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Abstract

Programmed cell death signaling networks are frequently activated to coordinate the process of cell differentiation, and a variety of apoptotic events can mediate the process. This can include the ligation of death receptors, the activation of downstream caspases, and the induction of chromatin fragmentation, and all of these events can occur without downstream induction of death. Importantly, regulators of programmed cell death also have established roles in mediating differentiation. This review will provide an overview of apoptosis and its regulation by Inhibitors of Apoptosis (IAPs) and Bcl-2 family members. It will then outline the cross-talk between NF-κB and apoptotic signaling in the regulation of apoptosis before discussing the function of these regulators in the control of cell differentiation. It will end on a discussion of how a DNA damage-directed, cell cycle-dependent differentiation program may be controlled across multiple passages through cell cycle, and will assert that the failure to properly differentiate is the underlying cause of cancer.

Keywords: Apoptosis; Programmed cell death; Inflammation; NF-κB signaling; Cell differentiation; DNA damage; Cell Metabolism; Cancer

Introduction

Since the discovery of apoptosis over 40 years ago, the signaling pathways that mediate this form of programmed cell death (PCD) have been extensively characterized. **(Kaczanowski, 2016)** These pathways can be activated by a variety of stimuli, such as the ligation of cell-surface death receptors, or following DNA damage, and this can lead to the coordinated destruction of the cell. **(Elmore, 2007)** Regulation of cell death frequently falls on Nuclear Factor kappa B (NF-κB) transcription factors, which are critical mediators of inflammation; however, as apoptosis is a developmentally regulated process, cell death signaling can reciprocally regulate proliferation and differentiation, as well. **(Brenner et al., 2015, Kaczanowski, 2016, Fernando & Megeney, 2007)**

A growing body of evidence suggests that apoptotic regulators mediate the process of cell differentiation, and it has even been suggested that caspase-dependent apoptosis is an extreme form of the differentiation program. **(Fernando & Megeney, 2007)** As most cell types are required to pass through cell cycle before differentiating, coordinating proliferative signaling with apoptotic signaling is likely critical to mediating differentiation. This paper will provide an overview of intrinsic and extrinsic apoptotic signaling, and it will discuss the regulation of these pathways in the control of cell death. It will then highlight the coordination between apoptotic and NF-κB signaling networks and discuss how cross-talk between these systems regulates cell survival vs. cell death. Then it will outline how apoptotic signaling machinery can be used by cells to coordinate a DNA damage-dependent, cell differentiation program, and briefly discuss its implications for cancer

Apoptosis

Apoptosis is a form of programmed cell death that is executed by a family of cysteine-aspartate proteases known as caspases. Caspases are normally present in the cell as inactive procaspases, but upon induction of apoptosis, procaspases undergo proximity-induced dimerization, and autocatalytic cleavage to their active caspase forms. **(Muzio et al., 1998, Boatright et al., 2003)** Caspases are often recruited to multi-protein complexes called caspase activation platforms, such as the Death-inducing Signaling Complex (DISC), or the Apoptosome, to accelerate caspase activations. **(Kischkel et al., 1995, Zou et al., 1999, Cain et al., 1999)**

Apoptotic caspases may be classified as either initiator or executioner caspases depending on their placement in apoptotic pathways. Caspases 8 and 9 are initiator caspases, and they are the first caspases activated during extrinsic, and intrinsic, apoptosis, respectively. Activated initiator caspases cleave and activate executioner procaspases, such as procaspases 3, 6, and 7. **(Cohen, 1997, Elmore, 2007)** These executioner caspases cleave additional caspases, as well as a variety of other targets, to initiate an irreversible cascade that culminates in the coordinated dismantling of the cell. The dismantled intracellular components then get packaged into apoptotic bodies for ingestion by local macrophages. **(Elmore, 2007, Fischer et al., 2003)** Importantly, while apoptosis is the best characterized form of programmed cell death, a variety of other biochemical pathways exist that can also lead to cell death. As dysregulation of apoptosis can lead to activation of these alternate pathways, some of them will be discussed below.

Extrinsic Apoptosis

Extrinsic apoptosis is initiated by the activation of members of Tumor Necrosis Factor (TNF) Superfamily, which include Fas, TNFR1/2, and TRIALR1/2 (DR4/5), among others. **(Locksley et al., 2001)** Fas is the stereotypical death receptor associated with extrinsic apoptosis,

and its ligation stimulates death receptor trimerization and results in the assembly of adaptor proteins at the receptor's cytoplasmic face. Fas-associated protein with death domain (FADD) is the predominant adaptor recruited to Fas, while TNFR1SF1A-associated via death domain (TRADD) is the main adaptor of TNFR1. **(Kischkel et al., 1995, Hsu et al., 1995)** FADD assembly results in the formation of a multi-protein DISC that recruits procaspase 8 to stimulate its cleavage and activation. **(Kischkel et al., 1995)** Distinct adaptor proteins can be recruited to different death receptors to change downstream protein activations. For example, in addition to FADD, both Daxx and Yes can to be recruited to Fas. **(Yang et al., 1997, Corsini et al. 2009)**

