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The molecular "Jekyll and Hyde" duality of PARP1 in cell death and cell survival

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1. ABSTRACT

The current literature clearly indicates that PARP1 but also PARP2 play a pivotal role in modulating the cellular responses to stress. Genetic and pharmacological studies demonstrated that overactivation of PARP1 is a key mediator of programmed-necrotic cell death *in vivo*. PARP1 appears to be also involved in programmed cell death processes others than necrosis, such as apoptosis or macroautophagocytotic cell death. On the other hand, growing evidence suggests that both PARP1

and PARP2 are multi-faced enzymes also playing important roles in cell survival processes. PARP1 and PARP2 were shown to be required for the maintenance of genomic integrity and to act as a survival factor for highly proliferating cells such as stem cells but also non-proliferating neuronal cells against cell death induced by oxidative stress under mild and moderate progressive damage *in vivo*. This review briefly summarizes the recent findings, which support a crucial role of PARP1 in different programmed cell death and cell survival processes. A special focus is placed on the proposed molecular mechanisms underlying the "Jekyll and Hyde" duality of PARP1 in cell death and cell survival pathways. A potential crosstalk between PARP1, PARP2 and other NAD^+-dependent ADP-ribosyling enzymes such as Sirtuins and CD38 in cell death and survival pathways is discussed.

2. INTRODUCTION

Poly-ADP-ribosylation reactions are phylogenetically ancient as evidenced by the poly-ADP-ribosylating activities reported in dinoflagellates and archaebacteria and the identification of *poly-ADP-ribose polymerase*-like genes in bacterial and archaeal genomes (reviewed in (1, 2)). Poly-ADP-ribose has a widespread occurrence and is present in most multicellular and unicellular organisms with few exceptions such as the yeasts *Saccharomyces cerevisae* and *Schizosaccharomyces pombe* (reviewed in (1-3)). In mammalians, poly-ADP-ribose synthesis and degradation occurs in all mitotic and post-mitotic cells (reviewed in (1-3)). Homeostasis of poly-ADP-ribose metabolism is thought to play an essential function in a wide range of physiological and pathophysiological processes, such as maintenance of genomic integrity, inter- and intracellular signaling, transcriptional regulation, cell differentiation and proliferation, energy metabolism and cell death (reviewed in (1, 2, 4, 5)).

Poly-ADP-ribose is a homopolymer of ADP-ribose units linked by glycosidic ribose-ribose 1'-2' bonds and synthesized by the poly-ADP-ribose polymerase family of enzymes (PARPs) (reviewed in (1, 2, 5)). Poly-ADP-ribose polymerases are an ancient family of enzymes. Six genes encoding "*bona fide*" PARP enzymes have been identified in mammalians: PARP1, PARP2, PARP3, PARP4/vPARP, PARP5/Tankyrases-1 and PARP6/Tankyrases-2 (reviewed in (1, 2, 5)). The best-studied PARP enzyme, PARP1, plays a primary role in the process of poly-ADP-ribosylation. PARP1 is a nearly ubiquitously expressed and highly abundant nuclear chromatin-associated enzyme. According to textbook models, on average, approximately one molecule of this enzyme is present per 1000 base pairs of DNA (reviewed in (1, 2, 5)). Like mono-ADP-ribose synthesis, poly-ADP-ribose synthesis requires nicotinamide adenine dinucleotide (NAD^+) as precursor and immediate substrate of the reaction with the release of nicotinamide. The constitutive levels of poly-ADP-ribose are usually very low in unstimulated cells. However, in response to mitogenic stimuli or genotoxic stress (i.e. in the presence of DNA strand breaks), the PARP activity and the levels of poly-ADP-ribose may increase 10- to 500-fold, while cellular NAD^+ levels are correspondingly reduced (reviewed in (1, 2, 4, 5)). Both constitutive and activated levels of poly-ADP-ribose are functions of the concentration of NAD^+ in cells. Most free or protein-associated poly-ADP-ribose polymers are rapidly degraded by poly-ADP-ribose glycohydrolase (PARG) in vivo. PARG is a key enzyme for poly-ADP-ribose catabolism by hydrolyzing both terminal ADP-ribose units from poly-ADP-ribose polymers via exoglycosidic activity and removing larger oligo-ADP-ribose fragments via endoglycosidic cleavage (reviewed in (1, 2, 4, 5)). The endoglycosidase activity releases larger poly/oligo-ADP-ribose fragments and could be of particular physiological importance due to the generation of various types of free poly/oligo-ADP-ribose (1, 2). These products are thought to be important signaling molecules involved in cell death and cell growth (reviewed in (1, 2, 4, 6)).

PARP1 and to a lesser extend PARP2 play a pivotal role in modulating the cellular responses to stress (reviewed in (1, 2, 5)). Based on careful analysis of the literature, overactivation of PARP1, initiated through severe stress, appears to be a key mediator of cell death in low or non-proliferating cells *in vivo*. Uncontrolled poly-ADP-ribosylation reactions can result in massive necrotic cell death and tissue damage, which in turn often lead to severe inflammatory or neurodegenerative disorders (reviewed in (1, 2, 4, 5, 7-9)). Overactivation of PARP1 has been implicated in the pathogenesis of several diseases, including stroke, myocardial infarction, diabetes, shock and allergy (reviewed in (1, 2, 4, 5, 7-9)). On the other hand, PARP1 and PARP2 were shown to be also required for the maintenance of genomic integrity and to protect highly proliferating cells such as stem cells but also non-proliferating neuronal cells against cell death induced by oxidative stress under mild and moderate progressive damage (10-15). The current literature clearly demonstrates that poly-ADP-ribosylation reactions play dual roles in cell death and survival pathways. The PARP/poly-ADP-ribosylation system was recently proposed to function as a sensor that integrates, in a "yin/yang"-like manner, information from the mitochondria and nucleus on the metabolic and oxidative states of cells, thus acting as a double-edged sword in ROS-dependent death/survival pathways (1, 2, 7, 16)). However, the exact molecular mechanisms of how poly-ADP-ribosylation/PARPs act in these pathways are still under debate. This review briefly summarize the recent advances on the physiological functions of PARP1 in cell death and cell survival pathways and highlights the proposed molecular mechanisms underlying its "Jekyll and Hyde" duality. A special focus is placed on the potential crosstalk between poly-ADP-ribosylation and other NAD+-dependent reactions in these pathways. For a detailed description of cell death and cell survival non-related functions of distinct PARP family members, the reader is referred to the accompanying review (Hassa and Hottiger, Frontiers in Bioscience 2008; (1)).

3. PROGRAMMED CELL DEATH MECHANISMS

Programmed cell death (PCD) is an essential physiological process used by metazoans for successful embryonic and postembryonic development, maintenance of tissue homeostasis as well as a defensive strategy against pathogens and cancer (reviewed in (17-26)). PCD is a genetically regulated process of cell suicide. During embryogenesis PCD plays a major role in sculpting the shape of organs and limbs (27). For instance, generation of specific cell types, morphogenesis and the development of the inner organs depend on PCD pathways (27). PCD is also necessary for proper development of the immune system and homeostasis of T- and B-cells (28-31). Negative selection of auto-reactive immature T-cells is a typical cell death process (29-33). PCD prevents the activation of irrelevant B cell clones and is required for affinity maturation during the antibody response process. PCD is also involved in cytotoxic killing of target cells such as tumor/malignant cells or virally infected cells by cytolytic activity of natural killer cells, granulocytes, or macrophages (28, 34-36). Abnormal regulation of PCD pathways often contributes to major pathologies, including many types of cancer, autoimmune diseases, neurodegenerative diseases and ischemic damage (reviewed in (37-40)). Defective apoptosis due to mutation or biochemical inhibition of pro-apoptotic gene products often results in uncontrolled cell proliferation, which in turn leads to autoimmunity or cancer (reviewed in (37-40)). Exaggerated PCD can cause hypotrophy, such as in ischemic damage (reviewed in (9, 41-43)). Excessive or defective PCD processes during embryogenesis may cause developmental abnormalities (44). Currently, PCD can be subdivided into at least four major types of cell death in mammalian cells, based on morphological and ultra-structural criteria, initiating death signal and the implication of caspases: apoptosis (type I), macroautophagy, (type II), programmed necrosis (type III) and mitotic catastrophe (type IV) (reviewed in (18, 19, 21, 22, 24, 25)). Other more restraining classifications, subdivide PCDs into classical apoptosis, apoptosis-like PCD, necrosis, necrosis-like PCD, classical macroautophagy-like PCD and mitotic catastrophe (reviewed in (18, 19, 21, 22, 24, 25)). Thus, the current classifications might soon change, based on our rapidly evolving understanding of programmed cell death processes.

3.1. Apoptosis

Classical apoptosis is the best-characterized form of PCD. Apoptotic cell death is an energy- (ATP)-dependent form of programmed cell death processes and characterized by the activation of caspases, cleavage of nuclear and cytoplasmic proteins, DNA fragmentation, nuclear chromatin condensation (pyknosis), membrane blebbing and cell fragmentation into apoptotic bodies (reviewed in (21, 29, 41, 45-47)). Apoptosis is generally restricted to single cells and associated with the retention of plasma membrane integrity, however induces cell surface changes that are important for the recognition and removal of apoptotic cells/bodies by phagocytic cells in a safe, non-inflammatory manner (reviewed in (48-53)). Clearance of apoptotic cells by phagocytic cells plays an essential role in the resolution of inflammation, protecting the surrounding tissue from harmful exposure to intracellular potentially pro-inflammatory substances of dying cells. Defective clearance of apoptotic cells is thought to contribute to inflammatory and autoimmune diseases (reviewed in (48-55)). Apoptosis is positively regulated by balancing pro-survival signals or negatively regulated by a diverse range of extracellular and intracellular signals (56). In mammals, apoptotic cell death consists of at least two phases, initiation and execution phase (reviewed in (21, 29, 41, 45-47)). The initiation phase can be subdivided in two major independently acting pathways: extracellular (extrinsic) death receptor pathways and intracellular (intrinsic) apoptotic pathways. Extrinsic apoptotic pathways are initiated by activated cell-surface-expressed death receptors, with signaling cascades orchestrated in part by the Bcl-2 and death receptors-mediated recruitment of procaspase-8 or procaspases-2/-10 (56-59). Intrinsic apoptotic pathways are mainly initiated following perturbation of intracellular organelle function (dysfunction of mitochondria and the endoplasmic reticulum) and induce the activation of caspase-9 via release of toxic mitochondrial proteins into the cytoplasm (i.e. cytochrome c) or inositol 1,4,5-trisphosphate (IP (3)-mediated Ca^{2+} release from the endoplasmic reticulum (56, 60-62). Both extrinsic and intrinsic pathways converge to the activation of procaspase-3 and of caspases-6 and -7, the canonical routes of caspase activation during the execution phase (56-62). For a detailed description of apoptotic cell death pathways, the readers are referred to the recent excellent reviews (21, 29, 41, 45-53, 56-62)

3.1. 1. Collective apoptotic cell death

A recent study provided evidence for a novel communal form of cell death, cellular mass-suicide in flies during development (63). Unlike conventional apoptotic pathways, this novel form of apoptosis is not restricted to single cells. However, this unique communal form of cell death appears to be controlled through the same apoptosome proteins regulating conventional apoptosis (63). It remains to be investigated whether collective apoptotic cell death might be a more widespread phenomenon and occur also in mammals, for example during development and in inflammatory/neurodegenerative disorders

3.2. Macroautophagocytotic cell death

A second distinct form of programmed cell death is the recently described macroautophagocytotic cell death. Controlled autophagy is a process of cellular "self-eating", a highly conserved ATP-dependent bulk degradation process in eukaryotes that usually promotes cell survival under metabolic stress (reviewed in (64-68)). Various types of metabolic stress, including nutrient and energy starvation, mitochondrial dysfunction or endoplasmic reticulum stress, oxidative stress, and infections can activate autophagy (reviewed in (64-68)). Controlled autophagy helps to maintain a balance between the synthesis, degradation, and subsequent recycling of cellular products. On the other hand, inappropriate activation of autophagy can trigger cell death under specific conditions (reviewed in (64-68). For instance, in starved cells,

autophagy works as an alternative energy source and promotes cell survival while various cytokines and chemicals have been shown to induce cell death caused by autophagy. At least four distinct pathways of autophagy are described: microautophagy, chaperone-mediated autophagy, macroautophagy and programmed macroautophagocytotic cell death (reviewed in (64-68)). Microautophagy and chaperone-mediated autophagy occurs directly into the lysosomes and is not involved in programmed cell death. In contrast, macroautophagy is characterized by the sequestration of bulk cytoplasm including excess or aberrant organelles, within double- or multi-membraned autophagic vacuoles, called autophagosome. There is growing evidence that, under certain conditions, organelles or even proteins can be selectively degraded by autophagy (69-72). Autophagosomes are delivered to the degradative organelle, the lysosome/vacuole, for bulk degradation and eventual recycling of the resulting low molecular weight degradation products (reviewed in (64-68)). Macroautophagocytotic cell death is a form of non-apoptotic, non-necrotic cell death (reviewed in (64-68)). However, the exact mechanisms by which autophagy contributes programmed cell death is only partially understood. Macroautophagy has been suggested to promote or execute programmed cell death especially under conditions when the apoptosis and programmed-necrotic cell death machineries are compromised (reviewed in (64-68)). Macroautophagocytotic cell death has been shown to play a prominent role in PCD during developmental processes and in both innate and adaptive immunity against intracellular bacteria and viruses by degrading the pathogens in autolysosomes (reviewed in (73-76)). In addition, recent reports suggested that abnormal regulation of autophagy could be a major cause for neurodegenerative diseases and cancer. For a detailed description of autophagic cell death pathways, the readers are referred to the recent excellent reviews (21, 44, 64-68, 73-77).

3.3. Programmed-necrotic cell death

Necrosis has been initially defined as an uncontrolled or default pathway of cell death (reviewed in (22, 24, 25, 41)). However several studies have clearly demonstrated that necrotic cell death is as well controlled and programmed as caspase-dependent apoptosis ((78, 79) and reviewed in (22, 24, 25, 80, 81)). Programmed-necrotic cell death largely lacks the features of apoptosis and autophagy and is characterized by swelling of cellular organelles, leading to loss of plasma membrane integrity, nuclear disintegration (pyknotic nuclei) and cell lysis (reviewed in (22, 24, 25, 80, 81)). Programmed-necrotic cell death is defined as an acute ATP/energy-independent type of cell death usually initiated following mitochondrial dysfunction, ATP depletion and energy loss. Necrosis is typically followed by pro-inflammatory signaling cascades, which are initiated through the active release of inflammatory cytokines when the cells lyse (reviewed in (22, 24, 25, 80, 81)). Unlike classical apoptosis, programmed necrosis is thought to be the result of extensive crosstalk between several signaling cascades. Several studies suggested that the serine/threonine kinase RIP1, a component of the death receptor TNFRI signaling complex, acts as a major initiator of signaling cascades contributing to necrotic cell death ((82-84) and reviewed in (85, 86). The propagation and execution phases of necrotic cell death includes calcium release, enhanced generation of reactive oxygen species (ROS) and activation of calpains and cathepsins (87, 88). In addition, upon ATP depletion, energy loss and permeabilization of the outer mitochondrial membrane, the mitochondrial flavoprotein apoptosis-inducing factor (AIF) often translocates to the nucleus under these conditions, where it participates in chromatin condensation and large-scale DNA fragmentation (reviewed in (2, 24, 89-92)). Mitochondria serve as the central relay station in cell death through the release of pro-apoptotic and pro-necrotic factors such as cytochrome c and AIF, which activate caspase-dependent and caspase-independent cell death signaling cascades, respectively (reviewed in (93)). AIF is a phylogenetically conserved 57kDa flavoenzyme that is restricted to the mitochondrial intermembrane space in healthy cells where it exerts protective functions in bioenergetic and redox metabolism (94-99). AIF exhibits both reactive oxygen species (ROS)-generating NAD (P)H oxidase and monodehydroascorbate reductase activity (95-99). The primary non-pathophysiological role of AIF appears to be the maintenance of a fully functional respiratory chain. However, under pathological conditions, opening of the outer mitochondrial transition pore is induced by high doses of genotoxic agents or NMDA, AIF is cleaved by a mitochondria-associated calpain-like protease, which mediates its release from mitochondria to the cytoplasm, where it combines with cyclophilin A (CypA) and EndoG to form an active DNAse (100-104). This activated complex then translocates to the nucleus and contributes to nuclear DNA fragmentation into 50-kbp fragments and chromatinolysis (105-108). AIF was shown to participate in both caspase-dependent and -independent cell death processes (105-108). The current models suggest that AIF serves as the main mediator of caspase-independent apoptosis/necrosis-like programmed cell death processes. The exact functional role of EndoG is still under debate since different knockout mice strains of the EndoG gene showed different defects in apoptosis, even opposite phenotypes (109, 110). Under normal physiological conditions AIF plays an important physiological role in mitochondria (111, 112). Studies using Aif knockout cells and Harlequin (Hq) mutant mice, which have only 20% AIF expression strongly suggested that AIF serves as a reactive oxygen species (ROS) scavenger (98, 111-115). Moreover, oxidative phosphorylation is compromised in cells with depleted AIF due to reduced expression of the electron transport chain complex I in the mitochondria (98, 111-115). Recent data strongly indicate, that the redox-active enzymatic region of AIF is associated with anti-apoptotic functions, while its DNA binding region possesses pro-apoptotic/necrotic activities ((105, 116) and reviewed in (117, 118)). Finally, a very recent genetic study, using knock-in mice expressing an AIF mutant that cannot be released from mitochondria and thus doesn't translocate into the nucleus, clearly demonstrated that AIF plays an active role during programmed cell death processes in the nucleus but not in the mitochondria (112). Reconstitution of mitochondrial AIF was not sufficient to rescue cell death (112).

