necrotic morphotype of the cells treated only with MG-132, in combination with the appearance of pMLKL signals in subsequent Western blot analyses suggests that MG-132 is capable of inducing necroptosis, in line with reports from the literature (Moriwaki and Chan 2016). Interestingly, Moriwaki and Chan suggest that the modification of RIPK3 by ubiquitin is mediating the induction of necroptosis that follows the treatment with MG-132. This is in contrast to the findings presented in this dissertation, since here, Nec-1s was able to prevent the induction of necroptosis by MG-132 (see Fig 3). Nec-1s inhibits the activity of RIPK1, which is known to act upstream of RIPK3 in the necroptotic cascade. Therefore, Nec-1s should not prevent necroptosis if the induction of necroptosis happens downstream and independent of RIPK1. This indicates that MG-132's ability to induce necroptosis might be based on a different mechanism than suggested by Moriwaki and Chan.

5.1.2 Immunoprecipitation of PGAM5 with K48-detecting Antibody

In the experiments performed for this dissertation, direct evidence for an ubiquitination of PGAM5 could not be provided, since the immunoprecipitation of PGAM5 by an antibody that is selective for K48-linked ubiquitin was not successful. As the most likely explanation, the antibody that was used to precipitate K48-ubiquitinated PGAM5 in this thesis did not detect K48-linked ubiquitin with sufficient affinity. This is indicated by the lack of high-molecular weight signals (Fig. 4A), which were still present in a previous experiment with a different lot of the same antibody (Fig. 4B). Additionally, the increase in low-molecular weight (that is, not ubiquitinated) PGAM5 levels during the time kinetic that can be observed in the experiment shown in Fig. 4B is the opposite of the previous results shown in Fig. 4A. Assuming an attenuated proteasomal degradation to be the cause of the accumulation of PGAM5 during necroptosis, a decrease in the amount of non-ubiquitinated PGAM5, as can be seen in Fig. 4A, was to be expected. The accumulation that was instead observed in the experiments performed (Fig. 4B), however, does indicate that the immunoprecipitation had failed at a technical level. Although it cannot be ruled out that this is due to mistakes in the experimental procedure, a change of operator did not change the results obtained in a new line of experiments (data not shown). The null-hypothesis, that is that PGAM5 is not constitutively ubiquitinated and degraded by the proteasome, can be ruled out (though it would explain the results of the immunoprecipitation experiments) because of the results that were obtained in the experiments with MG-132, Pyr-41 and the knockdown of PSMC4, which all support a constitutive degradation of PGAM5 by the UPS. In summary, results obtained in this line of experiments cannot be used to discard the hypothesis, that PGAM5 is continuously ubiquitinated and degraded by the UPS, nor do they validate it.

5.1.3 Inhibition of the ubiquitin-activating enzyme E1 increases PGAM5 levels

In the experiments with Pyr-41, a combination of Pyr-41 and zVAD-fmk did result in elevated PGAM5-levels, which was due to the induction of necroptosis (Fig. 5A). After the addition of the necroptosis inhibitors Nec-1s or GSK'872, necroptosis was abolished, but the increase in PGAM5 levels nevertheless persisted (Fig. 5B, C). This further substantiates the hypothesis that PGAM5 is constitutively degraded by the UPS, at least in the L929Ts C47 cells studied in this dissertation. The additional observation that the increase in PGAM5 levels occurred at later timepoints than in the experiments with MG-132 can be explained by the different mechanisms by which MG-132 and Pyr-41 cause their effects. MG-132 inhibits the proteasome directly and the increase in PGAM5 levels can be observed around 8 hours (at least in L929Ts cells). Pyr-41 however, inhibits the transfer of ubiquitin to the ubiquitin-activating enzyme E1, which is the first step in the attachment of ubiquitin to a protein. From there, the ubiquitin is transferred to the ubiquitin-conjugating enzymes E2 and subsequently to ubiquitin E3-ligases. Those ubiquitin-ligases in turn mediate the transfer of the ubiquitin chain to a

target protein (R. J. Dohmen and Martin Scheffner 2012). Therefore, a possible explanation for the later increase in PGAM5 levels might be that, when Pyr-41 is applied, a pool of already ubiquitinated E2- and E3-enzymes as well as already ubiquitinated proteins is remaining within the cell. This pool would have to be exhausted before proteins would cease to be degraded and hence accumulate. This would lead to the observed later accumulation between 8 and 24 hours after application of Pyr-41.