Following ligation of death receptors and formation of a DISC, the initiator procaspase 8 gets recruited and processed to active caspase 8, depicted in the upper left region (ULR) of Figure 1. Activated caspase 8, like caspase 9, is able to cleave procaspase 3 and a variety of intracellular targets to promote apoptosis**. (Fischer et al., 2003)** One notable caspase 8 target in extrinsic apoptosis is the pro-apoptotic Bcl-2 family member Bid. Bid is cleaved by caspase 8 to tBid (truncated), which enables tBid to translocate to the mitochondria to associates with pro-apoptotic Bcl-2 family members to promote the release of proteins from the mitochondrial inner membrane space (IMS). **(H. Li et al., 1998)** The release of IMS proteins is usually accompanied by a loss of mitochondrial membrane integrity, and the disruption of the proton gradient that maintains the Electron Transport Chain (ETC). Some of the key IMS proteins that are released to promote apoptosis include Cytochrome c, Endonuclease G (Endo G), Apoptosis-inducing Factor (AIF), and SMAC/Diablo, and their contributions will be discussed further below. **(L. Y. Li et al., 2001, Susin et al., 1996, Du et al., 2000)** Importantly, cleavage of Bid enables cross-talk between intrinsic and extrinsic apoptosis, as either pathway can impinge on Bcl-2 family member activity at the mitochondria.

Intrinsic Apoptosis and p53

Intrinsic apoptosis can be initiated by various types of cellular stress. DNA damage, as seen following irradiation or chemical treatment, can initiate intrinsic apoptosis if the lesions are irreparable. Growth factor/cytokine withdraw can also initiate intrinsic apoptosis, as can trophic factor withdraw, depending on the cell type in question**. (Elmore, 2007, Letai, 2006, Kummer et al., 1997)** These conditions lead to the activation of procaspase 9, the apical initiator caspase of intrinsic apoptosis. While caspase 9 can cleave and activate procaspase 3, the release of pro-apoptotic regulators from the mitochondrial IMS space is often required for cell death. These IMS proteins can promote procaspase activations, inhibit anti-apoptotic proteins, and help commit cells to apoptosis. For example, following its liberation from the IMS, cytosolic cytochrome c can bind to Apoptotic Protease Activating Factor-1 (Apaf-1) to promote the assembly of the apoptosome, a potent caspase activation platform that recruits and activates procaspase 9. **(P. Li et al., 1997)** Apoptosome assembly dramatically increases the amount of active caspase 9 in the cell and allows these proteases to accelerate cleavage and activation of downstream procaspases 3 and 7, as well as other family members. Active caspases 3 and 7 then cleave downstream targets to coordinate cellular destruction. **(Rodriguez & Lazebnik, 1999, Srinivasula et al., 1998, Martin & Green, 1995)**

The tumor-suppressor p53 is key regulator of intrinsic apoptosis, and it is activated following wide-ranging apoptotic stimuli, such as DNA damage, to coordinate the cellular response. Notably, p53 transactivates the cyclin-dependent kinase inhibitor (CKI) p21 to halt cell cycle through cyclin-dependent kinase (CDK) inhibition while the cell attempts to normalize the intracellular environment. **(Haupt et al., 2003)** However, if a cell is unable to restore homeostasis, p53 can also promote apoptosis by upregulating a variety of pro-apoptotic proteins,

such as the Bcl-2 family members Bax, Puma and Noxa. **(Haupt et al., 2003, Yu et al., 2003, Oda et al., 2000)** These Bcl-2 proteins can compromise the integrity of the outer mitochondrial membrane (OMM), and facilitate the release of the above-mentioned apoptogenic proteins from the IMS. Notably, p53 can promote death via transcription-independent mechanisms as well, as it can facilitate the translocation of pre-formed death receptors housed in the golgi apparatus to the plasma membrane. **(Bennett et al., 1998)**

Apoptotic DNA/Chromatin Modifications

Apoptosis results in characteristic modifications to the chromatin that facilitate its degradation, and caspase-mediated cleavage events facilitate these changes. **(Ura et al., 2001, Graves et al., 2001, Hu, et al., 2007)** For example, both extrinsic and intrinsic apoptotic stimuli lead to the activation of caspase 3, and this protease cleaves Inhibitor of Caspase-activated DNase (ICAD), which normally exists in a cytoplasmic complex with Caspase-activated DNase (CAD). Cleavage of ICAD by caspase 3 frees CAD to translocate into the nucleus to facilitate apoptotic DNA fragmentation. **(Sakahira et al., 1998)** CAD-mediated fragmentation generates double-strand DNA breaks (DSBs) that come concomitant with incorporation and phosphorylation at S139 of histone variant H2AX, referred to as γ-H2AX, into the DNA. **(Rogakou et al., 2000)** γ-H2AX is a hallmark of DSBs and is an important epigenetic modification involved in coordinating cellular DNA Damage Responses (DDR).