Recent studies provided evidence that there might be a continuum of apoptosis and necrosis in response to the same cell death stimulus. Indeed, many cell death inducing agents, such as MNNG induce apoptosis at lower doses and necrosis at higher doses (119). Treatment of thymocytes *in vitro* with peroxynitrite at low concentrations (10 μM) led to apoptosis, whereas higher concentrations (50 μM) resulted in programmed necrosis (120, 121). It was therefore suggested that features of both apoptosis and necrosis could coexist in the same cell. In addition, inhibition of apoptosis or autophagy can change the type of cell death to necrosis (reviewed in (22, 122)). Programmed-necrotic cell death occurs in both physiological and pathophysiological processes. Programmed necrosis is suggested to be crucial for the killing of tumor cells that have developed strategies to evade apoptosis. On the other hand, programmed-necrotic cell death plays a major role in inflammatory and neurodegenerative disorders and is therefore a major target for therapeutic strategies (8, 42, 91). For a detailed description of programmed-necrotic cell death, the readers are referred to the recent excellent reviews (2, 22, 24, 25, 80, 81, 85, 86, 122)

3.4. Mitotic Catastrophe

Mitotic catastrophe represents a caspase-dependent and caspase-independent form of programmed cell death that occurs during mitosis. Mitotic catastrophe is triggered by a combination of defective cell cycle checkpoints, accumulation DNA damage and the development of aneuploid cells (reviewed in (18, 123, 124)). Agents that disrupt the mitotic spindle also induce mitotic catastrophe. Thus, mitotic catastrophe is conceived as a molecular mechanism that protects against unwarranted (and possibly oncogenic) aneuploidization, which may participate in oncogenesis (18, 123-125). Mitotic catastrophe is a complex process that is suggested to be in large part dependent on the activation of caspase-2 and mitochondrial membrane permeabilization (126). However, the exact molecular events, which activate mitotic catastrophe remains to be elucidated. Recent studies provided preliminary evidence that mitotic catastrophe is tightly controlled by cell-cycle-specific kinases, cell cycle checkpoint proteins, such as Chk1, ATR/ATM, p53, members of the Bcl-2 family as well as survivin (127-136).

3.5. Senescence induced cell death?

A number of recent studies in the field of cellular senescence have provided preliminary evidence that cellular senescence or a subtype of it may represent an additional form of programmed cell death *in vivo (* (137-143) and reviewed in (144)). Cellular senescence was first described by Hayflick and Moorhead in 1961 as replicative senescence, a mechanism that irreversibly arrested the growth of normal human fibroblasts cells and prevented them from growing indefinitely in culture ("old cells") (145). According to Hayflick's model, normal somatic cells possess only a limited mitotic division potential. Later on, cellular senescence was subdivided in two major categories: (1) Replication history-dependent or telomere attrition-induced senescence, termed "replicative senescence" and (2) telomere attrition-independent or replication/proliferative history-independent senescence, termed premature or accelerated senescence (145-151). Premature "extrinsic" senescence can be induced by a large number of stressors, such as increased levels of ROS, in response to oncogenes, pro-inflammatory cytokines (e.g. TNF-α or IL-1), γ-radiation, UV light and accumulation of DNA strand breaks ("young cells") (reviewed in (151-157)). Both types of cellular senescence were suggested to serve as a tumor suppressor mechanism (reviewed in (151-157)). Indeed, numerous studies clearly demonstrated that both Rb and p53 tumor suppressors are key regulators of the senescence program, consistent with their roles in mediating cell-cycle checkpoints and tumor suppression (reviewed in (152-157)). On the other hand, senescence has also been shown to contribute to age-related pathology by disrupting local tissue integrity and stimulating tumorigenesis in aged mice and humans (reviewed in (151-157)). Senescent cells have been initially described as resistant to programmed cell death. However, apoptosis-resistance is not a general feature of senescent cells, which may also be apoptosis prone. Indeed, several studies provided evidence that both replicative and premature senescent cells may eventually undergo programmed cell death *in vivo* and *in vitro,* depending on the cell type and apoptotic stimuli ((137-143) and reviewed in (144)). For instance replicative and premature senescent human endothelial cells and porcine pulmonary artery endothelial cells can undergo apoptotic cell death when cultured for a prolonged period of time (141-143). Clearance of senescent neutrophils and eosinophils can occur through apoptosis- or necrosis-like programmed cell death and subsequent phagocytosis by macrophages (137-139, 158), while aged human senescent fibroblasts appears to eliminate themselves by macroautophagocytotic cell death (140). However the exact molecular mechanisms linking premature senescence to apoptotic, necrotic, autophagy- or mitotic catastrophe-like cell death remain to be investigated.

4. PARP1-MEDIATED CELL DEATH

Numerous genetic studies with *Parp1*- or *Parp2*-deficient mice and pharmacological approaches demonstrated that poly-ADP-ribosylation reactions play crucial roles in cell death pathways under lethal oxidative stress conditions (reviewed in (2, 5, 7, 8, 159-163)). Genetic knockout of the *Parp1* or *Parp2* gene or pharmacological inhibition of enzymatic PARP activity protects from several oxidative stress-dependent pathophysiological conditions, leading to aberrant cell death, in a variety of experimental models including endotoxin induced septic shock, streptozotocin-induced diabetes, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) toxicity, traumatic spinal cord injury, myocardial infarction, cerebral and cardiac ischemia (reviewed in (2, 5, 7, 8, 159-163)). The use of PARP inhibitors has been proposed as a protective therapy in decreasing cell death and other tissue damage in inflammatory and neurodegenerative disorders (reviewed in (2, 5, 7, 8, 159-163)). The involvement of nuclear and cytoplasmic isoforms of PARG in these processes is less well understood (164-168). However, recent studies using pharmacological inhibitors of PARG and *Parg* knockout

mice demonstrated that PARG could also enhance the pathogenesis induced by acute tissue injury, ischemia and inflammation ((169-173) and reviewed in (42, 159, 174-176)). Homeostasis of poly-ADP-ribosylation regulated by PARP1 and PARG has been proposed to be an important regulator of programmed cell death processes in multicellular organisms (177). The extent of poly-ADP-ribosylation appears to correlates with the severity of genotoxic stress and this might determine the cellular response. Under mild to moderate non-lethal oxidative stress conditions, it plays important roles in maintenance of genomic stability and transcriptional regulation in pro-inflammatory/cellular defense processes (reviewed in (2, 5, 7, 8, 42, 159-163, 174-176)). However, severe stress following acute neuronal injury, which causes the overactivation of PARP1 (i.e upon cellular damage by oxygen radicals or excitotoxicity) often results in unregulated poly-ADP-ribose synthesis, which in turn can lead to significant decrements in NAD^+, ATP depletion and widespread cell death (reviewed in (2, 5, 7, 8, 42, 159-163, 174-176)).

The exact mechanisms by which poly-ADP-ribosylation reactions lead to cell death are currently under debate. Several short term, primary mechanism were proposed, including poly-ADP-ribosylation-induced energy-failure and nuclear translocation of AIF in programmed-necrotic cell death or an increase in susceptibility of chromatin to cellular endonucleases and internucleosomal DNA fragmentation by poly-ADP-ribosylation of chromatin in the early stage of apoptosis. Poly-ADP-ribosylation-mediated cell death is currently thought to be solely manifested through programmed-necrotic cell death but this view is most likely too simple and not reflecting the (patho)physiological situation. Different alternative models are discussed in the following sections.

4.1. PARP1-mediated programmed-necrotic cell death.

In 1983, N. Berger suggested first that overstimulation of poly-ADP-ribosylation reactions might be linked to necrotic cell death and thus initiate cell-autonomous programmed necrosis (178-180). Indeed, recent studies using DNA damaging agents such as N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), hydrogen peroxide (H_2O_2) or peroxynitrite, which are well known to induce necrosis at high concentrations, showed that pharmacological inhibition of PARP activity or knockout of the *Parp1* gene blocks programmed-necrotic cell death induced by these agents (reviewed in (2, 5, 7, 8, 42, 159-163, 174-176)). Numerous reports from various laboratories indicate that inhibition or absence of PARP1 provides remarkable protection in disease models such as septic shock, diabetes, stroke, myocardial infarction and ischemia, which are characterized predominantly by programmed-necrotic cell death (reviewed in (2, 5, 7, 8, 42, 159-163, 174-176)). These studies clearly demonstrated that PARP1 and to a much lesser extend also PARP2 play an essential role in programmed-necrotic cell death. Remarkably, the contribution of PARP1- and PARP2-mediated poly-ADP-ribosylation reactions to programmed-necrotic cell death, appears to be dependent on the cell type, cellular metabolic states and on the expression levels and activity potential of PARP1, PARP2 and PARG. Poly-ADP-ribosylation reactions seem to play an important role in necrotic cell death of various endothelial and epithelial cells, as well as in several types of neuronal cells, whereas necrotic cell death caused by oxidative damage in other cell types, such as hepatocytes, is not dependent on PARP1 or PARP2 (reviewed in (2, 8, 80, 81, 93, 181-184)). However, the exact biochemical pathways and sequence of events leading from PARP1 activation to programmed-necrotic cell death are not fully understood. At least two (partially overlapping) models were proposed: The NAD-depletion/PARP1-suicide model proposed by N. Berger and the apoptosis-inducing factor (AIF) shuttling model, recently suggested by V. and T. Dawson (reviewed in (90, 92, 107, 178, 179, 185)).

4.1.1. NAD depletion /PARP1-Suicide Model

According to the „PARP-suicide" model proposed by N. Berger, lethal levels of DNA damage lead to overactivation of PARP (s) and a rapid decline of cellular NAD^+, which in turn affects the activities of the enzymes involved in glycolysis, the pentose phosphate shunt and the Krebs cycle thereby preventing glucose-dependent ATP production (178, 179). In an attempt to restore the NAD^+ pools, NAD^+ is re-synthesized with a consumption of 2-4 molecules of ATP per molecule of NAD^+ (depending on which salvage pathway it is used in the cell) and as a consequence cellular ATP levels become depleted, leading to subsequent cellular energy failure, cellular dysfunction and in necrotic cell death (178, 179). Several studies suggested that excessive poly-ADP-ribosylation by PARP1 contributes to mitochondrial failure and mitochondria permeability transition (MPT) (186-196). MPT results from the opening of a large conductance channel in the inner mitochondrial membrane and is a crucial factor leading to cell death during conditions of oxidative stress and energy depletion (186-193). This hypothesis could be partially confirmed in numerous studies by using novel PARP inhibitors, NAD^+ replenishment or cells from *Parp1* knockout mice. Repletion of intracellular NAD^+ through liposome-mediated delivery of NAD^+ partially preserved mitochondrial membrane potential, restored glycolytic function and rescues cells from PARP-mediated cell death (187, 189, 194-196). Moreover delivery of alternative substrates such as alpha-ketoglutarate, glutamate, glutamine and/or pyruvate that can be metabolized independent of cytosolic NAD^+ have been shown to reduced cell death from approximately 70% to near basal levels after PARP activation (78, 189, 195, 196). These studies also strongly indicate that PARP1 activation may only lead to rapid depletion of the cytosolic but not the mitochondrial NAD^+ pool (78, 189, 195-197). Thus, pharmacological inhibition of the enzymatic activity of PARPs or the complete absence of PARP1 significantly appears to improve the cellular energetic state and cell viability after exposure to necrotic cell death-inducing agents. DNA damage-induced NAD^+ depletion is a poly-ADP-ribosylation-dependent process that can be completed within 15 min, and therefore, precedes by far the execution of the apoptotic process (reviewed in (2, 4, 7, 198)). Since

ATP is required for the execution of apoptosis, overstimulation of poly-ADP-ribosylation reactions *in vivo* can result in necrotic cell death, even in a situation where the stimulus is definitely pro-apoptotic (reviewed in (78, 189, 195, 196, 199-201)).

Recent studies clearly demonstrated that the cellular metabolic state is a key factor in determining how ATP levels are affected by overstimulation of poly-ADP-ribosylation reactions (78, 189, 195, 196). The massive generation of poly-ADP-ribose in the nucleus by overactivation of PARP1 and, to a much lower extent, PARP2, has been suggested to preferentially deplete the nuclear and cytosolic pools of NAD^+, but not the mitochondrial pools, thereby inhibiting glycolysis but not oxidative phosphorylation (12, 78, 189, 195, 196). Thus, under conditions where glucose is the only available metabolic substrate, depletion of the cytosolic NAD^+ pool leads to cell death of highly glycolytic cells (78, 81, 189, 193, 195, 196). Actively proliferating cells use almost exclusively glucose through aerobic glycolysis for the production of ATP and die from NAD^+ and ATP depletion, as a consequence of overactivation of poly-ADP-ribosylation reactions. In contrast, non-proliferating cells can catabolize a mixture of metabolic substrates, including amino acids and lipids, and maintain ATP levels through oxidative phosphorylation in the mitochondria and are resistant or less sensitive to ATP depletion and cell death under the same conditions (78). These latter, not actively growing cells are therefore more sensitive to inhibition of the mitochondrial respiratory chain. The decision between cell death and survival after exposure to necrosis inducing agents is therefore mainly regulated by the availability of metabolic substrates and the expression levels or enzymatic activities of PARP1 and in part of PARP2 (78, 201, 202). Thus, the contribution of PARP1-mediated poly-ADP-ribosylation reactions to programmed-necrotic cell death depends on the cell type and cellular metabolic state of the cell. PARP1 appears to play an important role in programmed-necrotic cell death of various types of endothelial and epithelial cells, as well as several types of neuronal cells (reviewed in (2, 41, 81, 93, 184, 203, 204)). Taken together, there is clear evidence that poly-ADP-ribosylation reactions play a central role in programmed-necrotic cell death pathways (78, 80, 195, 201).