Evidence for the ability of Pyr-41 to induce necroptosis in combination with zVAD-fmk is provided by the presence of pMLKL signals in Western blot analysis, beginning 4 hours after treatment (Fig 5A). That finding was not entirely unexpected, since MG-132 had already been shown to be able to induce necroptosis (as well as other proteasome inhibitors) (Moriwaki and Chan 2016) and treatment with Pyr-41 (although by a different mechanism) ultimately also leads to a decrease in proteasomal degradation (Yang et al. 2007). Surprising however was the early timepoint at which the pMLKL-signals appeared in Western blot following the treatment with Pyr-41/zVAD, especially regarding the fact that the accumulation of PGAM5 did happen at a later timepoint, suggesting that Pyr-41 is able to induce necroptosis independent of PGAM5 accumulation. The Pyr-41/zVAD-mediated induction of necroptosis seems to require the activity of RIPK1, since addition of Nec-1s resulted in the loss of pMLKL-signals, identical to the findings with MG-132.

It should be noted that the findings on the ability of MG-132 and Pyr-41 to induce necroptosis were not a predefined aim of this dissertation but rather a coincidental observation made during the experimental phase. Therefore, the question of where this induction of necroptosis happens and by which molecular mechanism will have to be addressed in future experiments.

Further elucidation of the connection of Pyr-41 and MG-132 to necroptosis could on one hand prove valuable in the future, since both agents were suggested to be used in cancer-therapy (Yang et al. 2007; Moriwaki and Chan 2016). On the other hand, this connection between proteasomal inhibition and necroptosis may be of clinical relevance, helping to better understand the underlying mechanism of established treatments, since for example Bortezomib, a structural relative to MG-132 that is likewise able to induce necroptosis (Moriwaki and Chan 2016), was clinically approved in 2004 by the European Medicines Agency (EMA) for the treatment of multiple myeloma and mantle cell lymphoma (European Medicines Agency 2009). In general, it is accepted that proteasome inhibitors are a class of therapeutics with a large potential in the field of cancer therapy, since many of the escape mechanisms that tumour cells use to survive rely on the UPS (Yang et al. 2009). Therefore, a better understanding of the proteasomal activity and RCD is vital.

5.1.4 Knockdown of PSMC4 elevates PGAM5 levels

As stated above, PSMC4 emerged as a potential target for siRNA-inhibition because its downregulation was reported to allow an inactivation of the proteasome at the genetic level (Kumar et al. 2010) and

thus possibly circumvents unspecific effects that inhibitors like MG-132 or Pyr-41 might have (MG-132 is reported to act through the formation of reactive-oxygen-species (Han et al. 2010) which are known to have a toxic potential while Pyr-41 should have a variety of side-effects based on the fundamental role of ubiquitination not only in proteasomal degradation but in the internal signalling of the cell) (Dohmen and Scheffner 2012).

The knockdown of PSMC4 in this thesis resulted in an increase of PGAM5 levels in L929Ts C47 cells (Fig. 6A), which provides further evidence for the hypothesis of a constitutive degradation of PGAM5 by the UPS. In parallel, the knockdown of PSMC4 lead to an increase of spontaneous cell death (as seen in untreated cells, Fig. 6B). This spontaneous cell death was not necroptosis, since in Western blots, the lysates lacked signals for pMLKL. Instead, the cell death seemed to be apoptotic, since membrane blebbing, the morphological correlate of apoptosis was observed in the microscope (data not shown). After treatment with zVAD-fmk and TNF, the cell death was necroptotic since both the appearance of pMLKL (Fig. 6A) as well as the affiliated morphotype (swelling and rupture, data not shown)) were observed.