Two additional caspase-activated proteins that mediate apoptotic epigenetic modifications include Mammalian Ste20-like kinase 1 (Mst1) and the non-calcium-dependent Protein Kinase C δ isoform (PKCδ). Caspases cleave Mst1 in the cytosol to induce its nuclear import, while PKC is cleaved by caspase 3 in the nucleus to restrict its export. **(Ura et al., 2001, DeVries-Seimon et al., 2007)** Both Mst1 and PKCδ facilitate histone 2B serine 14 (H2BS14)

phosphorylation, and the caspase-cleaved protein acinus can bind to, and enhance the activity of, both of these kinases. **(Ura et al., 2001, Hu et al., 2007)** The H2BS14ph modification recruits the protein Regulator of Chromatin Condensation 1 (RCC1). **(Wong et al., 2009)** RCC1 is a Ran-GEF that controls nuclear transport by catalyzing the exchange of Ran-GDP for Ran-GTP, and its association with phosphorylated H2BS14 during early apoptosis disrupts this process, and by extension, nuclear transport. Disruption of nuclear transport impacts the subcellular distribution of both Mst1 and PKCδ, and it can even help exclude NF-κB transcription factors from the nucleus. **(Ura et al., 2001, DeVries-Seimon et al., 2007, Wong et al., 2009)** As NF-κB upregulates a variety of anti-apoptotic proteins, nuclear exclusion may potentiate apoptosis. NF-κB will be discussed in more detail below. It is important to note that the H2BS14ph modification requires prior deacetylation at H2BK15, and that the H2BS14ph modification co-localizes with γ-H2AX during apoptosis, which will also be discussed below. **(Ajiro et al., 2010, Solier & Pommier, 2009)**

Another important nuclear protein that regulates apoptosis is Poly (ADP-ribose) Polymerase 1 (PARP1). PARP1 activates following DNA damage and catalyzes the rapid NAD+-dependent addition of Poly-ADP-ribose (PAR) polymers, a process referred to as PARylation, to a variety of proteins, including itself. **(Huletsky et al., 1989, Ogata et al., 1981)** The addition of these highly electronegative PAR polymers to some DNA-binding proteins, such as histones, promotes their dissociation from DNA. **(Mathis & Althaus, 1987)** Conversely, PARylation of some DDR proteins, such as Checkpoint kinase 1 (Chk1), can facilitate recruitment to, and repair of, DNA breaks. **(Min et al., 2013)** PARP1 even promotes NF-κB activation following DNA damage. **(Stilmann et al., 2009, Hottiger, 2015)** During apoptosis, caspase 3 cleaves PARP1 to prevent NAD+/ATP depletion, while the enzyme PAR

Glycohydrolase (PARG) is responsible for degrading the PAR polymers. **(Kim et al., 2005)** Importantly, unrestrained PARP1 activity can lead to parthanatos, a PARP1-mediated form of cell death that does not depend on caspase activity. **(Fatokun, Dawson, & Dawson, 2014)**

AIF is an important apoptogenic protein that facilitates apoptotic chromatin condensation. AIF lacks nuclease activity, but it can may act through DNA-binding proteins to alter chromatin accessibility to various nucleases. **(Vařecha et al., 2009)** For example, AIF binds to the nuclease Macrophage Migration Inhibitory Factor (MIF) to promote PARP1-mediated DNA fragmentation, and an AIF-MIF association in the cytosol even facilitates nuclear accumulation of MIF. **(Wang et al., 2016)** Unlike many other IMS proteins, loss of mitochondrial membrane integrity is not sufficient to stimulate the release of AIF. Its release is frequently, though not exclusively, dependent on PARP1 and the calpain family of proteases. **(Vosler et al., 2009, Cao et al., 2007, Y. Wang et al., 2009)** Activation of PARP1 has been shown to promote Ca^{2+} mobilization, which leads to an accumulation of Ca^{2+} in the IMS. This activates the IMS-localized calpain 1, which cleaves AIF to tAIF, and enables tAIF to translocate into the nucleus. PAR polymers can directly binds to AIF promote this translocation. **(Wang et al., 2011)** Importantly, a fraction of AIF has also been found on the cytosolic face of the OMM in mouse brain cells, and this fraction's translocation into the nucleus can precede that of the IMS-localized AIF. **(Yu et al., 2009)**

Apoptotic Regulatory Families

Bcl-2 Regulation of Mitochondrial Membrane Integrity

Bcl-2 proteins are central regulators of mitochondrial membrane integrity, and they thereby control the mitochondrial amplification loop that accelerates caspase activations during

apoptosis. These proteins are subdivided into pro- and anti-apoptotic family members, and are categorized based of the number of Bcl-2 homology (BH) domains they contain. **(Luna-Vargas & Chipuk, 2016, Breckenridge & Xue, 2004)** The anti-apoptotic members Bcl-2, Bcl-X_L, and Mcl-1 are multi-region members that have four (BH1-BH4) domains, as do the pro-apoptotic members Bax and Bak. Bad, Bid, Bim, Bik, Noxa, and Puma are another subset of pro-apoptotic members that are BH3-only family members. **(Breckenridge & Xue, 2004)** Bcl-2 proteins function by forming heteromers with their family members, as well as other proteins such as p53, to promote or restrict mitochondrial membrane permeability (MMP), and mitochondrial membrane depolarization (MMD). **(Vaseva & Moll, 2009)**