4.1.2. AIF shuttling/signaling model

NAD^+/ATP depletion through excessive enzymatic activity of PARP1 has long been thought to be the sole mechanism of eliciting programmed-necrotic cell death upon genotoxic stress. However, recent genetic studies, using *Parp1* knockout mice, provided preliminary evidence that energy depletion alone might not be sufficient to mediate poly-ADP-ribosylation-dependent cell death (205, 206). Deadly NAD^+ depletion (60-95% of their normal levels) occurs only under very high levels of DNA damage, while under moderate levels of DNA damage the intracellular NAD^+ levels undergo a decrease of only 5-10%, thus other mechanisms are required for poly-ADP-ribosylation-dependent necrotic cell death (170). Several groups suggested that excessive activation of PARP1 most likely initiates a nuclear signal that spreads to the cytoplasm and triggers the release of AIF from the mitochondria into the cytoplasm and its translocation to the nucleus (reviewed in (2, 90-92)). Translocation of AIF has been shown to occur quickly after overactivation of poly-ADP-ribosylation reactions (reviewed in (2, 90-92)). Whether poly-ADP-ribosylation could also induce cytochrome c release is under debate. Some studies demonstrated that this process is only associated with the mitochondrial release of AIF, but not of cytochrome c (reviewed in (2, 90-92)). Pharmacological inhibition of the enzymatic activity of PARPs or the complete absence of PARP1 in different cells derived from *Parp1* knockout mice blocks the release of AIF and its translocation to the nucleus in various cell types (106, 108, 207-209).

Does poly-ADP-ribosylation-mediated programmed-necrotic cell death requires the activation of caspase or other proteolytical (calpain-like protease) signaling cascades? Previous studies did not support any role for caspases in PARP1 dependent neuronal cell death. On the other hand, as already mentioned, cleavage of AIF by a mitochondria-associated calpain-like protease is crucial for the release of AIF from mitochondria and is suggested to play a general role in mitochondrial cell death processes (79, 210-212). Calpain activation has been shown to contribute to necrotic cell death (213-216). Calpains are a family of cytoplasmic Ca^{2+}-dependent cysteine proteases. Indeed, using a panel of gene knockout cells, a very recent genetic study from the Susin lab clearly demonstrated that PARP1-mediated mitochondrial AIF release and programmed-necrotic cell death is a p53-independent mechanism, which requires calpains, but not cathepsins or caspases (79). Furthermore, these study revealed that the enzymatic activity of PARP1 could even regulate/induce calpain activation during alkylating DNA damage-induced programmed-necrotic cell death (79). However the exact molecular mechanisms underlying PARP1-mediated calpain activation remains to be elucidated.

4.1.2.1 Molecular mechanisms underlying the PARP1-mediated shuttling of AIF

Despite its pathophysiological importance and the tremendous efforts that has been made in the recent years, the exact poly-ADP-ribosylation dependent mechanisms by which PARP1 activation leads to mitochondrial dysfunction and release of AIF are still not clear. At present, more than 5 different, but partially overlapping nucleus-to-mitochondria signaling mechanisms were proposed. (1) Cytoplasmic and nuclear NAD^+/ATP depletion. (2) Cytoplasmic retention of AIF by HSP70 in *Parp1* knockout cells. (3) Free poly-ADP-ribose polymers serving as a nuclear-to-mitochondria signaling molecule and death signal. (4) Shuttling of nuclear pro-necrotic factors (potentially attached with poly-ADP-ribose) such as PARP1 itself or HMGB1 into cytoplasm or to mitochondria. (5) Activation of putative cytoplasmic isoforms of PARP1 and PARP2.

4.1.2.1.1. NAD/ATP depletion

Several studies support a model in which NAD^+ depletion as well as ROS-induced mitochondrial dysfunction may lead to mitochondrial permeability

transition (MPT) and trigger AIF-induced cell death (186-193). These studies demonstrated that PARP1-dependent depletion of NAD+/ATP levels appears to precede MPT and the release of AIF. Treatment of cells with PARP inhibitors, submicromolar concentrations of cyclosporine A, an inhibitor of MPT or with liposome-encapsulated NAD+ preserved mitochondrial transmembrane potential, rescued cellular respiration and ATP levels, blocked nuclear translocation of AIF and subsequent cell death in cells undergoing hyper-poly-ADP-ribosylation (186-193). These observations suggest that NAD+ depletion and MPT may be at least partially required for poly-ADP-ribosylation-mediated AIF translocation and cell death, under the tested conditions.

4.1.2.1.2. Enhanced cytosolic retention of AIF by HSP-70 in *Parp1* knockout mice

Several studies demonstrated that the expression levels of heat-shock protein HSP70 are increased in several types of primary cells derived from *Parp1* knockout mice when compared with wild-type mice (217-219). PARP1-mediated poly-ADP-ribosylation appears to repress heat shock factor-1 (HSF-1) activity and the heat shock response in these cell types (217-219). Transcription of the *Hsp70* gene requires HSF-1, the primary mediator of the heat shock response. Recent evidence suggests that HSP70, an inducible cytoprotectant protein can antagonize AIF-mediated cell death by both inhibiting mitochondrial AIF release and retaining leaked AIF in the cytoplasm (220-223). Although, HSP70 antagonizes apoptosis and programmed-necrotic cell death by interfering with multiple checkpoints in these cell death pathways, cytoplasmic retention of AIF has been suggested to be the major mechanism of HSP70-mediated cytoprotection. Interestingly, a very recent report provided preliminary evidence that the ATPase domain of HSP70 is critical for sequestering AIF in the cytoplasm under conditions of ATP depletion (224). Although the relative importance of the ATPase domain of HSP70 for sequestering leaked AIF in the cytoplasm remains controversial (220, 223-225), it is possible that the mitochondrial interaction between HSP70 and AIF might be indeed regulated through the ATP/NAD^+ levels, and thus implying that HSP70 may act as an ATP sensor under conditions of physiological injury.

4.1.2.1.3. Free poly-ADP-ribose could serve as nucleus-to-mitochondria signaling molecule and death signal

It has been recently suggested that certain types of free poly-ADP-ribose are involved in stress-dependent signaling processes in vivo (2, 92, 185, 198, 226). Free or protein-associated poly-ADP-ribose could activate pro-apoptotic/necrotic factors. This hypothesis is partially supported by three recent studies. Complete *Parg* loss-of-function in *Drosophila melanogaster* was shown to result in cytoplasmic accumulation of free or protein-associated poly-ADP-ribose and in severe neurodegeneration (227). This observation led several investigators to speculate whether a poly-ADP-ribosylation product could indeed directly serve as an AIF-releasing factor (2, 164, 198, 228). Remarkably, two recent reports provided preliminary evidence that poly-ADP-ribose polymers, produced by PARP1, may act directly as a non-proteinaceous AIF-releasing factor and cell death signal (164, 228). When exogenously added to neurons at very high concentration, poly-ADP-ribose polymers cause the translocation of AIF from the mitochondria to the nucleus in intact cells and subsequent cell death (164, 228). Exogenous delivery of poly-ADP-ribose polymers was also inducing necrotic cell death in primary cortical neurons isolated from *Parp1* knockout mice indicating that the presence of free or protein-associated poly-ADP-ribose polymers in the cytoplasm might indeed be essential for cell death signaling in programmed necrosis (164, 228). Furthermore, purified poly-ADP-ribose polymers, generated by PARP1 *in vitro* induced AIF release from isolated mitochondria *in vitro*. When cortical neurons were treated *ex vivo* with very high doses of N-methyl-d-aspartate (NMDA; 500μM) endogenous poly-ADP-ribose polymer accumulated primarily in the nucleus 15 minutes after NMDA receptor stimulation, but 30 and 60 min after treatment, poly-ADP-ribose polymers appears to colocalize in the cytoplasm and to mitochondria, though at very low levels (164, 228). Moreover, the Dawson lab recently identified a protein termed Iduna that could bind to and neutralize endogenous poly-ADP-ribose polymers accumulating in the cytoplasm, thereby fine-tuning the outcome of cell death (reviewed in (229)). Interestingly, poly-ADP-ribose polymer toxicity appears to be length-, structure- and dose-dependent. Increasing length and complexity of poly-ADP-ribose polymers resulted in increased cell death (164, 228). Thus, it seems that the size and structural complexity of poly-ADP-ribose produced by PARP1 is essential in MNNG-induced programmed-necrotic cell death. It remains to be elucidated whether poly-ADP-ribose polymers synthesized by the other members of the PARP family, such as PARP2, may also activate the AIF-mediated necrotic pathway.

However, despite the abundance of indications, further studies are required to confirm this hypothesis. The concentration of poly-ADP-ribose polymers used in these two studies to exogenously induce AIF release and translocation is unexpected high compared with the very low levels of endogenous poly-ADP-ribose polymer supposed to exist in the cytoplasm (164, 228). Indeed, endogenous PAR in the cytoplasm was barely detectable. Due to the extremely high NMDA concentration (500μM) used in the poly-ADP-ribose polymer localization studies (164, 228), one cannot exclude the possibility that NMDA could have indirectly lead to disruption of the nuclear envelope architecture and integrity and thus may result in a non-physiologically relevant release of the highly negatively charged, large and branched poly-ADP-ribose polymer to the cytoplasm. Indeed, based on the physico-chemical behavior of the highly negative charge and branched structure of poly-ADP-ribose polymers, one could even assume that poly-ADP-ribose polymers are most likely retained in the nucleus, indicating that the

Table 1. Potential PARP1-generated poly-ADP-ribose binding proteins in the cytoplasm and mitochondria

pADPr Target	Function in cell death	Functional relevance for poly-ADP-ribosylation	References
Calpains: Calpain I	Pro-apoptotic/necrotic	Activation of enzymatic activity?	NA
caspase family members:	Pro apoptotic	Inhibition/Activation of enzymatic activity?	NA
Kinases: ERK1/2, JNK1	Pro and anti apoptotic/necrotic, dependent on the conditions?	Inhibiton or activation of kinase activities?	(218, 248)
AIF Cytochrom c	Pro apoptotic	Enhance Release and nuclear translocation?	NA
PARPs: Tankyrases?	Pro and anti apoptotic/necrotic, dependent on the conditions?		Reviewed in (1, 2)
PARG isoforms:	Pro and anti apoptotic/necrotic, dependent on the conditions?		Reviewed in (1, 2)
Enzymes in NAD/ATP anabolism: NMNAT-2 NMNAT-3?	Anti-apoptotic/necrotic?	Inhibiton of nicotinamide mononucleotide adenylyl transferase activity?	(486-488)
GAPDH?	Pro and anti apoptotic/necrotic, dependent on the conditions?	Inhibiton of glyceraldehyde- 3-phosphate dehydrogenase activity?	(489-492)
Bcl-2 family members?	Pro and anti apoptotic		NA

observed cytoplasmic poly-ADP-ribose polymers may alternatively synthesized in the cytoplasm or mitochondria under these conditions (see next sections). In addition, no clear correlation in the kinetics between the accumulation of poly-ADP-ribose polymers in the cytoplasm and nuclear AIF shuttling were presented in these studies (164, 228). A recent study showed that PARP appears to be rapidly activated in two distinct phases, an initial immediate activation within the first 5-10 minutes and a late PARP activation between 60-240 minutes, dependent on the stimuli and cell type (230). On the other hand, it is quite possible that another AIF releasing event precedes the (weak!) accumulation of poly-ADP-ribose polymers in the cytoplasm. Thus it remains to be further investigated whether this shuttling will occur under pathophysiological more relevant conditions *in vivo*. It would be interesting to investigate whether pre- or post-treatment of cells with leptomycin B (LMB), an inhibitor of CRM-dependent nuclear protein export, could inhibit the cell death process in different cell types and under different pro-necrotic/apoptotic conditions (see also the next section). Moreover the mechanism by which poly-ADP-ribose polymers induces AIF release is not known. The authors of both studies proposed that the highly charged poly-ADP-ribose polymers could depolarize the mitochondrial membrane, which in turn would lead to permeability transition and subsequent AIF release (164, 228). An alternative scenario might be the activation of calpains or pro-apoptotic kinases mediated by direct protein-poly-ADP-ribose interactions in the cytoplasm or at the mitochondria, which then triggers AIF release from the mitochondria. Finally, it appears unlikely that poly-ADP-ribose polymers serve as a general nuclear/mitochondrial AIF-releasing signal in PARP1-dependent programmed-necrotic cell death since AIF-release was also observed independent of PARP1 ((2, 78, 81, 93), see also next sections). Potential poly-ADP-ribose target proteins in the cytoplasm and mitochondria are shown in Table 1.

4.1.2.1.4. Shuttling of nuclear proteinaceous pro-necrotic factors to mitochondria

A recent report provided first evidence that PARP1 might translocate to the cytoplasm under cytotoxic conditions (231). When cells were exogenously treated with high doses of purified HIV1-Vpr proteins, PARP1 shuttled to the cytoplasm in a glucocorticoid receptor complex-dependent manner (231). Several previous studies demonstrated that high levels of extracellular HIV1-Vpr exhibit cytotoxicity to uninfected bystander cells through apoptotic or necrotic mechanisms, resembling programmed-necrotic cell death (reviewed in (232)). Two other recent studies identified the linker histone variant H1.2 as an apoptogenic factor released from the nucleus to the cytosol and translocated to the mitochondria, exclusively in response to DNA double strand breaks (233, 234). Mitochondria-associated H1.2 co-localized with Bak and promotes the activation of pro-apoptotic Bcl-2 family proteins, mitochondrial cytochrome c release, and ultimately, cell death in a p53-dependent manner (233, 234). Although there is so far no clear evidence supporting such a hypothesis, certain poly-ADP-ribose-associated chromatin proteins, such as histones, high-mobility-group box proteins, or PARPs might also be used as AIF-releasing signals during apoptosis and programmed necrosis, respectively.

4.1.2.1.5. Activation of an unknown cytoplasmic isoform of PARP1 or PARP2

Most studies have localized PARP1 exclusively to the nucleus. However, several previous studies suggested the existence and activation of unknown mitochondrial or cytoplasmic potentially alternatively spliced isoforms of PARP1 and PARP2 ((194, 235-238) and reviewed in (239)). In fact, data from P. Mandels group provided preliminary evidence for the existence of cytosolic and mitochondria-associated poly-ADP-ribose polymerase and poly-ADP-ribose glycohydrolase activity in primary human liver cells, rat cortical neurons and mouse fibroblasts (235-238). Interestingly, PARP2 was recently suggested to be also perinuclear localized under normal physiological conditions but localized exclusively to the nucleus 6 h after irradiation at 0.5 Gy in *Parp1* (+/+) MEFs cells (240). Surprisingly in unirradiated *Parp1* (-/-) cells, PARP2 distribution was nuclear with no change after 0.5 Gy, indicating that PARP2 could shuttle between the cytoplasm and nucleus in a stimuli and PARP1-dependent manner (240). The possible existence of cytosolic and mitochondria-associated poly-ADP-ribose polymerase

activities may argue in favor of a direct interaction between PARP1 and AIF or mitochondrial components involved in MPT. This would also obviate any need for nuclear-to-mitochondria signaling molecules. However, one has to be cautious, despite that many groups have been actively involved in the search for a mitochondrial poly-ADP-ribosylation neither poly-ADP-ribose formation in the mitochondria nor localization of PARP1 protein to the mitochondria have been reported by other labs.

Taken together, it is possible that all suggested mechanisms of nuclear-to- (cytoplasmic)-to-mitochondrial communication could indeed serve as a trigger, dependent on the stimuli, metabolic state and on the cell lines in which PARP1 activation occurs. This could even taken place in the same cell in parallel or sequentially in waves.