The cell death observed in the PSMC4-downregulated and TNF/zVAD-treated cells exceeded the amount of death of the control cells transfected with a non-targeting siRNA that were treated in the same manner. This, together with the stronger signals for pMLKL in the PSMC4-downregulated cells does indicate that the impairment of the proteasome sensitizes L929Ts C47 cells to necroptosis induced by TNF/zVAD. This said, it will be interesting to elucidate whether that increase in sensitivity is (at least partially) mediated by accumulation of (polyubiquitinated) PGAM5 or rather is caused by another mechanism (e.g., the accumulation of members of the necroptotic signalling cascade).

Additionally, the results obtained in this set of experiments are consistent with the findings of Kumar et al. who claimed that the knockdown of PSMC4 does result in attenuated proteasomal activity (Kumar et al. 2010).

5.1.6 Elevated PGAM5 levels do not increase sensitivity to necroptosis induced by TNF/zVAD

To investigate whether increasing PGAM5 levels via siRNA inhibition of PSMC4 lead to an increased sensitivity to necroptosis, the PGAM5-deficient cell clone B11 was compared with the PGAM5-overexpressing clone C47 after induction of necroptosis by TNF/zVAD (Fig. 7). The missing signals for PGAM5 in Western blot of lysates from clone B11 validate the knockout of PGAM5, while the absence of signals for the subunit 6B indicate the success of the PSMC4-knockdown in both clones. The results of the single experiment performed in this thesis are preliminary but suggest no participation of PGAM5 in the sensitization of PSMC4-downregulated cells upon induction of necroptosis by TNF/zVAD. That can be said, because the cell death of the PGAM5-deficient L929Ts clone B11 was comparable to

that of the PGAM5-overexpressing L929Ts clone C47. The downregulation of PSMC4 seemed to be more effective in the clone B11 compared to the clone C47, as the appearance of a signal for subunit 6B in the PSMC4 siRNA-treated L929Ts C47 cells indicates. This could explain the higher cell death measured in untreated L929Ts B11 cells after siPSMC4-transfection. It must be mentioned that in this experiment (see 4.1.6), when comparing both control-transfected clones, the PGAM5-deficient clone B11 displayed higher levels of CD upon induction of necroptosis by TNF/zVAD than the PGAM5-overexpressing clone C47. However, this seemingly protective effect of PGAM5 was not seen in other experiments performed in the group of Prof. Dr. Dieter Adam that investigated the influence of PGAM5 on necroptosis (Fuchslocher Chico 2017; Voigt 2014), indicating that it is most likely an artifact of the single experiment that could be performed for this dissertation due to time constraints.

5.1.7 Conclusion on the accumulation of PGAM5 and its function

Overall, it could be demonstrated in this dissertation that PGAM5 does accumulate when the proteasome is inhibited (MG-132), the attachment of ubiquitin is attenuated (Pyr-41) or the proteasomal activity is reduced (PSMC4-knockdown). This, together with the finding that both endogenous as well as transfected PGAM5 levels are increasing during necroptosis, suggests an attenuated proteasomal degradation to be the cause of the PGAM5-accumulation during necroptosis. However, the functional meaning of this PGAM5 accumulation remains unclear, since it was shown that increasing PGAM5-levels do not influence the number of dying cells when necroptosis is induced by combination of zVAD-fmk and TNF. This strongly opposes the idea of any form of direct participation of PGAM5 in necroptotic signalling or cell death. However, the necroptosis-induced accumulation of PGAM5 may have other consequences, as PGAM5 is involved in other processes, such as the ROS-response (via the PGAM5-KEAP1-NRF2-axis) (Lo and Hannink 2008), inflammation (via ASK1, JNK or P38 pathway) (Takeda et al. 2009) or apoptosis (via Bcl-xL (Ishida et al. 2012; Yang et al. 2017), or BAX (Xu et al. 2015)).