Bcl-2 protein heteromers function largely by associating with Voltage-dependent Anion Channel 1 (VDAC1) at the cytosolic face of the OMM, or by directly opening a channel in the mitochondrial membrane. **(Tsujimoto & Shimizu, 2000, Shamas-Din, Kale, Leber, & Andrews, 2013)** The pro-apoptotic members, such as Bax and Bak, can oligomerize at the OMM to promote the release of IMS-localized proteins, and this often occurs alongside MMD. **(Eskes et al., 2000, Dewson et al., 2009)** Conversely, the anti-apoptotic Bcl-2 proteins can heterodimerize with their pro-apoptotic counterparts to keep them sequestered in the cytoplasm, they can bind VDAC1 to restrict to restrict pro-apoptotic family member associations, or they may bind to pro-apoptotic members that are inserted into the membrane to restrict pore formation. **(Oltval et al., 1993, Yang et al., 1995, Tsujimoto & Shimizu, 2000, Luna-Vargas & Chipuk, 2016)** Interestingly, Hexokinase II, the commonly expressed isoform in cancer, can bind VDAC1 to prevent Bcl-2 family member associations, and likely functions in the regulation of these events. **(Pastorino & Hoek, 2008)**

Inhibitors of Apoptosis

Inhibitors of Apoptosis (IAPs) are a family of proteins that regulate cell death signaling by restricting caspase activity and by promoting NF-κB survival signaling. X-linked Inhibitor of Apoptosis (XIAP), the most potent IAP, is able to bind to and inhibit caspases 3, 7, and 9. **(Deveraux et al., 1997, Deveraux et al., 1998)** Cellular IAP1 (cIAP1) and cIAP2, in contrast, don't directly inhibit caspases, but they can restrict the assembly of caspase activation platforms to limit caspase activity. **(Tenev et al., 2011)** All three of these proteins play important roles in facilitating NF-κB activation, which is predominantly anti-apoptotic, and each can be upregulated by NF-κB transcriptional activity. **(Stehlik et al., 1998)** While there are many IAP family members, this article will focus on cIAP1/2, XIAP, and survivin. **(Deveraux & Reed, 1999)**

XIAP, cIAP1, and cIAP2 each contain three N-terminal baculovirus IAP-repeat (BIR) domains and a C-terminal RING domain. The BIR domains enable these IAPs to interact with processed caspases and other proteins, frequently through interaction with IAP-binding motifs (IBMs), while their RING domains enable them to ubiquitinate a variety of targets, including themselves. **(Chai et al., 2000**, **Hegde et al., 2002, Yigong, 2002, Vaux & Silke, 2005)** IAP ubiquitination events can modify the functions of the target proteins, generate scaffolds to recruit signaling regulators, or direct proteins to the proteasome for degradation. The BIR domains of cIAP1/2 enable them to associate with components of the TNFR1 complex, while their RING domains mediate ubiquitination events that are important for proper NF-κB activation. **(Samuel et al., 2006, Mahoney et al., 2008)** XIAP can also play a role in NF-κB activation through its BIR1 and RING domains, which will be discussed below. **(Galbán & Duckett, 2009)**

Unlike the IAPs discussed above, survivin only contains 1 BIR domain and lacks a RING domain. **(Verdecia et al., 2000)** In response to various cell death stimuli, survivin regulates cell

death via associations with cIAP1/2, XIAP, Smac/DIABLO, and the cellular protein hepatitis B X-interacting protein (HBXIP), among others. **(Dohi et al., 2004, Song et al., 2003, Marusawa et al., 2003)** Survivin's association with XIAP helps stabilize XIAP by restricting its Ub-mediated targeting to the proteasome. (**Dohi et al., 2004)** By binding to HBXIP, survivin can also inhibit procaspase 9 recruitment to Apaf-1 to restrict apoptosome formation and apoptosis. **(Marusawa et al., 2003)**

IAP antagonists

IAP antagonists are a group of proteins that are often localized to the IMS and are released when pro-apoptotic Bcl-2 proteins compromise the OMM. Smac/DIABLO and Omi/Htr2 are two mammalian IAP antagonists released during apoptosis. In the cytosol, Smac binds to either the BIR2 or BIR3 domain of different IAPs and directs their activity. **(Chai et al., 2000)** For example, cytosolic Smac/DIABLO may bind to cIAP1/2 to stimulate their degradation, or it may associate with XIAP to remove XIAP-mediated caspase inhibition. **(Yang & Du, 2004)** Unlike Smac/DIABLO, an alternatively spliced isoform known as Smac3 can bind to XIAP to promote its autoubiquitination and proteosomal degradation. **(Fu et al., 2003)** While XIAP directly inhibits caspases 3, 7, and 9, it can also indirectly promote its own induction via NF-κB signaling, which will be outlined below. **(Deveraux et al., 1997, Deveraux et al., 1998, Jin et al., 2009)** Survivin can also be induced by NF-κB signaling alongside XIAP, and survivin can simultaneously restrict the induction of apoptosis via HBXIP, and bind XIAP to prevent Smac-mediated inhibition of XIAP. **(Wang et al., 2010)** Mitochondrial Survivin can also directly bind to Smac in the IMS to prevent it release in response to apoptotic stimuli. **(Ceballos-Cancino et al., 2007)**

Other IMS-localized proteins are able to promote apoptosis following their release from the mitochondria. For example, Omi/Htr2 is an IAP antagonist with serine protease activity that is released in response to some apoptotic stimuli. Unlike Smac, Omi/Htr2 is able to directly cleave its substrates during apoptosis. **(Suzuki et al., 2001)** Some of these target include IAPs such as cIAP1, cIAP2, and XIAP, but they are not restricted to apoptotic regulators, as Omi/Htr2 also cleaves eukaryotic initiation factor 4GI (eIF-4GI), elongation factor 1-alpha (EF-1α) and various cytoskeletal proteins, among others. **(Vande Walle et al., 2007)** The activity of IAP antagonists removes IAP-mediated inhibition of caspases, shuts down NF-κB signaling, and promotes lethal levels of caspase activity to induce apoptosis.