4.1.3. Phosphorylation-dependent activation of PARP1 in programmed-necrotic cell death

A recent study from the Swanson lab provided first evidence that the activity of PARP1 is controlled by phosphorylation of PARP1 during programmed-necrotic cell death (241). Using a rat model of hypoglycemic brain injury, these authors demonstrated that PARP1-mediated programmed-necrotic cell death of neuronal cells induced by NMDA, peroxynitrite, or DNA alkylation agents is blocked by pharmacological inhibition of the extracellular signal-regulated kinase-1 and 2 (ERK1/2) inhibitors and by siRNA knockdown of ERK2 expression (241). *In vitro* kinase assays with recombinant proteins and *in vivo* studies using PARP1 mutants revealed that direct phosphorylation of PARP1 by ERK1/2 on S372 and T373 is required for maximal PARP1 activation after DNA damage. Inhibition of PARP1 phosphorylation by ERK1/2 was proposed to be the major mechanism by which inhibitors of the ERK2 signaling cascade reduce cell death rates following ischemia–reperfusion (241). Furthermore, based on genetic approaches and pharmacological inhibition, two other recent reports showed that c-Jun N-terminal kinase-1 (JNK-1), but not the other groups of mitogen-activated protein kinases (MAPK), is required for H_2O_2- and MNNG induced PARP1-mediated mitochondrial dysfunction, AIF translocation and subsequent cell death (84, 242). However, the data of these studies are at least in part conflicting. It is not yet clear whether JNK-1 activation occurs upstream or downstream of PARP1. The first study demonstrated that MEFs derived from knockout mice of JNK-1, receptor-interacting protein 1 (RIP1) and tumor necrosis factor receptor (TNFR)-associated factor 2 (TRAF2) are highly resistant to PARP1-induced cell death *in vitro (*84). This study also suggested that JNK-1 acts downstream of PARP1 as the main executor in MNNG-induced programmed-necrotic cell death and comprises together with RIP1 and TRAF2 a pathway to mediate the signaling from PARP1 over-activation to mitochondrial dysfunction (84). The rapid activation of the JNK-1 cascade was blocked in presence of non-specific PARP inhibitors while pharmacological inhibition of JNK and deletion of the *Jnk1* gene did not prevent the activation of PARP1 (84). This observation is also partially supported by previous reports using non-specific PARP and kinase inhibitors (243-248). Both, RIP1 and TRAF2 appears to act upstream of JNK-1 in this pathway (84). In contrast, the second study demonstrated using the same pharmacological or genetic approach that H_2O_2-mediated PARP1 activation is dependent on JNK-1 activation (242). JNK-1 was also previously shown to act as the main executor in H_2O_2-induced programmed-necrotic cell death. Pharmacological inhibition of JNK-1 or genetic deletion suppressed the sustained activation of PARP-1 induced by H_2O_2, suggesting that JNK-1 acts upstream of PARP1 (242). Similar to ERK1/2, JNK-1 appears to promote the sustained activation of PARP1 activation through a direct protein-protein interaction and phosphorylation of PARP1 (242). This discrepancies might be explained by the different cell types and stimuli used in these studies. Indeed the high concentration of MNNG used in the first study (500 μM), MNNG could directly activate JNK-1 or induce mitochondrial depolarization, including direct facilitation of mitochondrial permeability transition, independent of PARP1 ((119, 249) and reviewed in (250)). Alternatively, one cannot fully exclude the possibility that a overlooked positive poly-ADP-ribose-dependent feed back loop may exist, which could be triggered by PARP1, PARP2 or another PARP family member.

Interestingly, in the last 6 years, several studies provided preliminary data, indicating that PARP1-catalyzed poly-ADP-ribosylation might negatively affect cytoprotective kinase signaling pathways, which play a significant role in cell survival and cell death (243-248, 251). Inhibitor studies demonstrated that several different non-specific PARP inhibitors may enhance the endotoxin-induced or ischemia-reperfusion-induced activation of phosphatidylinositol 3-kinase-AKT/protein kinase B (PKB) and p38 mitogen-activated protein (MAP) kinase (p38-MAPK) in *ex vivo* and *in vivo* models (243-248, 251). Moreover, PARP inhibition causes activation of ATM, which is known to be involved in the DNA DSB response (252, 253). However, the exact molecular mechanism remains to be investigated. There are no combined biochemical and genetic studies reported, which could support the hypothesis that PARP1-catalyzed poly-ADP-ribosylation reactions negatively affect cytoprotective kinases through direct modulation of distinct kinase activities. The specificity of PARP inhibitors is in general very questionable due to their ADP-ribosylation non-related off-target activities, including ROS scavenging (reviewed in (1)). Thus, it is not yet clear whether the observed effect is directly mediated through PARP1-catalyzed poly-ADP-ribosylation or indirectly through PARP1 independent mechanisms. Whether poly-ADP-ribosylation directly affects these pathways can therefore only be addressed *in vivo* by using knock-in mice models expressing enzymatic mutants of distinct PARP enzymes and *in vitro* by subsequent poly-ADP-ribose-binding and kinase activity assays.

4.1.4. PARP1 independent AIF mediated cell death processes

Necrotic cell death caused by oxidative damage in other cell types, such as hepatocytes, does not depend on poly-ADP-ribosylation reactions (reviewed in (2, 41, 81, 93, 184, 203, 204)). Indeed, mitochondrial AIF release and

shuttling of AIF to the nucleus is not exclusively dependent on poly-ADP-ribosylation and PARP1 or other PARPs. Several reports suggested that programmed-necrotic cell death can be also mediated independent of PARP activation or caspase activity, but through other not yet fully understood pathways (254-258). As already mentioned above, high concentration of MNNG (>100μM) can directly induce mitochondrial depolarization, including facilitation of mitochondrial permeability transition, independent of PARP1 ((119, 249) and reviewed in (93, 250)). MNNG is used as a standard tool for the functional characterization of PARP1 activation during PCD. However, the concentration of MNNG used for PARP studies varies between 100 and 500 μM, wich is 10-50 times the IC_{50} value of MNNG for most cells. Indeed, recent studies demonstrated that MNNG induced NAD^+ depletion and cell death can be prevented by inhibition of the permeability transition pore opening. MNNG appears to act as an inhibitor of respiration and promotes opening of the permeability transition pore in intact cells independent of PARP1 (249). These studies suggest that DNA damage and PARP1 activation are not in general the primary events in the sequence leading to MNNG induced NAD^+/ATP depletion and cell death (249). Thus, mitochondrial dysfunction appears to be the cause rather than the consequence of MNNG induced NAD^+ depletion ((119, 249) and reviewed in (93, 250)).

The activities of other factors such as p53 and caspase-2 were also shown to be required for this process to a similar extent ((254-258) and reviewed in (2, 24, 25, 41, 93)). Recent studies demonstrated that both AIF and caspase-12 dependent PCD pathways are coactivated during degenerative processes in an animal model of retinitis pigmentosa, independent of poly-ADP-ribosylation and PARP1. The co-translocation to the nucleus of caspase-12 and AIF appears to occur in response to two endoplasmic reticulum (ER) stresses: protein misfolding and disruption of calcium homeostasis (255, 256). AIF has been suggested to primarily control programmed cell death caused by changes in calcium homeostasis while caspase-12 may have a main role in programmed cell death induced by protein misfolding (255, 256). The production of reactive oxygen species (ROS) appears to be a key factor for these processes, in presence or absence of poly-ADP-ribosylation and caspase activation (reviewed in (89, 93, 259-261)). Moreover, it is not yet investigated whether the short isoforms of AIF (AIFsh and AIFsh2) (262, 263) and the AIF-related factor, AIF-homologous mitochondria-associated inducer of death (AMID) (264-267), might also be (partially) poly-ADP-ribosylation-dependent. The new pro-apoptotic/necrotic isoform AIFsh, when overexpressed, translocates to the nucleus and leads to a caspase-independent necrotic cell death. AMID is a pro-apoptotic flavoprotein with similarity to AIF, possessing NAD (P)H oxidase activity and is localized to the outer mitochondrial membrane and the cytosol (264-267). Several studies suggested that the mechanism is similar to that induced by AIF, but appears to be independent of caspase-12 and p53 (264-267). Thus, it will be important to elucidate the factors, which determine whether a programmed-necrotic cell death process is PARP dependent or independent.

Taken together PARP1-mediated poly-ADP-ribosylation reactions appear to play an important but not exclusive role in AIF-dependent programmed-necrotic cell death pathways. It was recently proposed that the PARP/poly-ADP-ribosylation system and AIF may function together as a mitochondria-nucleus interconnected Yin/Yang-like sensor of metabolic or oxidative state of cells, acting as a double-edged sword in ROS-dependent death/survival pathways (1, 2, 16). One edge would protect the animal from the development of tumors by turning off the anti-apoptotic functions of certain kinases, such as AKT/PKB and switching the dual functions of AIF towards apoptosis, thereby limiting the development of cancer. By the opposite edge, uncontrolled poly-ADP-ribosylation reactions can result in massive necrosis and tissue damage, which in turn often leads to severe inflammatory or neurodegenerative disorders.

4.1.5. Secondary necrosis in the surrounding tissue as a result of poly-ADP-ribosylation mediated release of HMGB1?

High-mobility group protein box 1 (HMGB1) is a nuclear non-histone chromatin-associated protein with widely studied functions as a transcription factor (reviewed in (268, 269)). HMGB1 has been reported to be actively secreted by activated monocytes and macrophages as a damage signal (alarmin) and to function as a late mediator of inflammation (reviewed in (270-273)). Recent studies indicate that HMGB1 undergoes nuclear-cytosolic relocalization during early stages of DNA-damage induced programmed-necrotic cell death and is "passively released" from necrotic cells into the extra-cellular milieu at late stage of necrosis (reviewed in (268-273)). HMGB1 translocation into the cytoplasm of neurons was also observed *in vivo* within the ischemic brain of mice (274, 275). HMGB1 is released in the culture media of murine neurons and astrocytes upon treatment with pro-necrotic but not pro-apoptotic stimuli (274, 275). Indeed, apoptotic cells bind HMGB1 irreversibly to their chromatin (reviewed in (270-273)). Extracellular released HMGB1 can act as a potent pro-inflammatory cytokine by promoting inflammation and participating to the pathogenesis of diverse inflammatory and infectious disorders in peripheral organs (reviewed in (268-273)). For instance, microinjection of HMGB1 into the brain of mice was shown to increase the levels of pro-inflammatory mediators and sensitizes the tissue to the ischemic injury (274, 275). HMGB1 has been suggested to act as a messenger of death, thereby signaling the necrotic state of cells to the surrounding tissue (reviewed in (268-273)). HMGB1 signaling occurs via the receptor for advanced glycated-end-products and via members of the Toll-like receptor family (reviewed in (268-273)). The HMGB1 mediated inflammatory responses include the production of multiple pro-inflammatory mediators, chemoattraction of certain stem cells, induction of vascular adhesion molecules and impaired function of intestinal epithelial cells (reviewed in (268-273)). HMGB1 has been shown to serve as a late mediator of endotoxin lethality in mice and as a mediator of post-ischemic brain damage by increasing excitotoxic as well as ischemic neuronal death *in vitro* and *in vivo (*276, 277). Indeed, HMGB1 is a crucial therapeutic

Table 2. Potential poly-ADP-ribose binding proteins in the nucleus and cytoplasm serving as alarmin

pADPr Target	Function in programmed cell death	Functional relevance for poly-ADP-ribosylation	References
Heat shock protein: HSP60, HSP70 HSP110	Pro-apoptotic /necrotic, dependent on the conditions?	Inhibition of DNA binding or enzymatic activity?	Reviewed in (1, 2)
High mobility group (HMG) proteins: HMGB1	Pro-apoptotic /necrotic, dependent on the conditions?	NA	(273, 274, 282) and reviewed in (1, 2)
Histones H2A H2B	Pro-apoptotic /necrotic, dependent on the conditions?	NA	Reviewed in (1, 2, 497, 498)

target in experimental models of ischemia/reperfusion, rheumatoid arthritis, endotoxin induced septic shock and cancer ((274-277) and reviewed in (268-273)).

The active secretion of HMGB1 by stimulated macrophages or monocytes requires acetylation of HMGB1, which in turn induces its relocalization to the cytosol into secretory lysosomes and subsequent release into the extracellular environment (278, 279). Activation of monocytes and macrophages by inflammatory signals shifts the balance towards chromatin acetylation and leads to hyperacetylation of HMGB1 (278, 279). Acetylation of HMGB1 at two specific lysines was shown to interfere with nuclear import but not with nuclear export of HMGB1 (278, 279). Hyperacetylated cytosolic HMGB1 is then concentrated into secretory lysosomes and secreted when monocytic cells receive an appropriate second signal (278, 279). However, the exact mechanism of how HMGB1 is released from chromatin during necrotic cell death was not known, until recently. In addition to acetylation, HMGB1 can be post-translationally modified by lysine and arginine methylation, phosphorylation, and glycosylation, which were also suggested to regulate its relocalization and extracellular release (280, 281). It was speculated that PARP1 mediated poly-ADP-ribosylation might also be required for the nuclear export and release of HMGB1 during programmed-necrotic cell death (2). Indeed, in a recent study redistribution of HMGB1 was identified to be dependent on PARP1 and on its enzymatic activity (282). Following MNNG induced DNA damage and/or mitochondrial stress, relocalization of HMGB1 was only observed in wild-type cells, while HMGB1 remained nuclear in *Parp1* (-/-) or in the presence of the general PARP inhibitor DHIQ (282). It has been suggested that the release of HMGB1 from chromatin and its relocalization is regulated by the attachment of poly-ADP ribose to HMGB1 and chromatin thereby destabilizing the association of HMGB1 with chromatin (282). However the exact molecular mechanism remains to be further investigated. Potential poly-ADP-ribose-binding proteins in the nucleus and cytoplasm that may serve as alarmin are presented in Table 2

4.1.6. The Role of other PARP family members and PARGs in cell death

PARP1 appears to be the major PARP member playing a crucial role in programmed cell death processes. However several studies provided evidence that PARP2 and both Tankyrase-PARPs, TANK1 and TANK2, may also function as a novel executor of cell death pathways. Thus, further investigations are needed to determine if poly-ADP-ribosylation reactions mediated by family members other than PARP1 are also required for AIF shuttling. On the other hand there is already good evidence demonstrating that PARG and its alternatively spliced isoforms are crucial players in programmed cell death pathways.

4.1.6.1. PARP2

Similar to PARP1, PARP2 has been suggested to synthesize poly-ADP-ribose polymers in a DNA-dependent manner. PARP2 has been suggested to contribute only 5% to 10% of total PARP activity in response to DNA damage (15, 283). Thus, PARP2 may indeed account for the residual poly-ADP-ribose synthesis observed in *Parp1* knockout cells. PARP2 is mainly expressed in actively dividing tissues during mouse development, similar to PARP1, but to a much smaller extent (15, 283-285). A recent study indicate that PARP1 and PARP2 may possess both overlapping and non-redundant functions in programmed cell death pathways (12). PARP2 has been shown to function, at least in part, as a novel executioner of cell death pathways in focal cerebral ischemia (12). However, since PARP activity is reduced by maximal 10% in most *Parp2* (-/-) cells tested, its unlikely that PARP2 activity could directly contribute to the NAD depletion during programmed necrosis in focal cerebral ischemia. Unfortunately, these studies did not provide any information regarding the amount of NAD^+ depletion after genotoxic stimuli in *Parp2* (-/-) cells or mice (12). Moreover, it remains to be investigated whether poly-ADP-ribosylation reactions mediated by PARP2 may also induce AIF shuttling during programmed necrosis in focal cerebral ischemia. A recent study using immortalized MEFs derived from *Parp2* (-/-) and *Parp2* (+/+) mice suggested a PARP2 and p53-independent role of PARP1 in MNNG-induced AIF release from mitochondria (79). However, no direct evidence is available at the moment to rule out that activation of PARP2 does induce AIF translocation *in vivo*, in a stimuli and cell type specific manner. Moreover, these preliminary observations have to be confirmed by an additional independent *Parp2* KO model since the observed effects could be caused by a dominant negative activity of a potentially expressed N-terminal DNA binding fragment of PARP2 in the current *Parp2* knockout mice model (15).