The involvement of PGAM5 in the response to ROS is of interest in this context not only because ROS have been shown to be released during necroptosis at least in L929 cells and MEF (Kim et al. 2007; Goossens et al. 1999), but also because the increase in ROS might be the cause of PGAM5's accumulation. The group of Lo and Hannink found PGAM5 to tether a ternary complex, consisting of PGAM5, KEAP1 and Nrf2, to the outer membrane of the mitochondria (Lo and Hannink 2008), where KEAP1 mediates the ubiquitination (and subsequent degradation) of Nrf2 and PGAM5 in the absence of ROS. When ROS are present, however, KEAP1 ubiquitinates itself instead of the transcription factor Nrf2, leading to the accumulation of Nrf2 (Eggler et al. 2005). This results in the upregulation of the Nrf2-controlled anti-oxidative gene products and thus protects the cell against oxidative damage. Lo

and Hannink could demonstrate in 2006, that the presence of ROS (induced by tert-butylhydroquinone) does not only inhibit the ubiquitination of Nrf2 but also that of PGAM5 (Lo and Hannink 2006), which then accumulates likewise. Increasing levels of PGAM5 could therefore be a correlate to the mitochondrial ROS-release during TNF-induced necroptosis. On the other hand, the findings of Lo and Hannink also suggest a role of PGAM5 in regulation of the Nrf2-response, since the upregulation of PGAM5 was shown to decrease the expression of the Nrf2 controlled anti-oxidant gene products, thus sensitising the cells to ROS-induced damage (Lo and Hannink 2006). To further address this issue, future experiments could investigate whether the increase of PGAM5-levels during necroptosis persists when ROS-release is inhibited (e.g., by N-acetyl-L-cysteine).

Since necroptosis is a proinflammatory type of cell death, another possible function for the accumulation of PGAM5 during necroptosis could be the activation of ASK1 by PGAM5, feeding into the proinflammatory pathways controlled by p38 as well as JNK (Takeda et al. 2009) in order to further enhance the inflammatory reaction. Concerning the connection of PGAM5 to apoptosis (Ishida et al. 2012; Xu et al. 2015; Yang et al. 2017), the increase of PGAM5 levels during necroptosis might represent a convergence point to intrinsic apoptosis.

5.2 PARL does not influence the outcome of necroptosis

One aim of this thesis was to provide insights to the question whether PARL expression has an influence on the outcome of TNF-induced necroptosis in MEF. The obtained results argue against a participation of the uncleaved form of PGAM5 (as found in PARL-deficient MEF) and also not its cleaved, shorter form in TNF-induced necroptosis. This can be said, since PARL-deficient and PARL-reconstituted MEF showed equal sensitivity to necroptosis upon treatment with zVAD-fmk and TNF with or without the addition of CHX (Fig 8C). The results also support the assumption of Saita *et al.* that PARL is not required for the proteolytic maturation of HtrA2 (Saita et al. 2017). Considering that HtrA2 has been previously shown to participate in necroptosis (Suzuki et al. 2001; Blink et al. 2004; Vande Walle et al. 2010; Sosna et al. 2013; Fuchslocher Chico 2017) in MEF, one would expect a diminished reaction upon induction of necroptosis in PARL-deficient MEF if PARL is required for the proteolytic maturation of HtrA2.

5.3 Role of PGAM5 in MNNG-induced parthanatos

Due to PGAM5's implication in ROS response (Lo and Hannink 2008) and the role of PGAM5 cell death following H₂O₂- and TBO treatment, reported by Wang and colleges (Wang et al. 2012), it was investigated whether PGAM5 expression has an influence on the outcome of parthanatos. For this purpose, PGAM5-deficient L929Ts cells were compared against PGAM5-overexpressing L929Ts C47 cells as well as wildtype L929Ts cells with regard to their sensitivity for parthanatos. Some of the

PGAM5-deficient clones had to be excluded due to their slow growth rates. This exclusion seemed to be necessary because the amount of DNA damage caused by MNNG is likely to strongly depend on the grade of chromatin condensation (in correlation to the cellular turnover rate) that amongst other things influences the DNA's accessibility for enzymes such as PARP1 or agents such as MNNG (Mansidor and Risca 2022). Therefore, MNNG could be less efficient in inducing parthanatos in slow growing cells, similar to the known diminished toxicity of chemotherapeutics in treatment of (tumour-) cells with slow division rates (Moore et al. 2012).