Nuclear Factor-kappa B and the TNFR1

Nuclear Factor-kappa B (NF-κB) proteins are a group of transcription factors that include RelA (p65), RelB, c-Rel, p105/p50, and p100/p52 in mammalian cells. These proteins are critical mediators of inflammation, but they also regulate proliferation, differentiation, and apoptosis, as well. **(Zhang, Lenardo, & Baltimore, 2017, Zhang et al., 2012**) To mediate this, NF-κB proteins form distinct dimers that preferentially induce different downstream targets. These dimers are normally held in the cytoplasm by Inhibitor of kB (IκB), but following their liberation from this complex, they translocate in the nucleus to induce their respective targets. p65/p50 is the stereotypical dimer that activates classical NF-kB signaling, while RelB/p52 typically activates non-canonical/alternative NF-kB signaling.

NF-kB signaling is intimately linked to apoptotic signaling, and the cross-talk between these pathways enables fine-tuned control of cell death. Classical NF-κB signaling can lead to the induction of cIAP1/2, XIAP, and survivin. **(Stehlik et al., 1998, Wang et al., 2010)** These IAPs operate within a feedback loop with NF-κB whereby they simultaneously promote

maintenance of NF-κB signaling, and restrict cell death. NF-κB transcription factors can also induce Bcl-2 and Bcl-X_L, which restrict pro-apoptotic Bcl-2 family members from compromising the integrity of the OMM, to prevent intrinsic apoptosis. **(Tamatani et al., 1999)** While the majority of evidence supports an anti-apoptotic role of NF-κB signaling, these transcription factors can also unpregulate pro-apoptotic molecules, such as Fas, TRIALR1/2 (DR4/5). **(Liu et al., 2012, Mendoza et al., 2008, Shetty et al., 2005)** After outlining the signaling events that activate NF-κB in response to TNFR1 ligation and DNA damage, a discussion of the cross-talk between these signaling networks will outline their co-regulation in more detail.

NF-κB activation via TNFR1 Ligation

The TNFR1 is a stereotypical death receptor that may promote proliferation, differentiation, or apoptosis, depending on the cell type and context of activation. **(Widera et al., 2006, Rastogi et al., 2012, Chomarat et al., 2003)** As with Fas ligation, activation of TNFR1 leads to caspase processing; however, TNFR1 signaling also liberates NF-κB to induce its downstream targets, which can restrict cell death. **(Brenner et al., 2015)** The balance between these countervailing signals enables cell survival under conditions of active apoptotic signaling

Ligation of the TNFR1 by TNF-α results in trimerization of the receptor, and assembly of various adaptor proteins at its cytoplasmic face. **(Brenner et al., 2015)** As depicted in the upper right region (URR) of Figure 1, these adaptors include TRADD, TRAF2/5, Ripk1, and cIAP1/2, which together form the membrane-associated Complex I. **(Hsu et al., 1995, Hsu et al., 1996, Ting et al., 1996, Hsu et al., 1996, Mahoney et al., 2008)** The ubiquitin ligase activity of the cIAPs allows them to add K11-, K48- and K63-polyubiquitin chains to several adaptors, notably Ripk1 and cIAP1, which recruits LUBAC to add M1-polyubiquitin chains to Ripk1. **(Mahoney**

et al., 2008, Haas et al., 2009, Dondelinger, et al., 2016) These polyubiquitin chains function as scaffolding for NF-κB-activating proteins to assemble on. Notably, one complex consisting of TGF-β-activated kinase 1 (TAK1) and TAB2/3, and another consisting of IKKα, IKKβ, and NEMO (IKKγ) get recruited to the polyubiquitin chains. (**Kanayama et al., 2004, Ea et al., 2006)** Following recruitment, TAK1 phosphorylates IKKβ, which allows IKKβ to phosphorylate and inactivate Inhibitor of κBα (IκBα). This liberates NF-κB transcription factors to translocate into the nucleus. **(C. Wang et al., 2001, DiDonato et al., 1997)** TAK1 can also promote the activation of the kinases JNK and p38 alongside NF-κB. Importantly, the ubiquitin ligase activity of the cIAPs is necessary for maintaining canonical NF-κB signaling, and depletion of these proteins, as seen following release of Smac/DIABLO from the IMS, may instead activate non-canonical NF-κB signaling through stabilization of NF-kappa-B-inducing kinase (NIK). **(Yang & Du, 2004, Zarnegar et al., 2008)**