4.1.6.2. Tankyrases

PARP5/tankyrase-1 (TANK1) and PARP6/tankyrase-2 (TANK2) were identified as components of a telomeric complex and functions both as oligo-ADP-ribosyltransferases ((286, 287) and reviewed in

(1, 2)). Human Tankyrase-1, PARP5/TANK1 binds the telomere-binding protein TRF1 and increases telomere length when overexpressed in human cells (287). Surprisingly, in contrast to PARP5/TANK1, PARP6/ TANK2 causes programmed-necrotic cell death when highly over-expressed in human immortalized cells (286). Programmed-necrotic cell death, induced by PARP6/TANK2 can be blocked by non-specific PARP inhibitors, suggesting that either the enzymatic activity of PARP6/TANK2 is required or PARP6/TANK2 may modulate the enzymatic activity of PARP1 (286). On the other hand, PARP5/tankyrase-1 appears to protect cells from genotoxin induced cell death and injury through inhibition of PARP1-mediated NAD depletion and cell death (288). Thus, Both PARP5/tankyrase-1 and PARP6/tankyrase-2 may modulate PARP1 or PARP2-mediated programmed cell death pathways in an antagonistic manner.

4.1.6.3. Poly-ADP-ribose glycohydrolase (PARG)

The major enzyme rapidly hydrolyzing poly-ADP-ribose polymers formed by PARP1 and PARP2 is the well-characterized poly-ADP-ribose glycohydrolase (PARG) (289, 290). The endoglycosidase activity of PARG releases free poly-ADP-ribose from PARP1 and PARP2, suggested to serve as signaling molecules involved in cell-death (2, 92, 198). The mammalian *Parg* gene encodes for at least five alternative spliced isoforms; the nuclear mPARG-110/hPARG-111 isoform representing the full-length 110/111 kDa PARG protein in human and mice, the cytoplasmic and nuclear localized isoform mPARG-63/hPARG-60, the strictly mitochondrial localized mPARG-59/hPARG-55, and the two cytoplasmic isoforms PARG-102, PARG-99, mainly characterized in human (166, 168, 289-291). Mice with a targeted deletion of exons 2 and 3 of the *Parg* gene, which results in depletion of the nuclear PARG-110 protein and the cytoplasmic isoforms PARG-101 and PARG-99, are viable and phenotypically normal but show an increased sensitivity to alkylating agents and ionizing radiation (169, 170). In addition these mice were susceptible to streptozotocin-induced diabetes or endotoxic shock and showed an enhancement of ischemic brain injury, most likely due to abnormal regulation of the nuclear and cytoplasmic poly-ADP-ribosylation metabolism and accumulation of poly-ADP-ribose (169, 170). Surprisingly, ATP depletion was found to be similar in Parg-Δ2-Δ3/Δ2-Δ3-knockout and wild type mice after ischemia, indicating that impairment of PARG-110-, PARG-102- and PARG-99-dependent poly-ADP-ribosylation catabolism does not significantly affect the brain's energy dynamic during hyper-poly-ADP-ribosylation. Remarkably, the activity of both the mPARG-63/hPARG-60 isoform and PARP1 in intact cells is increased in *Parg-Δ*2-Δ3/Δ2-Δ3-knockout cells upon oxidative stress (166). Mice with a targeted deletion of exons 3 and 4 of the *Parg* gene, resulting in a complete depletion of all isoforms, show early embryonic lethality and increased sensitivity to alkylating agents and ionizing radiation (172). The lethality results from the failure to hydrolyze poly-ADP-ribose. These PARG-deficient cells accumulate very high levels of poly-ADP-ribose and undergo increased cell death. Conversely, transgenic neuronal cells overexpressing PARG appears to be partially resistant to programmed necrosis *in vivo*, after focal ischemia (164, 228). These genetic studies suggest that a precise coordination of PARPs and PARG activities is important for cellular responses under normal physiological as well as cytotoxic stress conditions. The disruption of poly-ADP-ribose homeostasis due to a failure to degrade poly-ADP-ribose polymers appears to have deleterious consequences in pathological processes. Strangely, other pharmacological and genetic studies provided evidence that PARG may also mediates oxidative and excitotoxic neuronal death (171, 292, 293). Mice deficient for the nuclear PARG-110 isoform appears to be protected against renal ischemia/reperfusion injury, under the tested conditions (166, 171, 292, 293). Moreover, siRNA-mediated *Parg*-knockdown in immortalized human cells results in an increased resistance to oxidant-induced apoptosis concomitant with a delayed PAR degradation and transient accumulation of ADP-ribose polymers longer than 15-mers at early stages of drug treatment (294). Surprisingly no increased resistance or sensitivity was observed in response to the DNA alkylating agent NMNG (294). This discrepancy strongly indicates that the specific outcome (pro- or anti-apoptotic) could be mediated by the putative opposite functional roles of distinct PARG isoforms, dependent on the cellular context, species and stimuli.

4.2. PARP1-mediated poly-ADP-ribosylation in Apoptosis

PARP1 and its poly-ADP-ribosylation activity have been initially proposed to play an important pro-apoptotic role. Several studies reported a transient burst of poly-ADP-ribosylation occurs early in various cell lines undergoing apoptosis, such as in human osteosarcoma HL-60 cells, human Jurkat T cells, mouse 3T3-L1 and immortalized fibroblasts derived from PARP1 wild type mice ((295-297) and reviewed in (6, 298-300)). Thus, it was suggested that an early transient burst of poly-ADP-ribosylation in the nucleus prior commitment to death is required for apoptosis to proceed (295-297). However, the majority of studies using *Parp1* (-/-) mice and primary cells derived thereof suggest that PARP1 appears not to play a mayor role in apoptosis. These studies have shown that primary *Parp1* (-/-) cells, including hepatocytes and thymocytes display normal susceptibility towards ceramide, dexamethasone, etoposide or TNFα-mediated apoptosis (reviewed in (7, 163, 301-304)). On the other hand, a few studies have demonstrated that PARP1-depleted human Jurkat T-cells and certain immortalized cell lines derived from *Parp1* (-/-) mice are resistant to CD95/FasL induced apoptosis ((305-308) and reviewed in (7, 163, 301-304)), indicating that PARP1 could indeed activate apoptosis under some, most likely non-genotoxic conditions. However, the exact role of the enzymatic activity of PARP1 remains conflicting. Several pro- and anti-apoptotic factors were suggested to bind poly-ADP-ribose. For instance, the apoptosis-associated Ca^{2+}/Mg^{2+}-dependent DNA endonuclease and topoisomerase-I and -II might be kept inactive by attached poly-ADP-ribose polymers during the initiation phase of apoptosis (309-313). It was suggested that poly-ADP-ribose polymers are in turn

removed from the endonuclease and topoisomerase II during the execution phase of Apoptosis (314). Thus, poly-ADP-ribosylation might coordinate apoptotic processes under certain conditions. Potential pro- and anti-apoptotic poly-ADP-ribose binding proteins in the nucleus are presented in Table 3.

4.2.1. Caspase-mediated cleavage of PARP1, a proposed hallmark of apoptosis

During the execution phase of apoptosis, PARP1 and also PARP2 are specifically cleaved by caspases to generate N-terminal DNA-binding and C-terminal catalytic fragments, thereby inactivating PARP1 and PARP2 (315-320). However, the N-terminal apoptotic fragments of PARP1 and PARP2 have been thought to retain their strong DNA-binding activity upon cleavage (315). The functional consequence of this proteolytic event is still under debate. The rapid cleavage of PARP1 into the N-terminal 25 kDa DNA-binding domain (DBD) and the C-terminal 89 kDa proteolytic fragment was suggested to enhance the apoptosis process (reviewed in (6, 298-300)). The DBD of PARP1 could be irreversibly recruited to sites of DNA breaks during early stages of the execution phase and thereby preventing subsequent genome repair events (6, 298-300). Caspase-mediated inactivation of PARP1 and PARP2 has been proposed to prevent depletion of NAD^+ and ATP, which are required for full execution of apoptosis (6, 298-300, 315). Several studies, overexpressing the apoptotic DNA-binding domain of PARP1 in various human cells and mouse fibroblasts provided preliminary evidence that the apoptotic DNA-binding domain of PARP1 may indeed inhibit the endogenous catalytic activity of uncleaved PARP1 in a dominant-negative manner, *in vivo* (321-329). Thus, proteolytic cleavage of PARPs by caspases is thought to be a hallmark and regulatory event for apoptotic cell death. Subsequent studies using *Parp1* (-/-) fibroblasts stably expressing either wild type PARP1 or a caspase-noncleavable PARP1 mutant, containing a point mutation in the cleavage site DEVD (214) partially confirmed these initial observations (307, 321, 330, 331). Cells expressing this mutant PARP exhibited an accelerated necrotic cell death upon treatment with staurosporine, TNFα and genotoxic stress (307, 321, 330, 331). This accelerated necrotic cell death was suggested due to NAD^+ depletion. Thus PARP1 cleavage was proposed to prevent the induction of necrosis during apoptosis and ensures appropriate execution of caspase-mediated programmed cell death (307, 321, 330, 331). Moreover, PARP1 cleavage was suggested to function as a molecular switch between apoptotic and necrotic cell death (307, 321, 330, 331). However these data have to be cautiously interpreted. The recent generation of a *Parp1* knock-in mice model, expressing a caspase-noncleavable PARP1 mutant (*Parp1mutD214N/mutD214N*) could not confirm these *in vitro* data (308). While *Parp1mutD214N/mutD214N* mice developed normally, they were highly resistant to endotoxic shock and to intestinal and renal ischemia-reperfusions due to the compromised production of specific inflammatory mediators (308). No direct effect of PARP1 cleavage on the preservation of cellular pools of NAD^+/ATP and rate of cell death could be observed *in vivo* (308). This study clearly demonstrated that PARP1 cleavage is physiologically relevant in the regulation of an inflammatory response but very unlikely directly modulating programmed cell death processes, *in vivo*.

4.2.2. Poly-ADP-ribosylation induced changes of chromatin structure during apoptosis

A recent study suggested that internucleosomal DNA fragmentation, mediated by apoptosis/necrosis inducers such as DNA-damaging agents (i.e. UV light and chemotherapeutic drugs) correlated with enhanced poly-ADP-ribosylation of chromatin (297). Remarkably internucleosomal DNA fragmentation could be prevented when cells were treated with nonspecific PARP inhibitors (297). Thus, poly-ADP-ribosylation of chromatin in the early stages of apoptosis could facilitate internucleosomal DNA fragmentation by increasing the susceptibility of chromatin to cellular endonucleases under certain conditions. Over 20 years ago, several groups demonstrated that poly-ADP-ribosylated chromatin adopts a more relaxed structure than its native counterpart ((332-335) and reviewed in (2)). When isolated polynucleosomes of interphase chromatin were poly-ADP-ribosylated *in vitro* by a highly purified preparation of PARP1 at low and moderate ionic strengths, the solenoid structure (30-nm fiber) unwound into the 10-nm fiber and adopted the fully extended "beads-on-a-string" structure characteristic of H1-depleted chromatin ((333, 335-340) and reviewed in (2)). Poly-ADP-ribosylation of polynucleosomes rendered chromatin more susceptible to micrococcal nuclease digestion ((332, 333) and reviewed in (2)). Thus, poly-ADP-ribosylation-induced relaxation of the chromatin structure, observed *in vitro*, may explain the partial dependency of internucleosomal DNA fragmentation on poly-ADP-ribose under certain conditions that occurs during apoptosis *in vivo*.

4.2.3. Poly-ADP-ribosylation/PARG-mediated activation of TRPM calcium channels during oxidant induced apoptosis

Recent studies demonstrated that TRPM2, a cation channel, widely expressed in neuronal cells, blood cells and in pancreatic cells, plays an essential role in programmed apoptotic cell death (reviewed in (341-343)). TRPM2 can be activated by oxidative stress (H_2O_2) or TNFα (reviewed in (341-343)). Once activated, TRPM2 enables influx of Na^+ and Ca^{2+}, sustained elevation in intracellular free Ca^{2+} concentration, increased activity of caspase 3, 7, 8 and 9, and subsequent apoptotic cell death (344, 345) and reviewed in (341-343)). Free ADP-ribose, which can serve as a second messenger, has been recently identified as a major regulator of TRPM2 channel gating ((346-349) and reviewed in (350)). ADP-ribose binds to a Nudix homology domain in the cytosolic C-terminus of TRPM2. However, The molecular mechanism leading to accumulation of free ADP-ribose in the cytosol is not fully clear. It has been thought that the mayor source for accumulation of free ADP-ribose in the cytoplasm under condition of oxidative stress is the degradation of poly-ADP-ribose-polymers by poly-ADP-ribose glycohydrolase (PARG isoforms) and ADP-ribosyl-protein-lyase activities. Remarkably several recent reports provided first evidence

that TRPM2 channel opening in response to oxidative stress might be indeed dependent on activation of poly-ADP-ribose polymerase (349, 351-353). Oxidant-induced TRPM2 gating and subsequent TRPM2-mediated apoptosis was blocked when cells were treated with the non-specific PARP inhibitory compounds SB750139-B, PJ34 and DPQ (349, 351-353). However more detailed studies including genetic approaches will be required to elucidate the exact roles of PARP/PARG enzymes in TRPM2 mediated, oxidant induced apoptosis.

4.2.4. PARP/PARG-mediated hyperglycation and glyoxidation could induce apoptotic cell death

In addition to the suggested role as a second messenger and regulator of TRPM2 channel gating, free ADP-ribose and poly/oligo-ADP-ribose may also induce programmed cell death pathways through its non-enzymatic reactivity towards protein side chains and formation of protein glycation/glycoxidation products at high concentrations. The massive accumulation of poly-ADP-ribose-polymers produced by PARP1 upon oxidative stress, are in turn rapidly hydrolyzed into free ADP-ribose by PARG (reviewed in (2, 4)). Several Studies suggested that non-enzymatic ADP-ribose-mediated glycation is initiated by the reaction of free ADP-ribose with lysines through Schiff bases or with cysteines to form an ADP-ribosyl-thiazolidine (354-360). This is often followed by a complex chemistry leading to protein glycoxidation products referred to as advanced glycosylation end products (AGE) (356-360). Although the reactions are favored at higher pH 8-9.0, the cross-linking of histones and the formation of AGE were also detectable *in vitro* at pH values in the physiological range ((354-360) and reviewed in (2)). *In vitro* studies with nuclear proteins demonstrated that the lysine-rich histone H1 appears to be a preferential glycation and glycoxidation target of this intranuclear non-enzymatic ADP-ribosylation (356-360). However, the glycation/glycoxidation reactions *in vivo* are poorly understood due to the technical difficulties to measure glycation/glycoxidation products *in vivo* ((354-360) and reviewed in (2)). Preliminary data indicate that oxidative stress i.e. nitric oxide (NO) may enhance non-enzymatic ADP-ribosylation of proteins such as histones, β-actin and glyceraldehydes 3-phosphate dehydrogenase in the presence of free ADP-ribose *in vivo* (356, 361, 362). Although the intracellular levels of ADP-ribose under normal physiological conditions are not known, the intracellular concentrations of free ADP-ribose achieved upon oxidative stress have been proposed to be in the range of up to 100 μM (356). Recent studies demonstrated that ADP-ribose concentrations of 50-500 μM resulted in efficient histone glycation and glycoxidation in *vitro* (356, 360).

Glycation leads to the formation of protein-bound free radicals, which may further attack on proteins and could lead to the formation of protein-bound amino acid oxidation products, peptide cross-linking or peptide bond cleavage (356-360). Indeed, glycation of proteins and glycoxidation has been linked to the pathophysiology of aging and a number of specific diseases, especially diabetes, and Alzheimer's disease (363-367). Massive non-enzymatic protein glycation and glycoxidation by ADP-ribose has been proposed to induce protein damage and eventually apoptotic cell death (2, 358, 359). The cytotoxity of free ADP-ribose is counteracted *in vivo* by the housecleaning enzymes, members of the super family of Nudix hydrolases that catalyze the hydrolysis of free ADP-ribose to AMP and ribose 5'-phosphate (reviewed in (368-371)). The mammalian ADP-ribose-specific pyrophosphatases NUDT9 and NUDT5 are thought to serve as protective enzymes to prevent non-enzymatic ADP-ribosylation by limiting the intracellular accumulation of free ADP-ribose during oxidative/nitrosative stress (reviewed in (368-371)). However, the exact molecular mechanism remains to be elucidated.