Despite the exclusion of some PGAM5-deficient clones, the response of the remaining PGAM5-deficient clones upon MNNG-treatment was inconsistent, as some clones seemed to be protected while others showed increased vulnerability (Fig. 9B, C). Those findings allow neither an acceptance of the hypothesis that PGAM5 participates in parthanatos, nor its rejection. Possible explanations for this observation are amongst others, differing growth rates between the cells.

In order to validate the hitherto obtained results, the PGAM5-deficient clones which had shown signs of protection were retransfected with PGAM5 to investigate whether this would reinstall sensitivity to MNNG-treatment. After reconstitution of PGAM5, an increase in basal levels of cell death with features of apoptosis (e.g., membrane-blebbing) was observed in regular microscopical controls (data not shown). This basal cell death persisted during the following experimental steps, as the readout showed an increase in cell death in both the untreated as well as in the MNNG-treated fractions of the PGAM5-retransfected clones (Fig. 10B). The fact that high basal death rates were observed in both fractions of PGAM5-transfected, but not in clones transfected with a control vector, indicates that the cause of that increase in cell death is related to the transfection of PGAM5 itself, but not to a difference in the reaction to MNNG-treatment. Therefore, it can be said in conclusion that the results obtained in this dissertation strongly suggest that PGAM5 does not play an important role in the execution of parthanatos induced by MNNG.

5.4 Role of HtrA2 in MNNG-induced parthanatos

The observed strong difference of HtrA2-deficient and -reconstituted cells in their sensitivity towards MNNG-induced parthanatos (Fig. 11B) indicates an important role for HtrA2 in parthanatos. Notably, untreated HtrA2-deficient cells did show much higher rates of basal cell death in the initial experiments (Fig. 11B), which cannot be explained with certainty, though this might be mediated by the lack of other functions of HtrA2 in the deficient clone (e.g., HtrA2's role in the homeostasis of mitochondria or its chaperone function). The validation that the cells did not die from necroptosis seemed necessary

since a) HtrA2 was recently shown to participate in necroptosis (Suzuki et al. 2001; Blink et al. 2004; Vande Walle et al. 2010; Sosna et al. 2013; Fuchslocher Chico et al. 2017) and b) necroptosis and parthanatos were historically seen as two subsets of the same regulated cell death entity by some authors (Nagley et al. 2010) and have resembling morphotypes.

Due to the clear protection of the HtrA2-deficient cells upon treatment with MNNG, the experimental setting was expanded to include wildtype L929Ts cells to exclude that the observed susceptibility of the HtrA2-retransfected cells was an artifact mediated by the reconstitution. These additional experiments (Fig. 12) further corroborated the finding that HtrA2 is important for the execution of parthanatos. While wildtype L929Ts cells proved to be even more sensitive to the treatment with MNNG, arguing against a transfection-associated vulnerability driven by the reconstitution of HtrA2, the protection of the HtrA2-deficient clone persisted. Though, in the second line of experiments, the HtrA2-deficient clone did show an increase of 9 % in cell death upon treatment with MNNG and wasn't immune to the treatment, as the first experiments suggested. Therefore, the question whether HtrA2 fulfils an essential function in the parthanatic machinery remains unanswered, since the increase of cell death by only 9 % could either be explained by off-target-toxicity of MNNG, especially since the new experiments were made with twice the concentration of MNNG or with a strong but incomplete inhibition of parthanatos. Further and more detailed investigations must address this theory of an essential role for HtrA2 in parthanatos.

In summary, it can be concluded that HtrA2 is influencing the outcome of parthanatos induced by MNNG in L929Ts cells in a strong and consistent manner and therefore is part of the parthanatic cascade. The corresponding results obtained in this thesis are therefore the first description of a pro-parthanatic function of HtrA2.