Following signal transduction events at the membrane-associated TNFR1 Complex I, deubiquitination of Ripk1 results in its dissociation from Complex I, and the formation of either Complex IIa or Complex IIb in the cytoplasm. **(Bertrand et al., 2008, Micheau & Tschopp, 2003, Wang et al., 2008)** Either of these complexes can process procaspase 8. **(Wang et al., 2008)** The TRADD-dependent Complex IIa consists of TRADD, FADD, and either a procaspase 8/c-FLIP$_L$ heterodimer, or a procaspase 8 homodimer. Complex IIa assembly results in the cleavage of Ripk1, Ripk3, and the Ripk1 deubiquitlyase Cylindromatosis (CYLD). **(Wang et al., 2008, Brenner et al., 2015, Wright et al., 2007)** With cIAP depletion following Complex I assembly, the Ripk1-dependent Complex IIb forms instead. This complex consists of a Ripk1, Ripk3, FADD, and either a procaspase 8/c-FLIP$_L$ heterodimer, or a procaspase 8 homodimer. Both Complex IIa and Complex IIb have overlapping targets, such as Ripk1, Ripk3, CYLD, and

procaspase 8, and the assembly of either complex can lead to apoptosis. Interestingly, the depletion of cIAP1/2 and XIAP promotes the formation of the Ripoptosome, a Ripk1/FADD/Caspase 8 complex that is reminiscent of Complex IIb; however, this caspase activation platform is unique in that it can form without prior assembly of Complex I. **(Tenev et al., 2011)** If caspase 8 activity is inhibited, and Ripk1/3 are not cleaved, the these kinases both auto- and trans-phosphorylate one-another. **(Berghe, et al., 2014)** This leads to the assembly of the necrosome, a filamentous complex consisting of Ripk1, Ripk3, FADD and inactive caspase 8. This necrosome phosphorylates Mixed Lineage Kinase Domain-like (MLKL) proteins, which leads to their oligomerization, and allows them to disrupt plasma membrane integrity to mediate pro-inflammatory cell death. **(Berghe, et al., 2014)**

c-FLIP Proteins

Cellular FLICE (FADD-like IL-1β-converting enzyme)-inhibitory protein (c-FLIP) is an important regulator of apoptosis that is structurally similar to caspase 8, but lacks a functional catalytic domain. **(Irmler et al., 1997)** This protein is expressed as either a long, short, or Raji isoform, notated as c-FLIP$_L$, c-FLIP$_S$, and c-FLIP$_R$, respectively, though c-FLIP$_L$ and c-FLIP$_S$ are the major isoforms present in mammalian cells. **(Safa, 2012)** All of these isoform have 2 N-terminal death effector domains (DED) that mediate caspase interactions, while only c-FLIP$_L$ has 2 C-terminal caspase-like domains. The c-FLIP$_S$ and c-FLIP$_R$ splice variants are truncated at the C-terminus and lack caspase-like domains. c-FLIP isoforms can also be alternatively processed by caspases, and the cleavage fragments can display unique regulation of apoptosis, as well. **(Safa, 2012, Golks et al., 2006)**

c-FLIP primarily regulates apoptosis by modulating caspase activity and promoting NF-κB signaling. c-FLIP$_L$ can compete with procaspase 8 for dimerization at various caspase

activation platforms to alter the potency of the signaling complexes, and caspase 8 substrate specificity. **(Micheau et al., 2002, Micheau & Tschopp, 2003, Feoktistova et al., 2011)** At the DISC for example, the ratio of c-FLIP proteins to procaspase 8 proteins critically regulates the stoichiometry of DISC components. **(Schleich et al., 2012)** Low levels of c-FLIP$_L$ allow for DED-mediated assembly of multiple procaspase 8 molecules at the DISC, referred to as Death Effector Filaments (DEF), which can lead to cell death. High levels of c-FLIP$_L$ instead prevent this assembly and reduce the stoichiometry of FADD:c-FLIP$_L$:Caspase 8 to1:1:1. This allows for limited substrate processing at the DISC, which can inhibit apoptosis. **(Hughes et al., 2016, Schleich et al., 2012)** For example, Ripk1, an essential component of the TNFR1 Complex I, Complex IIb/Ripoptosome, and the necrosome, is cleaved at the membrane-restricted DISC; this may restrict TNFR1 signaling during active Fas signaling, and also prevent the formation of the cytosolic Ripoptosome, which does not require prior assembly of Complex I. **(Micheau et al., 2002, Feoktistova et al., 2011)**

The recruitment of c-FLIP proteins to cytosolic caspase activation platforms also directs downstream death responses. As mentioned, c-FLIP$_L$ recruitment to Complex IIa promotes the cleavage of Ripk1, Ripk3, and CYLD, and as Ripk1 and Ripk3 are essential components of Complex IIb and the necrosome, their cleavages likely restrict the formation of these complexes. **(Brenner et al., 2015, Li et al., 2012)** CYLD can also mediate deubiquitlyation of Ripk1, which is important for necrosome assembly. The assembly of Complex IIb, which is often seen alongside cIAP inhibition, similarly leads to Ripk1 and Ripk3 cleavages. Unlike c-FLIP$_L$, however, c-FLIP$_S$ can both promote the formation of the Ripoptosome, and simultaneously inhibit caspase 8 activity within this complex. This can instead promote death via the necrosome. **(Feoktistova et al., 2011)**

c-FLIP proteins can also be processed by caspases to generate distinct cleavage fragments that control cell death. Interestingly, procaspase 8 can cleave c-FLIP$_{L/S}$ into an N-terminal p22-FLIP, and elevated ratios of c-FLIP$_L$/procaspase 8 lead to higher levels of p22-FLIP formation. **(Golks et al., 2006)** p22-FLIP is able to bind to both the Fas-assembled DISC to restrict cell death, and to NEMO to promote NF-κB signaling. While procaspase 8 processes c-FLIP$_L$ to p22-FLIP, caspase 8 processes c-FLIP$_L$ to p43-FLIP. p43-FLIP is able to promote NF-κB survival signaling and restrict cell death, but p22-FLIP appears to be the more potent NF-κB activator. **(Golks et al., 2006, Safa, 2012)**