4.2.5. Proposed role of poly-ADP-ribosylation of p53 in apoptosis

Many studies have been shown that p53 can be associated with poly-ADP-ribose polymers generated by PARP1 (226, 372-374). It is still not yet clear whether poly-ADP-ribose is covalently attached to an acceptor protein or simply associated in a non-covalent manner. It has been speculated that PARP1-mediated attachment of poly-ADP-ribose polymers onto p53 may influence its pro-apoptotic function (226, 372-374). Interestingly, a recent report claimed to have identified the major sites in p53 covalently poly-ADP-ribosylated by PARP1 *in vivo* (375). In mouse p53, three glutamic and aspartic acid residues, namely E255, D256 and E268 have been suggested to be covalently poly-ADP-ribosylated by PARP1 (375). PARP1-mediated poly-ADP-ribosylation has been proposed to inhibit the interaction between p53 and the nuclear export receptor CRM1, resulting in nuclear accumulation of p53 (375). These authors claimed that nuclear accumulation and upregulation of the transactivation activities of endogenous p53 in response to DNA damage require PARP1-mediated poly-ADP-ribosylation of p53 (375). Thus, poly-ADP-ribosylation of p53 may promote its pro-apoptotic function.

However, these data are quite conflicting and thus have to be very cautiously interpreted. Kanai and colleagues mainly used overexpressed GFP fusion proteins and observed this effect also in complete absence of PARP1 activation (375). Moreover, since no mass spectrometric data were presented in this study it remains still elusive whether poly-ADP-ribose is covalently attached to p53 on the proposed acceptor sites. Several reports clearly demonstrated that free and PARP1-bound poly-ADP-ribose polymers target p53 for strong non-covalent salt-, acid-, and detergent-resistant interaction ((372, 376) and reviewed in (2, 226)). The polymer binding sites could be mapped to two amino acid sequences in the sequence-specific core DNA binding domain of p53. The same studies demonstrated that poly-ADP-ribose can bind in a non-covalent manner to proteins that carry 20 to 30 amino acid long stretches containing a cluster rich in basic amino acids and a pattern of hydrophobic amino acids interspersed with basic and in part, with acidic residues (376). Remarkably, the proposed poly-ADP-ribose acceptor sites are exactly located within the C-terminal boundary of a poly-ADP-ribose-binding motif located in the DNA

binding domain of p53 (375)! The simultaneous amino acid exchange from E/D to A of all three putative sites could indeed strongly affect the protein structure in this domain of p53, thereby disrupting the non-covalent interaction of p53 with poly-ADP-ribose. Moreover, the suggested functional consequences appear not to be supported by other studies (226, 372-374, 377-381). Several independent previous reports even suggested the contrary (226, 372-374, 377-381). For instance, PARP inhibition has been demonstrated to sensitize p53-deficient breast cancer cells to doxorubicin-induced apoptosis, an observation that is in sharp contrast to the findings of Kanai and colleagues (382). Moreover several *in vitro* studies, using purified recombinant PARP1 and p53 proteins or nuclear extracts derived from early apoptotic cells suggested that poly-ADP-ribosylation suppressed p53 interaction with its DNA consensus sequence in p53-dependent promoters (226, 372-374). This observation is partially supported by gene expression studies using freshly isolated primary *Parp1* (+/+) and *Parp1* (-/-) mouse embryonic fibroblasts (MEFs) (226, 372-374, 377-381). On the other hand, the expression levels of tumor suppressor p53 and Rb1 proteins have been reported to be strongly reduced in immortalized long passaged, partially senescent *Parp1* (-/-) cells (383-386).

One possibility for this discrepancy could be a stimuli and cell type specific regulation of p53 by PARP1: For instance, treatment of MEFs derived from *Parp1* (-/-) with the single alkylating agent 2'-methyl-2'-nitrose-urea (MNU) has been shown to result in rapid and sustained accumulation and activation of p53 in *Parp1* (-/-) cells, while accumulation and activation was strongly reduced in *Parp1* (+/+) cells (387). In contrast primary *Parp1* (-/-) MEFs display delayed p53 accumulation and activation upon exposure to γ-irradiation, when compared to *Parp1* (+/+) cells (387). Moreover, overactivation of PARP1 by hyperoxia was recently reported to be associated with activation of p53 and premature senescence in immortalized long passaged human cells (383) (see also next sections). On the other hand nuclear translocation of p53 appears to be normal in all different primary *Parp1* (-/-) cells, tested so far (378-382, 387). Taken together, due to these conflicting data, the exact mechanism how poly-ADP-ribosylation regulates 53 has to be carefully re-evaluated.

4.2.6. PARP1-mediated transcriptional upregulation of pro-apoptotic genes

In addition to PARP1-mediated short-term cell death mechanisms discussed above, several long-term mechanisms such as regulating of cell death inducing transcriptional processes were also proposed (reviewed in (1, 2, 7, 388)). It is now widely established that PARP1 can also act as transcriptional coactivator for different transcription factors, which play a crucial role in inflammatory and neurodegenerative disorders (reviewed in (1, 2, 7, 388)).

PARP1 has been shown to play a key role in AP-1 and NF-κB-mediated gene expression of pro-inflammatory mediators (reviewed in (1, 2, 7)). PARP1 interacts directly with both subunits of NF-κB, p50 and RelA/p65 (388-390). NF-κB was initially thought to act generally cytoprotective toward apoptotic or necrotic PCD by inducing the expression of genes encoding anti-apoptotic and anti-oxidizing proteins in response to a variety of death-inducing stimuli (reviewed in (391, 392)). However, recent evidence strongly indicates that depending on the specific cell type and the stimulus involved, NF-κB can also sensitize cells to death-inducing stimuli and enhance programmed cell death (PCD) via apoptosis or necrosis (reviewed in (391, 392)). The exact molecular mechanisms are not fully understood. Recent studies suggested that pro-apoptotic transcriptional target genes of NF-κB including the death receptors Fas (CD95), TRAIL, DR4-6, the death-inducing ligands FasL, TNF and TRAIL as well as pro-apoptotic Bcl-2 family members may be required for this effect (reviewed in (391, 392)). Moreover, NF-κB can also function indirectly through upregulation of other transcription factors such as p53 or IRF-1, which acts along with NF-κB to enhance the expression of pro-apoptotic genes (reviewed in (391, 392)). Whether PARP1 is also required for the expression of these pro-apoptotic NF-κB transcriptional target genes remains to be investigated. In addition to its role as a transcriptional coactivator for the upregulation of pro-inflammatory and potentially pro-apoptotic genes, PARP1 may also serve as a transcription repressor for the transcriptional downregulation of anti-apoptotic proteins such as Bcl-2. Indeed, atypical activators of NF-κB such as the tumor suppressor protein ARF that is activated in response to DNA damage, UV-C radiation, and the chemotherapeutic drugs daunorubicin, and cisplatin, have been found to convert the transactivating property of RelA into a promoter-specific transcriptional repressor of anti-apoptotic factors such as Bcl-xL, XIAP and A20 (reviewed in (391, 392)). However, the exact mechanisms to achieve this pro-apoptotic shift in NF-κB activity remain to be elucidated. Both cisplatin and alternative reading frame protein (ARF) have been suggested to promote the recruitment of histone deacetylase-1 (HDAC1) to actively repress expression of the anti-apoptotic proteins and sensitize cells to apoptosis (reviewed in (391, 392)). PARP1 has been recently found to interact with histone deacetylases (HDACs) 1-3, *in vivo* (390). These findings suggest a model in which PARP1 might function as a promoter-specific corepressor/coactivator exchange factor for NF-κB-mediated gene activation (1, 2, 7, 388-390, 393). Whether the enzymatic activity of PARP1 is required for the observed effects remains elusive. Pharmacological studies suggested that the enzymatic activity of PARP1 appears to be required for NF-κB-mediated transcriptional activation in a context and promoter-specific manner.

4.3. Role of PARP1 in programmed macroautophagocytotic cell death

There is preliminary evidence suggesting that PARP1 may also play an important role in autophagocytotic programmed cell death processes, thereby contributing to caspase- and AIF-independent PCD of macrophages. Treatment with lipopolysaccharide (LPS) in the presence of chemical cysteine protease inhibitors like zVAD-fmk or specific siRNA-mediated casp8 knockdown

can cause macroautophagocytotic cell death instead of necrosis in certain cell types such as human Jurkat T cell lymphoma, L929 mouse fibrosarcoma, U937 monocytoid cells and macrophages ((394, 395) and reviewed in (122)). A recent study strongly indicates that Toll/interleukin-1 receptor-domain-containing adaptor inducing IFN-beta protein (TRIF), receptor-interacting protein 1 (RIP1), ROS production and PARP1 activation are involved in macroautophagocytotic programmed cell death of activated macrophages (394). Treatment of macrophages with LPS in the presence of zVAD-fmk resulted in reactive oxygen species (ROS) production, PARP1 activation and an increase in poly-ADP-ribose polymer formation. Genetic and pharmacological approaches revealed that TRIF, RIP1 and ROS acts upstream of PARP1 (394). PARP1 activation occurs downstream of ROS production but upstream of autophagic body formation in macrophages (394). Interestingly, it has been recently shown that cause of ROS accumulation upon caspase inhibition is the selective autophagic degradation of the major enzymatic ROS scavenger, catalase (72). The molecular basis of selective protein targeting of autophagy is not yet known. One cannot exclude that even an endogenous inhibitor of PARPs could be selectively degraded by autophagy. It will be of great interest to determine the exact role of PARP1 in programmed autophagocytotic cell death using *Parp1*-deficient mice.

4.4. PARP1 may regulate senescence-induced programmed cell death

During the last decade evidence has been arising that PARP1 may serve as an important factor regulating premature senescence. The first report suggested that PARP1 activity could regulate post-translational activation of p53 protein in immortalized very long-passaged aging cells (383). Surprisingly, inhibition of PARP activity with a broad range inhibitor increased cellular lifespan while overactivation of PARP1 by hyperoxia was associated with activation of p53 and premature senescence (383). Subsequent studies using immortalized very long-passaged aging MEF cells derived from *Parp1* (+/+) and *Parp1* (-/-) mice demonstrated that the expression levels of tumor suppressor p53 and Rb1 proteins were strongly reduced in immortalized long passaged/aging *Parp1* (-/-) cells (384-386). Moreover when these long passaged/aging *Parp1* (+/+) and *Parp1* (-/-) fibroblasts were treated with colcemid, a mitotic spindle disrupting agent, *Parp1* (-/-) cells showed an drastic increased escape from mitotic arrest within 24 h compared with *Parp1* (+/+) cells (384) (385, 396). Moreover, while both *Parp1* (+/+) and *Parp1* (-/-) fibroblasts genotypes arrived in G1 tetraploid state, only the G1 tetraploid *Parp1* (+/+) cells were eliminated by apoptotic cell death (384, 385). Remarkably stable re-expression of PARP1 in these long passaged/aging *Parp1* (-/-) fibroblasts restored p53 and Rb protein expression levels and susceptibility to G1 tetraploidy checkpoint-induced programmed cell death (384, 385, 396). These data strongly suggest a PARP1-dependent negative selection of cells with these genetic aberrations. Indeed, several studies clearly demonstrated that premature senescent cells could become polyploid by endomitosis and endoreduplication (reviewed in (143, 144, 397, 398)). Most of these cells die through mitotic catastrophe-induced apoptotic cell death. However, a few of the tetraploid senescent cells with genetic mutations or epigenetic alterations in the senescence pathway may escape cell death and undergo neosis (reviewed in (144)). Neosis is a parasexual, somatic, reductive division that occurs only in senescent, multinucleate and/or polyploid giant cells, formed during the replicative ore premature senescent phase of normal or tumor cells (reviewed in (144)). Neosis is characterized by production of aneuploid daughter cells via nuclear budding (399, 400). The immediate neotic aneuploid progeny are termed the Raju cells, which seem to transiently display stem cell properties (144, 399, 400). Raju cells were suggested to immediately undergo symmetric mitotic division and mature into tumor cells (reviewed in (144)). Taken together these results suggest that PARP1 may fulfill its role as a guardian of the genome even without exogenous DNA damage through its role as a cell death-promoting factor in mitotic and post-mitotic G1 tetraploidy checkpoints during senescence-induced programmed cell death. However, the exact molecular mechanisms of how PARP1 might suppress neosis of senescent cells are not understood and remain to be investigated.

5. PARPS AS SURVIVAL FACTORS

The current literature clearly demonstrates that PARP1 and to a lesser extend PARP2 play important roles in programmed cell death processes. However, growing evidence suggest that PARP1 and PARP2 are multi-faced enzymes involved in both programmed cell death and cell survival processes. The precise molecular and cellular mechanisms regulating this "Jekyll and Hyde" duality of PARP1 in programmed cell death and cell survival are unclear. Under normal physiological conditions and mild progressive damage, PARP1 and PARP2 appear to play a protective role as survival factors while under severe oxidative stress conditions, in which free radicals can damage DNA, PARP1 and PARP2 overactivation results in programmed cell death. Several mechanisms were proposed for their protective roles: (1) promoting cell cycle progression upon cellular stress, (2) direct inhibition of DNA damage-induced apoptotic cell death and (3) regulator of ROS levels (reviewed in (1, 2)).

5.1. PARP1 is essential for cell cycle progression of highly proliferating cells upon cellular stress.

Experimental data have demonstrated that all three different *Parp1* knockout mice as well as mice carrying a targeted disruption of the *Parp2* gene are hypersensitive to whole body γ-irradiation (10, 11, 15, 401, 402). Remarkably, *Parp1* (-/-)/*Parp2* (-/-) double knockout mice are not viable and die at the onset of gastrulation due to massive cell death, suggesting that PARP1 and PARP2 are both essential during early embryogenesis (14). Both *Parp1* and *Parp2* knockout mice showed gastrointestinal failure due to massive cell death and hemorrhage in villi of the small intestine and systemic dehydration, suggesting that the absence of PARP1 and PARP2 could promote the gastrointestinal lethality (GI) syndrome (10, 11, 15). Whole body γ-irradiation preferentially damages endothelial cells of the gut microvasculature in the gastrointestinal tract

(403-406). Intestinal epithelium is one of the most proliferative tissues in the mammalian body and its stem cells show extreme sensitivity to low-level genotoxic stress (403-406). Several studies implies a two-compartment model for the irradiation-induced death of intestinal cells: endothelial cells in the gut microvasculature die first, followed by epithelial stem cells that depend on the endothelial cell support (405-410). This is consistent with a two-compartment model for normal growth of tissue, which seems to depend on the prior expansion of endothelial cells and angiogenesis. Expansion of the endothelial cell population in the microvasculature of several tissues is required before these tissues can grow, expand or regenerate. PARP1 has been therefore suggested to act as a survival factor for intestinal stem cells *in vivo* at low DNA damage levels (10). These observations/hypothesis are supported by *ex vivo* and *in vitro* studies, which clearly demonstrated that deletion of the *Parp1* gene drastically increases the sensitivity of highly proliferative cells like splenocytes and bone marrow cells to apoptosis induced by mono-functional alkylating agents or γ-irradiation (reviewed in (7, 163, 301, 304). Remarkably, susceptibility to γ-irradiation is not significantly increased *ex vivo* in various normal proliferating cell types derived from *Parp1* (-/-) mice when compared with wild type control cells (reviewed in (7, 163, 301, 304)). Thus PARP1 appears to play a crucial role in the regulation of cell cycle progression of highly proliferating cells (10). This would be consistent with the observation that PARP1 expression levels strongly correlate with cell proliferation ((411-413) and reviewed in (7, 10, 163, 301, 304)). Moreover, pharmacological and genetic studies have been recently demonstrated that inhibition of PARPs or complete absence of PARP1 reduces angiogenesis induced by growth factors, such as VEGF and FGF, in *ex vivo* and *in vivo* models (414-416, 499). The observed effects derived from a decrease of endothelial cell migration, proliferations and tube formation (414-416).