After reviewing the literature to find a connection of HtrA2 to an essential member of the parthanatic cascade (e.g., PARP1, PAR, AIFM1 or MIF), the protein XIAP (X-linked Inhibitor of Apoptosis) emerged as a possible connection between HtrA2 and AIFM1. XIAP is the first described member of the family of inhibitor of apoptosis proteins (IAPs) and is well known to be cleaved and thereby inactivated by HtrA2 upon its release from the mitochondria (Yang et al. 2003). This mitochondrial release of HtrA2 is the consequence of MOMP or of opening of the permeability transition pore (PTP) (Kroemer et al. 2007) and has been shown to take place during parthanatos (Yu et al. 2002). The link between XIAP and AIFM1 is based on the finding that AIFM1 is a substrate of XIAP under physiologic as well as apoptotic conditions (Wilkinson et al. 2008; Lewis et al. 2011). Wilkinson *et al.* report that XIAP's BIR2-domain is responsible for binding AIFM1 and that this interaction can be disrupted by SMAC/DIABLO. They further propose the ubiquitination of AIFM1 by XIAP's E3-ligase activity to prevent DNA-binding

of AIFM1 (Wilkinson et al. 2008) which in turn is necessary for the AIFM1-mediated chromatin degeneration during parthanatos (“AIF-mediated cell death”) (Lewis et al. 2011). Lewis *et al.* could later substantiate their findings by demonstrating that the ubiquitination of AIFM1 by XIAP does result in decreased cell death upon treatment with MNNG as well as attenuated DNA-binding of AIFM1 (Lewis et al. 2011). This connection between XIAP and AIFM1 could explain the pro-parthanatic role of HtrA2 that is proposed in the present dissertation. HtrA2 could proteolytically inactivate XIAP during parthanatos, thus lifting the inhibitory effect of XIAP onto AIFM1 and therefore increase (or reinstall) the sensitivity of the cell to die in this manner. This would ascribe HtrA2 an important regulatory function in parthanatos. However, the data obtained in this part of the dissertation formed the basis for a subsequent study showing that the protease function of HtrA2 is dispensable for its role in parthanatos, refuting this hypothesis. In the same study, it was also shown that HtrA2 is not leaving the mitochondrion during parthanatos (Weiß et al. 2023).

The molecular connection of the serine protease HtrA2 to parthanatos is also of clinical interest because both HtrA2 as well as parthanatos have been associated with the pathogenesis of Parkinson’s disease. HtrA2 was identified as a disease-related gene, designated PARK13, in Parkinson’s disease (Strauss et al. 2005). Hitherto, that association of HtrA2 with Parkinson’s disease was often attributed to the role of HtrA2 in mitophagy, based on an interaction between PINK1 and HtrA2 that has been reported (Plun-Favreau et al. 2007). Though the association of HtrA2 (in parallel to PINK and Parkin) with Parkinson’s disease is increasingly doubted by some authors (Krüger et al. 2011), the identification of HtrA2’s role in parthanatos in this dissertation could be of interest for further investigations concerning the mechanism of neurodegeneration in Parkinson’s disease as well as in other forms of neurodegeneration.

6 Summary

The understanding of cell death has been changing heavily in recent years. What was originally understood as a simple dualism of programmed death, called apoptosis, and accidental cell death, referred to as necrosis, has become a whole set of different cell death pathways. Necroptosis and parthanatos, two members of those distinct regulated cell death pathways, which supposedly play a role in different clinical settings, such as hypoxia-induced damage or neurodegenerative diseases, are in the focus of this dissertation.