NF-κB activation through Nuclear Signaling

Various types of genotoxic stress are able to induce NF-κB through nuclear signaling events. Treatment with chemotherapeutic drugs, exposure to UV/IR radiation, or generation of ROS can all lead to DNA damage-induced activation of NF-κB. **(Jin et al., 2009, Janssens & Tschopp, 2006, Morgan & Liu, 2011)** Importantly, as with death receptor activations, nuclear NF-κB-activating stimuli converge on the activation of the cytoplasmic IKK complex, and IAP ubiquitination events are critical to this process.

Following DNA damage, several nuclear complexes assemble to coordinate the DDR with NF-κB activation, which are depicted in the lower right region (LRR) of Figure 1. One important complex, the PARP1 signalosome, consists of ATM/PARP1/PIASγ/NEMO. **(Stilmann et al., 2009)** Ataxia telangiectasia mutated (ATM) is an important mediator of the DDR that phosphorylates a variety of proteins, such as Checkpoint kinase 2 (Chk2) and p53, to coordinate and amplify the DDR signal. **(Shiloh & Ziv, 2013, Matsuoka et al., 2000, Saito et al., 2002)** PIASγ (Inhibitor of Activated STATγ) is unique E3 ligase that post-translationally sumoylates proteins by conjugating SUMO (small ubiquitin-like modifier) groups onto lysine

residues of its target proteins. **(Mabb et al., 2006)** PARP1 initially associates with damaged DNA to coordinate repair, where it recruits DDR mediators. However, PARP1 autoPARylation causes it to disassociate from the DNA, and this promotes the assembly of the nuclear ATM/PARP1/PIASγ/NEMO complex. **(Stilmann et al., 2009)** PARP1 initially recruits ATM and PIASγ, after which PIASγ sumoylates NEMO at residues K277/K309, and ATM phosphorylates NEMO at S85. **(Huang et al., 2003, Mabb et al., 2006, Wu et al., 2006)** After phosphorylation, NEMO is mono-ubiquitinated to displace the SUMO groups, and cIAP1 can mediate this ubiquitination through its RING domain. Both the Ub-modified NEMO and ATM then translocate into the cytoplasm to facilitate IKK activation. **(Jin et al., 2009, Wu et al., 2006, Hinz et al., 2010)**

Additional cytoplasmic interactions are required to activate NF-κB following DNA damage. Cytoplasmic ATM associates with TRAF6 to promote TRAF6 K63-polyubiquitination. **(Hinz et al., 2010)** This results in the recruitment of cIAP1, TAB2/TAK1, and the IKK complex, and results in TAK1 phosphorylation. In addition to TAK1 activation, NEMO undergoes an essential mono-ubiquitination event at K285, which can be mediated by an ATM/TRAF6/cIAP1 complex. Ripk1 also complexes with IKK and promotes IκB degradation, and this activity is independent of TRAF2 and Ripk1 kinase activity. **(Hinz et al., 2010, Hur et al., 2003, Yang et al., 2011)**

XIAP can also facilitate NF-κB activation in response to some stimuli. For example, XIAP facilitates an important IKKβ phosphorylation event following treatment with etoposide or camptothecin by associating with a TAB1/TAK1 complex via an interaction with its BIR1 domain, and recruits IKK to be phosphorylated. **(Jin et al., 2009, Lu et al., 2007)** Importantly, this provides a point at which NF-κB signaling can be shut down because, following its

liberation from the IMS, Smac can interact to XIAP in this complex and promotes its dissociation. **(Lu et al., 2007)** This restricts IKK activation and NF-κB activity.

PIDD and the PIDDosome

Another protein complex that assembles in response to genotoxic stress and coordinates with the PARP1 signalosome consists of PIDD-C/Ripk1/NEMO. **(Janssens et al., 2005)** In response to DNA damage, PIDD (p53-induced with Death Domain) translocates into the nucleus and undergoes autocatalytic cleavage to enable the formation of different protein complexes. **(Janssens et al., 2005, Tinel et al., 2007)** The PIDD-C/Ripk1/NEMO complex, like the PARP1 signalosome, is proposed to facilitate NEMO sumoylation and NF-κB activation. However, neither PIDD nor Ripk1 was not detected as part of the PARP1/ATM/PIASγ/NEMO complex, and their precise contributions to NEMO sumoylation are not currently known. **(Stilmann et al., 2009, Janssens et al., 2005)** It is interesting to note that despite rapid formation of the PARP1 signalosome, a delay in NEMO sumoylation was apparent. As such, it seems likely that the PIDD-C/Ripk1/NEMO complex mediates additional PARP1 signalosome-independent interactions that are important for NEMO sumoylation. **(Stilmann et al., 2009, Janssens et al., 2005)**

While small amounts of DNA damage result in PIDD promoting NF-κB activation, large-scale DNA damage elicits a distinct response. In this instance, PIDD-C undergoes an additional cleavage event and is processed to PIDD-CC, which assembles with RAIDD (RIP-associated ICH1/CED3-homologous protein with Death Domain) into a caspase activation platform known as the RAIDD-PIDDosome. **(Tinel et al., 2007)** This RAIDD-PIDDosome is cytoplasmic, and is able to recruit and process procaspase 2. Caspase 2 is able to cleave Bid to tBid, and Ripk1, as well. Importantly, PIDD processing appears to be sequential such that PIDD-