The exact molecular mechanisms are not yet clear but a functional role of PARP1 as transcriptional coactivator of genes required for cell cycle progression under cellular stress was suggested (7). Indeed, a recent study demonstrated that the expression of genes required for cell cycle progression or mitosis, DNA replication or chromosome assembly are down-regulated in immortalized *Parp1* (-/-) fibroblasts (386). For instance, microarray hybridization, immunoblot analysis revealed a reduction in expression of $p55^{CDC}$ (CDC20), cyclins A and B1 in immortalized *Parp1* (-/-) fibroblasts when compared with wild-type cells under the tested conditions (386). PARP1 was suggested to serve as coactivator for the transcription factor E2F1 and c-Myc (386, 417). Moreover, two recent studies identified PARP1 as a critical coactivator of HIF-1-dependent gene expression and regulation of cell death in both neoplastic and ischemic conditions (418, 499). PARP1 appears to regulate the expression of critical HIF-1 target genes expression of genes such as *GLUT-1, CAIX* and *VEGF*. Remarkably, for at least a subset of PARP1-dependent HIF-1 target genes, the enzymatic activity of PARP1 was necessary for full activation (499).

5.2. PARP1 and PARP2 are essential for repression of DNA damage induced apoptotic cell death.

Several genetic studies strongly suggested that PARP1 and PARP2 might participate as a component of molecular complexes with other molecules involved in genome surveillance, such as Ku70/86, BRCA1/2 or ATM ((419, 420) and reviewed in (1, 162)). Human Ku is an abundant heterodimeric nuclear protein, consisting of 70- and 86-kDa tightly associated DNA binding subunits, which is together with the DNA-dependent protein kinase involved in DNA repair mediated by nonhomologous end-joining (NHEJ) (reviewed in (1)). Recent studies have revealed that PARP1 can interact with Ku70, Ku86 and ATM *in vitro* ((421-424) and reviewed in (1)). The tumor suppressor genes BRCA1 and BRCA2 appear to be required for DNA repair processes mediated by homologous recombination (HR) (reviewed in (1)). Indeed, haploid loss of *Parp1* is sufficient to induce lethality of *Brca1*-deficient cells. Haploinsufficiency of *Parp1* in *Brca1 (mut/mut)* embryos dramatically accelerates BRCA1-associated chromosome aberrations, centrosome amplification, and telomere shortening, leading to apoptosis and embryonic lethality caused by *Brca1* deficiency (420). Moreover, homozygous disruption of both the *Parp1* and *Ku80* genes cause embryonic lethality and display an increased level of apoptosis around the gastrulation stage (425, 426). Mice lacking *Ku80* are viable but growth retarded and show deficiency in V (D)J recombination. The early embryonic lethality observed in *Parp1*/*Ku80* double-knockout mice was explained by an increase in DNA damage induced cell death (425, 426). Remarkably, the phenotype of the *Parp1*/*Ku80* double-knockout mouse resembles the phenotype of *Parp1*/*Atm* double-knockout mice (162). A similar function has been observed for PARP2 (reviewed in (1)). A recent report provided evidence that in the absence of PARP2 but not of PARP1, the survival of DP thymocytes undergoing TCR-alpha recombination is affected despite normal amounts of Bcl-xL (13). Thus, PARP2 has been suggested to be an important mediator of T-cell survival during thymopoiesis by preventing the activation of DNA damage-dependent apoptotic response during the multiple rounds of TCR-alpha rearrangements preceding a positively selected TCR (13).

The exact molecular mechanism underlying these observations remains to be elucidated. Absence of PARP2 leads to transcriptional upregulation of the pro-apoptotic proteins Noxa and Puma DP thymocytes (13). Thus, both PARP1 and PARP2 were proposed to participate as a component of transcriptional corepressor/coactivator complexes in transcriptional DNA damage response pathways (1, 2). The proposed function of DNAPK-Ku70/86-PARP1 coactivator/corepressor complexes in genomic stability processes is supported by biochemical evidence. PARP1 was identified as a regulated promoter-specific exchange factor and component of Ku70/86-DNAPK/Rad50/topoisomerase-I and-II-containing complexes ((421-424) and reviewed in (1)). Recent studies found that the corepressor-silencing mediator for retinoid and thyroid hormone receptor (SMRT) can associate with a DNA-PK/Ku70/Ku86/PARP1 complex (421-424). The

SMRT/HDAC-3 complex was required for the transcriptional repressive property of the Ku70 subunit and for cellular recovery from DNA DSBs induced by ionizing radiation or DNA damage-inducing drugs (421-424). Unliganded thyroid hormone receptor (TR) can function as a transcriptional repressor of specific cellular target genes by acting in concert with a corepressor complex harboring histone deacetylase (HDAC) activity (421-424). Thus, it was proposed that DNA-PK promotes the establishment of transcriptionally repressive chromatin at TR target promoters by enhancing the HDAC activity of the TR-bound SMRT/DNA-PK/Ku70/86/PARP1 corepressor complex in response to DNA DSBs ((421-424) and reviewed in (1, 2))

5.3. PARP1 and PARP2 serve as survival factors for neuronal cells by modulating ROS levels

Recent studies suggested a dual function of PARP1 and PARP2 in neuronal cell death pathways (12, 427). Inactivation of PARP1 activity in primary cortical neurons by pharmacological inhibition or by siRNA can either enhance or prevent apoptotic death, depending on the degree of oxidative damage (427). PARP1 appears to protect neurons against apoptosis induced by mild oxidative stress (427, 428). There is preliminary evidence that PARP2 has also differential effects on cell survival depending on the experimental model and mode of cell death (12). PARP2 appears to act as an executioner of cell death pathways in focal cerebral ischemia with energy failure and a largely necrotic pathology, while it could also function as a survival factor after global ischemia with a delayed apoptosis-like cell death (12). However the molecular mechanism by which PARP1 and PARP2 provides protection against neuronal cell death is not known (12). Surprisingly two recent studies using *Parp1* knockout mice and *Parp2*-deficient plants lines of *Arabidopsis thaliana* and oilseed rape (*Brassica napus*) indicate, that both PARP1 and PARP2, may play a role in ROS homeostasis, probably in a antagonistic manner (428, 429). Remarkably, adult *Parp1* knockout mice were found to exhibit a strong increase in accumulation of ROS levels (25%) and lipid peroxidation in their serum with age compared with wild type mice (428). Moreover, microarray studies revealed that approximately 40% of genes found to be misregulated in *Parp1* knockout mice under normal conditions are known to be influenced by oxidative stress or are known to modify levels of cellular ROS (428). The increased DNA damage induced cell death and genomic instability observed in *Parp1* knockout mice might be a direct result of this increase of ROS (428). On the other hand, *Parp2*-deficient plant lines showed a delayed, reduced or even completely abolished expression of many oxidative stress-related genes, including the superoxide-producing NADPH respiratory burst oxidase homolog C (429). The observed impaired oxidative stress response and decrease of superoxide levels during stress have been suggested to be caused by a more efficient scavenging of ROS in the *Parp2*-deficient plant lines (429). However the exact molecular mechanism is not known. In addition it remains to be investigated whether mammalian PARP2 has a similar function (12).

5.4. ADP-ribose-AMP/cAMP-mediated AMPK / survival signaling

Metabolic stress often leads to a decline of ATP/ADP ratios with a subsequent increase in AMP concentrations (430), which in turn can stimulate the activity of the AMP-dependent kinase (AMPK) (reviewed in (431)). The AMPK cascade has been suggested to act as a cellular energy sensor system, which is conserved in all eukaryotic cells (432, 433). AMPK has been shown to promote cell survival under conditions of growth-factor withdrawal and metabolic stress ((434, 435) and reviewed in (431)). AMPK is an important regulator of diverse cellular pathways in the setting of energetic stress and has been suggested to act as a cellular fuel gauge by promoting ATP generating pathways, including glucose transport, glycolysis, and fatty acid oxidation, while inhibiting energy/ATP-consuming anabolic pathways (431, 434-438). Genetic and pharmacological studies using transgenic mice expressing a kinase dead form of AMPK or 5-aminoimidazole-4-carboxamide ribonucleoside, a chemical activator of AMPK demonstrated that both isoforms AMPKα-1 and AMPKα-2 are responsible for activation of glucose uptake and glycolysis during low-flow ischemia *in vivo*. Both AMPKα-1 and AMPKα-2 play an important protective role in limiting damage and apoptotic activity associated with ischemia/reperfusion in the heart and type 2 diabetes (434, 435). Ischemia and reperfusion have been recently shown to stimulate autophagy in the mouse heart *in vivo* (reviewed in (436)). Autophagy during ischemia appears to be protective, but detrimental during reperfusion (436). Remarkably autophagy induced during myocardial ischemia *in vivo* is strongly dependent on AMPK while reperfusion after ischemia promotes autophagy through AMPK-independent mechanisms (436). Thus, the coordinated action of PARP1, PARG and NUDT5/NUDT9-ADP-ribose pyrophosphatases, which results in an increase in cellular AMP levels, could indeed activate AMPK-dependent survival pathways. In addition, there might also exist a feedback regulation of PARP1 by AMPK. A recent study provided preliminary evidence that AMPK could physically interact with PARP1 and that phosphorylation of PARP1 by AMPK may modulate positively or negatively PARP1 activity (439). Moreover, moderate increased levels could also activate adenylate cyclase/cAMP cAMP-dependent protein kinase pathways that play an essential role in cell survival. Indeed, several studies provided evidence that the breakdown of ATP and ADP ribose can lead to an up to 30-fold increase in cAMP levels during ischemia and inflammation (430, 440). Taken together a moderate increase of free ADP ribose and subsequent AMP levels could eventually activate cAMP- and AMP-dependent protein kinase survival pathways while under high genotoxic stress conditions the detrimental effects of free poly-ADP-ribose polymers, free ADP-ribose and NAD depletion will overcome the beneficial effect of cAMP and AMP.

6. POLY-ADP-RIBOSYLATION/PARP1-MEDIATED SWITCHES BETWEEN PCD AND CELL SURVIVAL

Tissue homeostasis requires a carefully orchestrated balance between cell proliferation, cellular senescence and cell death. Cell death and survival

pathways are tightly regulated through genetic and epigenetic networks (for a detailed overview and discussion of these networks, the reader is referred to the excellent reviews (19, 24, 26, 46, 47, 56, 392, 441)). Accumulating evidence indicates that the metabolic state of the cells mainly determines whether they undergo cell death or cell survival. High ATP levels promote cell survival, while lowering the ATP levels in cells can make them more susceptible to cell death. Several studies have been suggested that the enzymatic activity of PARP1 is also regulated by the metabolic state of the cell. Physiologic concentrations of ATP (1 to 10 mM (442)) can inhibit the auto-poly-ADP-ribosylation activity of PARP1 (443) (about 3- to 5-fold over a physiological range of ATP concentrations) *in vitro* using purified proteins and in isolated nuclei. Interestingly, nearly complete inhibition of PARP1 enzymatic activity is only observed at higher concentrations of ATP (6 to 10 mM (443)). In contrast, the transfer of poly-ADP-ribose polymers to histones is only slightly affected by ATP, indicating that the massive auto-ADP-ribosylation but not trans-ADP-ribosylation activity of PARP1 might be suppressed in bioenergetically intact cells (413, 444). The inhibitory site of ATP on PARP1 was identified as arginine 34 in the first zinc finger of the DNA binding domain of PARP1 (444). In addition, these studies also demonstrated that ATP could directly activate the enzymatic activity of PARG in vitro using purified proteins and in isolated cell nuclei (443, 444). Thus under normal physiological condition with high ATP levels, the reduced PARP1 activity may promote cell survival and cellular proliferation of bioenergetically intact cells. In contrast, under stress conditions and lower ATP levels in cells the enzymatic activity of PARP1 could increase and promote programmed cell death processes. Whether this proposed mechanism will exist *in vivo* remains to be investigated.

6.1. A PARP1/poly-ADP-ribosylation-mediated switch between different PCD pathways?

Co-activation and crosstalk of different types of PCD pathways have been observed in several systems. However, the molecular mechanisms have not been well characterized. It has been suggested that the type and exposure time of the initiating stimulus and the metabolic status/ "history" of the cell mainly influence the switch between apoptotic, macroautophagocytotic and programmed-necrotic cell death processes (reviewed in (20, 24, 25, 122)). Decreases in cellular level of ATP can induce a switch in the death mode apoptotic to necrotic cell death. High levels of ATP are crucial for the enzymatic activities of caspases and optimal activation of caspase-mediated execution of apoptosis (reviewed in (20, 24, 25, 122)). Indeed, inhibition of the caspase cascade with zVAD-fmk has been shown to induce a switch from apoptosis to programmed-necrotic cell death (reviewed in (122)). In addition, the intracellular level of ROS could also be major factor directly related to the switch mechanism from apoptosis to programmed-necrotic cell death (445-448). Nitric oxide, which has been shown to inhibit caspases *in vitro* by covalently modifying the prosthetic cysteine group, can induce a switch from apoptosis to programmed-necrotic cell death *in vivo (*449-451). The regulatory mechanism underlying the switch from apoptosis to macroautophagocytotic cell death or between programmed-necrotic cell death and macroautophagocytotic cell death are far more complicated and not yet well understood (reviewed in (20, 24, 25, 122)).

The role of PARP1 in these crosstalk and switch processes is only poorly understood. It is not yet clear whether PARP1 plays an active role in these switch processes or is just a target downstream of these regulatory events. Preliminary data suggest both mechanisms may exist *in vivo.* Pretreatment of cells upon with the PARP inhibitors can resulted in a switch from necrosis to apoptosis in cells depending on the stimuli and cell types (reviewed in (8, 120, 121, 184, 203, 204)). At least two molecular mechanisms underlying this PARP-mediated switch have been proposed: (1) Inhibition of PARP1 could retain NAD^+ and ATP levels required for apoptosis. (2) Rapid intracellular acidification induced by alkylating DNA damage has been shown to be strongly decreased in the presence of PARP inhibitors or in *Parp1* (-/-) fibroblasts, indicating that PARP1 activation and poly-ADP-ribose production is the major cause of acidification (452). Intracellular acidification has been suggested to suppress apoptosis while permitting necrotic death of cells with extensively damaged DNA (452). On the other hand, inhibition of the caspase cascade with zVAD-fmk switched the apoptotic response to necrotic cell death, accompanied by a strong activation of PARP1 activity (307, 453-455). Prior treatment of cells with broad range PARP inhibitors preserved intracellular ATP levels and prevented the cells from undergoing necrotic cell death (307, 453-455). These findings indicate that PARP1 acts downstream of these regulatory events as an executioner enzyme of programmed-necrotic cell death process.

6.2. A poly-ADP-ribose code for Cell death and Cell Survival

Given the complexity of poly-ADP-ribose structures observed *in vitro* and the existence of distinct PARP enzymes *in vivo*, it has been suggested that most of these poly-ADP-ribose structures may also exist *in vivo* and vary depending on the cellular context and stimuli (reviewed in (2)). In analogy to the "glyco code" hypothesis of the highly diverse oligosaccharide moieties of glycoproteins, glycolipids, proteoglycans, and polysaccharides, a putative "poly-ADP-ribose code" has been recently proposed to exist *in vivo* and to dictate the outcome of distinct poly-ADP-ribose signaling pathways to reflect the different signaling functions of PARP family members (2). Distinct types of free poly-ADP-ribose polymers could regulate stress-dependent signaling processes *in vivo* and could recruit or regulate the activities of pro- or anti-apoptotic/necrotic signaling factors (2). Interestingly, poly-ADP-ribose polymer toxicity appears to be length-, structure- and dose-dependent (164). High-molecular-weight poly-ADP-ribose polymers resulted in increased programmed-necrotic cell death with polymers >60 ADP-ribose units inducing >80% cell death while polymers of 16 and 30 ADP-ribose residues induce only a small amount of cell death, at an equivalent poly-ADP-ribose polymer concentration (164). It remains to be elucidated whether poly-ADP-ribose polymers synthesized

by the other members of the PARP family, such as PARP2, may also activate the AIF-mediated necrotic pathway. Finally the specific enzymatic activities of distinct PARG isoforms and endogenous cofactors regulating the specific branching activities of different poly-ADP-ribose polymerases *in vivo* could also determine whether programmed cell death or cell survival pathways are activated.