Necroptosis was described in 2005 and was later found to critically rely on the function of RIPK3 and MLKL. Next to those essential proteins, multiple other factors have been implicated in the regulation of necroptosis. Among these, PGAM5 (phosphoglycerate mutase family member 5) was initially described as a key factor. Although subsequent studies argue against an essential function of PGAM5

in necroptosis, it could fulfill an accessory function during necroptosis as it was shown in this thesis that PGAM5 strongly accumulates in murine L929Ts C47 fibrosarcoma cells during necroptosis. Since this equally applied to endogenous as well as transfected PGAM5, it appeared unlikely that this was due to transcriptional regulation but rather pointed to a proteolytic regulation. To validate this hypothesis, L929Ts cells were either treated with the proteasome inhibitor MG-132, with the ubiquitin-activating enzyme E1 inhibitor Pyr-41 or with siRNA against PSMC4 (encoding the ATPase A4 of the 26S proteasome regulatory subunit 6B). Every single method of proteasomal inhibition that was tested in this dissertation resulted in a strong increase of PGAM5 levels, hence the hypothesis of a proteasomal regulation of PGAM5 must be accepted. The question of the exact role of the increase in PGAM5 levels during necroptosis could not be answered completely, though the obtained data suggest a facilitation of cell death (or necroptosis) by elevated PGAM5-levels.

A related aim of this project was to investigate the role of the mitochondrial rhomboid protease PARL in necroptosis. PARL normally cleaves PINK-1, but, upon loss of the mitochondrial membrane potential, it can change substrates and cleave PGAM5 instead. Mouse embryonic fibroblasts deficient for PARL or stably retransfected with a PARL-FLAG construct were tested for their sensitivity to necroptosis. However, in this thesis, no difference between the two types of MEFs was found, suggesting that the function of PARL is dispensable for necroptosis.

Parthanatos, which is driven by PARP1 (poly(ADP-ribose)-polymerase 1) was termed in 2008. In this thesis, it was investigated whether PGAM5 or the mitochondrial serine protease HtrA2 are taking part in the execution of that cell death pathway. L929Ts cells deficient for either PGAM5 or HtrA2 were therefore treated with MNNG, a strong alkylating agent, to induce parthanatos. While the expression of PGAM5 did not influence the outcome of parthanatos in L929Ts cells, it could be shown that deficiency for HtrA2 strongly inhibits parthanatic cell death triggered by MNNG and is therefore likely to be implicated in this cell death entity and a promising target for further research.

7 References

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8 List of Publications

- Hoyer, J., Fuchslocher Chico, J., Davarnia, p., Voigt, S., Adam, D. (2017): Proteolytic regulation of PGAM5 in non-apoptotic cell death. 21. Joint Meeting of the Signal Transduction Society (STS), Weimar, Deutschland, 8.11. – 10.11. 2017
- Heib, M., Weiß, J., Saggau, C., Hoyer, J., Fuchslocher Chico, J., Voigt, S., Adam, D. (2022): Ars moriendi: Proteases as sculptors of cellular suicide. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research* 1869, 119191
- Weiß, J., Alp, G., Hoyer, J., Adam, D. (2022): Role of HtrA2/Omi in parthanatos. Gordon Research Conference: Cell Death-Associated Inflammation in Disease, Les Diablerets, Schweiz, 15.05. – 20.05.2022
- Weiß, J., Alp, G., Hoyer, J., Adam, D. (2019): Role of HtrA2/Omi in parthanatos. 23rd meeting on signal transduction - receptors, mediators, and genes, Weimar, Deutschland, 4.11.2019 – 6.11.2019

9 Eidesstattliche Versicherung

Hiermit erkläre ich, Justus Hoyer, geboren am 14.09.1992 in Berlin, dass ich die vorliegende Dissertation mit dem Titel: “Proteolytic regulation of PGAM5 in non-apoptotic cell death” nach den Regeln guter wissenschaftlicher Praxis eigenständig verfasst und keine anderen als die angegebenen Hilfsmittel und Quellen benutzt habe. Dabei habe ich keine Hilfe, außer der wissenschaftlichen Beratung durch meinen Doktorvater Prof. Dr. rer. nat. Dieter Adam in Anspruch genommen. Diese Arbeit wurde bisher an keiner Stelle im Rahmen eines Prüfungsverfahrens vorgelegt. Teile dieser Arbeit wurden bereits publiziert.

Kiel, im Mai 2024

Justus Hoyer

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