6.3. Gender effects

Surprisingly, recent studies provided preliminary evidence that the role of PARP1 in neuronal cell death and survival appears to be gender specific, at least in mice. Protective effects of *Parp1* gene disruption on neuronal cell death are only observed in males ((456, 457) and reviewed in (458)). In contrast, PARP1 appears even to protect tissue damage in female in systemic inflammatory response and upon stroke (456, 457). Pharmacological inhibition of PARP even appears to result in a worsening of the outcome of stroke and endotoxin-treatment in the female animals (456). In addition, female 'tolerance' is also strongly mediated through nNOS (457). Loss of either of these enzymes strongly enhances infarction in female animals (456, 457). A similar observation was made for the susceptibility to NMDA toxicity and focal ischemia. However the molecular mechanisms underlying this observed phenomena is not known (457). Whether activation of these enzymes directly protects against cell death or if the observed protection is indirect, remains to be elucidated. Several recent studies indicated that female sex hormones might be responsible for these observations (459-461), at least in shock- or inflammation-related studies: *Parp1* gene disruption resulted in reversal of the neuroprotective activity by the female sex steroid, 17beta estradiol ((462) and reviewed in (458)). In addition, pretreatment of mice with 17beta-estradiol appears to prevent endotoxin-induced hepatic injury and reduces poly-ADP-ribosylation *in vivo* in male but not female mice (457, 462). Thus, 17beta estradiol has been suggested to be partially responsible for this gender difference (457). Other reports even observed major differences between male and female neurons grown separately in cell culture (463). Moreover differences in the degree of PARP1 activation were observed between cells incubated in male versus female serum (457, 462). However, 17beta estradiol does not directly inhibit the enzymatic activity of recombinant PARP1 *in vitro* (462). An additional mode of action might be the antioxidant property of the female sex hormones, which can exert cytoprotective effects, even at very low concentrations. Thus, further investigations are required for defining the exact molecular mechanism responsible for the observed gender differences.

6.4. Crosstalk of ADP-Ribosylation and Other NAD+-Dependent Reactions in cell death and survival pathways

Cell survival upon genotoxic stress is determined by a counterbalance of pro- and anti-death factors. It has been recently proposed that PARPs and other NAD^+ consuming ADP-ribosylating enzymes could either directly regulate each other in a positive and/or negative manner through trans-ADP-ribosylation or indirectly through modulation of the NAD^+ levels and/or ADP-ribose metabolites (2, 16). Indeed, *Parp1* (-/-)/*Parp2* (-/-) double mutant mice are not viable and die at the onset of gastrulation (14). PARP5/tankyrase-1 appears to protect cells from genotoxin induced cell death and injury through inhibition of PARP1-mediated NAD^+ depletion and cell death (288) while PARP6/tankyrase-2 causes rapid necrotic cell death when highly over-expressed in human immortalized cells (286). Thus, PARP1, PARP2, PARP5/tankyrase-1 and PARP6/tankyrase-2 may modulate each other in an antagonistic or synergistic manner during cell death and survival processes. It has been recently suggested that NAD^+/nicotinamide levels could serve as converging points for interactions of PARP/poly-ADP-ribosylation reactions and SIRT-dependent pathways (2, 16, 464, 465). Crosstalk of SIRT1 and poly-ADP-ribosylation reactions has been suggested to provide the balance between cell survival and cell death, longevity, and senescence (2, 16). Sirtuins (SIRTs) encompass a family of NAD^+-depended deacetylases and mono-ADP-ribosyltransferases that have been implicated in mediating cell survival and growth, longevity, and genome stability (reviewed in (466)). For example, *Sirt1*-deficient cells exhibited p53 hyperacetylation after DNA damage and increased ionizing radiation-induced thymocyte apoptosis (467). Acetylation of p53 at lysine residues K320 and K373 was shown to be essential for the upregulation of p53-dependent pro-apoptotic genes (467-469). Moreover, SIRT1 deacetylates the DNA repair factor Ku70, causing it to sequester the pro-apoptotic factor Bax from mitochondria and thereby inhibiting stress-induced cell death (470, 471). Inhibition of SIRT1 enhances acetylation of Ku70 and induces release of Bax, allowing it to translocate to mitochondria and trigger cytochrome c release, leading to caspase-dependent cell death (470, 471). Thus, different poly-ADP-ribosylation reactions could modulate the NAD^+-dependent deacetylation of proteins by SIRTs via the NAD^+/nicotinamide connection. The decline of NAD^+ levels and the rise of nicotinamide on activation of poly-ADP-ribosylation reactions may downregulate the activity of SIRTs since the enzymatic activity of SIRT1 is dependent on high concentrations of NAD^+ and inhibited by low physiological levels of nicotinamide ($IC_{50} < 50$ μM) (reviewed in (2, 16)). This hypothesis is indeed, at least partially supported by three independent studies, which provided preliminary evidence for a functional link between SIRT1 and PARP1 (472-474). The first two studies provided preliminary evidence that increased activity of PARP1 upon genotoxic stress, associated with depletion of cellular NAD^+ levels may reduce SIRT1 deacetylase activity in myocyte cells, contributing to myocyte cell death during heart failure (473, 474). Remarkably, PARP1-mediated myocyte cell death is protected by replenishing cellular NAD^+ levels as well as by activation of SIRT1 (473, 474). Conversely, the third report showed a drastic increase in poly-ADP-ribosylation in immortalized *Sirt1*-deficient cells upon DNA damage (472). The unbalanced regulation of PARP1 in the absence of SIRT1 resulted in AIF-mediated cell death (472). These findings provide the first evidence that the two NAD^+-dependent enzymes SIRT1 and PARP1 might modulates each other's enzymatic activity under pathophysiological

conditions. However, the exact molecular mechanisms and hierarchy of regulation underlying this putative antagonistic crosstalk remain to be elucidated. There is so far no genetic study that could support these *in vitro* observations. Indeed, one cannot rule out the possibility that SIRT1 and PARPs are even acting synergistically under normal physiological conditions but antagonistically under pathophysiological conditions. If such a scenario would be true, these effects will be only detectable in conditional double knockout mice for *Sirt1* and *Parp1* or *Sirt1* and *Parp2*.

A recent report demonstrated that during the exposure of activated T cells to NAD^+, the ADP-ribosyl cyclase/cyclic-ADP-ribose-hydrolase CD38 is modified by ecto-mono-ADP-ribosyltransferases (e-mART)-specific for arginine residues (475). E-mART-mediated mono-ADP-ribosylation of CD38 on arginine residues inactivates both cyclase and hydrolase activities and causes a decrease in intracellular cyclic-ADP-ribose and a subsequent decrease in Ca^{2+} influx, resulting in apoptosis of the activated T-cells (475)**.** On the other hand, PARPs might be indirectly regulated by the ADP-ribosyl cyclase/cyclic-ADP-ribose hydrolase CD38 through the modulation of NAD^+ levels under normal physiological conditions. CD38 seems to be a key regulator of cellular NAD^+ levels under normal physiological conditions (476), while PARP1 appears to be the key factor determining intracellular NAD^+ levels under genotoxic stress conditions (1, 2). Indeed, several studies demonstrated that tissue levels of NAD^+ in *Cd38*-deficient mice are up to 10-fold higher than that in wild-type animals (476-479). Moreover, the endogenous activity of SIRT1 was several-fold higher in nuclear extracts from *Cd38* knockout mice when compared to wild type nuclear extracts (477-479), strongly indicating that the non-membranous nuclear localized CD38 isoform (480, 481), linked to structural nuclear proteins of the inner nuclear matrix is a major regulator of cellular/nuclear NAD^+ level, and SIRT1 activity (477-479). It will be interesting to investigate using genetic approaches, how these three crucial players PARP1, SIRT1 and CD38 could act together. It would be not surprising if they may even function together antagonistically as well as synergistically, depending on the cell type, metabolic state and stimuli.

Taken together, PARP1, SIRT1 and nuclear CD38-dependent pathways may provide a unified network for multicellular eukaryotes to deal with nutritional supply and environmental stress (1, 2). The net result, such as survival or death, proliferation or terminal differentiation, will depend on the equilibrium between specific pathways and the local cellular environment (2).

7. CONCLUSIONS AND FUTURE PERSPECTIVES

Since N. Berger proposed the „PARP-suicide" model in 1983, a tremendous amount of work has been done to elucidate the molecular mechanism how PARP1-mediated ADP-ribosylation reactions can promote programmed cell death. The current literature indicates that PARP1-mediated poly-ADP-ribosylation reactions play a predominant role in programmed-necrotic cell death processes. Moreover, new studies clearly demonstrate that energy collapse is not the sole mechanism by which PARP1 contributes to programmed-necrotic cell death. A nucleus-to-mitochondria death-signaling cascade between PARP1 and AIF has been established. The contribution of PARP1 to programmed cell death may fulfill its role as a guardian of the genome under controlled non-pathophysiological conditions. PARP1-mediated poly-ADP-ribosylation reactions has been suggested to protect the animal from the development of tumors by switching the dual functions of AIF towards programmed cell death, thereby limiting the development of cancer (2). The PARP/poly-ADP-ribosylation system and AIF may function together as a sensor that integrates, in a "yin/yang"-like fashion, information from the mitochondria and nucleus on the metabolic and oxidative states of cells, acting as a double-edged sword in ROS-dependent death/survival pathways (2). On the other hand, uncontrolled PARP1-mediated poly-ADP-ribosylation reactions can also result in massive necrotic cell death and tissue damage, which in turn often lead to severe inflammatory or neurodegenerative disorders. Overactivation of PARP1 has been implicated in the pathogenesis of Parkinson and Alzheimer diseases, stroke, myocardial infarction, heart failure, diabetes mellitus, circulatory shock, colitis and allergy. Growing evidence suggest that both PARP1 and PARP2 are also involved in cell survival pathways. PARP1 appears to act as a survival factor for stem cells and neuronal cell types at low levels of oxidative stress and DNA damage *in vivo*.

However, despite the tremendous progress that has been made in the last decade, several crucial questions remain: How are these distinct roles of poly-ADP-ribosylation between programmed cell death and cell survival regulated? Is the outcome of poly-ADP-ribosylation reactions between life and death determined by the proposed "poly-ADP-ribose code" or, alternatively, by a shift in the homeostasis of the poly-ADP-ribosylation metabolism? Considering that even small changes in poly-ADP-ribose levels might be physiologically meaningful such changes might disturb untargeted pathways. How many different structural types of poly-ADP-ribose do exist *in vivo* and what are their exact structures? Moreover, if the existence of such a poly-ADP-ribose code can be confirmed *in vivo*, it will be important to identify the putative cofactors regulating elongation and branching activities of PARPs *in vivo*.

Another aspect is the nucleus-to-mitochondria death-signaling cascade. How do PARP1-mediated poly-ADP-ribosylation reactions in the nucleus trigger events in mitochondria? It remains unclear whether ROS-induced PARP1 activation and depletion of cellular NAD^+, may also indirectly cause MTP opening and AIF release via inhibition of glycolysis and ATP depletion. Despite the growing evidence, which suggests that poly-ADP-ribose may directly serve as death signal, it is still elusive how such highly negative charged and branched poly-ADP-ribose polymers could shuttle from the nucleus to the cytoplasm/mitochondria. Thus the role of NAD^+ depletion and poly-ADP-ribose as death signal in AIF shuttling needs to be carefully re-evaluated.

An even more crucial and conflicting issue is the a recent hypothesis that highly negative charged and branched poly-ADP-ribose polymers could be covalently attached to p53 by PARP1 and inhibit the interaction between p53 and the nuclear export receptor CRM1, which may in turn result in nuclear accumulation of p53 and increased p53-dependent apoptosis. Indeed, such a scenario would have fare reaching negative consequences for the application of PARP inhibitors in cancer treatment, because both programmed-necrotic cell death and apoptosis, would be blocked by PARP inhibitors. Thus, the data concerning the putative covalent poly-ADP-ribosylation of p53 and its functional consequences have to be re-evaluate *in vivo* using mass spectrometry approaches combined with genetic studies (i.e. knock-in of modification mutants of p53 and/or enzymatic inactive mutants of PARP1)

Several recent studies provided preliminary evidence that a crosstalk between different programmed cell-death pathways may exist *in vivo* (reviewed in (25, 122, 482-485). Future investigations of the interdependency of these programmed cell-death pathways will certainly answer the question whether PARP1 or another PARP family member might be involved in such a crosstalk.

Regarding the clinical application of pharmaceutical PARP inhibitors, the most important questions remain: How important is the presence of PARP1 for cell survival pathways? And what is the exact molecular mechanism by which PARP1 mediated poly-ADP-ribosylation reactions may lead to cell survival?

An aspect that might be underestimated and certainly needs more attention in future studies is the role of other PARPs in programmed cell death and cell survival pathways. What is the exact molecular mechanism underlying the observed crosstalk between PARP1 and PARP2 in cell survival? Do PARP2 mediated poly-ADP-ribosylation reactions also contribute to programmed cell death processes or is it just the physical presence of PARP2, i.e. through the formation of PARP1/PARP2 hetero-oligomers, which may promote or modulate PARP1-dependent poly-ADP-ribosylation reactions?

A may be rather obscure but just as important observation are mitochondria-associated poly-ADP-ribosylation reactions in mammalian cells. Although, mitochondrial poly-ADP-ribosylation has been reported repeatedly, the responsible PARP isoform could not be identified. The discovery of two mitochondria-associated PARG isoforms capable of preventing poly-ADP-ribose accumulation in close proximity to, or possibly within mitochondria, led to the hypothesis that full mitochondria-associated poly-ADP-ribosylation cycles may exist in mammalian cells. Genetic studies indicate that these two mitochondria-associated PARG isoforms could block AIF release from mitochondria and eventually cell death, thus acting as survival factors. On the other hand it has been suggested that the novel PARG isoforms could contribute to oxidative stress induced cell death through the release of free ADPR from the mitochondria and specific activation of the cell membrane Ca^{2+} channel TRPM2. Therefore, the existence of an entirely mitochondrial poly-ADP-ribose metabolism is of great interest. Selective knockout and knock-in studies will be required to elucidate the exact roles of the mitochondria localized ARH3 and PARG isoforms in programmed cell death and cell survival pathways.

An additional interesting issue, which needs further careful investigations, is the proposed crosstalk of different NAD^+-metabolizing enzymes in cell death and cell survival processes. Could NAD^+/nicotinamide levels serve as a converging point for interactions of poly-ADP-ribosylation reactions and other NAD^+-metabolizing pathways? Since recent studies claimed that SIRT1-dependent mechanisms could be modulated by poly-ADP-ribosylation and *vice versa*, it will be important to test whether conditional double knockouts of *Parp1/Sirt1* or *Parp2/Sirt1* could rescue the lethality phenotype observed with single knockout mice for the *Sirt1* gene.

Taken together a powerful method to establish the physiological relevance of the proposed functions PARPs and PARGs *in vivo* might be the generation of knock-in mice expressing enzymatic mutants of PARP family members, PARG isoforms as well as of ARH3. Genetic studies using combinations of double knockouts and knock-in mice expressing enzymatic dead mutants of different PARPs are needed to elucidate potential roles of poly-ADP-ribosylation in programmed cell death and cell survival processes associated with aging and cancer *in vivo*. Such approaches should also clarify whether mono-ADP-ribosylation reactions, mediated by Pl-mARTs or SIRTs, might be involved in these processes and regulate PARP1-mediated poly-ADP-ribosylation reactions. Targeted genetic approaches will also provide suitable *in vivo* platforms for the development of highly selective isoform-specific pharmaceutical inhibitors of PARPs and PARGs.

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