C generation, and activation of pro-survival NF-κB signaling, is expected to precede PIDD-CC-mediated caspase activations. **(Tinel et al., 2007)**

ATM and Checkpoint kinase 1 (Chk1) have been found to be important regulators of RAIDD-PIDDosome formation. **(Ando et al., 2012)** ATM regulates PIDD's pro-survival to pro-death decisions by phosphorylating PIDD on T788 in PIDD's death domain. While this does not affect PIDD-C to PIDD-CC processing, it does allow PIDD to recruit RAIDD and procaspase 2 instead of Ripk1. Active Ch1 signaling, however, restricts this phosphorylation event and suppresses the formation of the RAIDD-PIDDosome, and this may also restrict apoptotic signaling during an active DDR. **(Ando et al., 2012)** Interestingly, T788-phosphorylated PIDD is subject to mitotic regulation, where PIDD gets recruited to the sites of checkpoint signaling by BubR1. This interaction restricts the recruitment of RAIDD and the assembly of the caspase 2 activation platform.

Death and Differentiation

Apoptotic signaling events have been suggested to be an extreme form of the differentiation program, and many of the proteins discussed above have been shown to regulate cell differentiation in a variety of cell types; these proteins include death receptors, caspases, calpains, Bcl-2 proteins, IAPs, and NF-κB transcription factors, among others. **(Fernando & Megeney, 2007)** These proteins are often subject of differentiation-specific regulation that allows them to promote cell specialization as opposed to cell death. For example, transient protein activations, attenuated levels of activations, and spatiotemporal control of these proteins are the commonly proposed mechanisms by which these death-associated proteins control cell differentiation. **(Fischer et al., 2003)**

Among the many protein families discussed above, caspases have well-established roles in mediating differentiation. Caspase 8, for example, has been shown to be necessary for monocyte differentiation into macrophages, as well as for the differentiation of osteoblasts and lymphocytes. **(Sordet et al., 2002, Mogi & Togari, 2003, Alam et al., 1999)** Caspase 9 is involved in lens epithelial cell differentiation, which involves modulating the expression of Bcl-2 family members and IAPs, and also sees cytochrome c release. **(Weber & Menko, 2005)** Caspase 3 has likewise been shown to be required for the differentiation of myoblasts, neurons, embryonic stem cells (ESCs), hematopoietic stem cells (HSCs) and bone marrow stromal cells. **(Fernando et al., 2002, Rohn et al., 2004, Fujita et al., 2008, Janzen et al., 2008, Miura et al., 2004)** While these proteins often cleave and activate canonical death substrates during differentiation, the stage of the differentiation program that requires caspase activity is cell type-specific. **(Weber & Menko, 2005)** Importantly, not all cells require caspase activity to differentiate. Some cell types, such as Dendritic Cells, can differentiate even in the presence of broad-spectrum caspase inhibitors. **(Sordet et al., 2002)**

Both upstream and downstream caspase regulators have also been shown to promote cell differentiation. Various death receptors, including Fas, TNFR1, TRAILR1/2, and NGF, among others are implicated in the regulation of cell survival and differentiation of stem cells. **(Solá, Morgado, & Rodrigues, 2013)** In the hematopoietic system, regulation of the Fas-FADD-Caspase 8 signaling axis by c-FLIP is critical to the activation of Jurkat T cells, with downstream induction of IL-2. **(J. Zhang et al., 1998, Kataoka et al., 2000)** However, Jurkat cells have constitutive Akt signaling, and studies of the DO11.10 T cell line indicate that both Akt and Fas signaling activity are critical to T cell activation. **(Fang et al., 2004)** Outside of the hematopoietic system, Fas activation in neural stem cells (NSCs) promotes survival and

differentiation without recruitment of FADD; instead, these cells recruit the Src family member Yes, which activates a PI3K/Akt/GSK3β-mTOR pathway. **(Corsini et al., 2009)** TNFα activation of canonical NF-κB also controls the induction of NSC differentiation, as well as the asymmetric division of these cells. **(Y. Zhang et al., 2012)** TRIAL Receptor can also induce the differentiation of several cell types, such as osteoclasts, and intestinal cells. **(Rimondi et al., 2006, Yen et al., 2008)** As the combined activation of cell death and cell growth signaling is observed during both T cell activation and NSC induction of differentiation, dissecting the co-regulation of these signaling networks in the control of cell differentiation should be a focus of future research.

Bcl-2 family members, such as Bcl-X_L, Bax, Bid and Bim can regulate neural differentiation by directing precursor cells down distinct lineages, though the molecular mechanisms that allow these proteins to direct cell fate are poorly defined. **(Solá et al., 2012)** Bcl-X_L, for example, is essential to neuronal survival during brain development and in the central nervous system, and its expression can increase neuronal progenitor proliferation and inhibit the differentiation of glial precursors. **(Motoyama et al., 1995, Parsadanian et al., 1998, Liste et al., 2007)** Bax expression correlates with astrocyte formation, and it can direct embryonic cortical precursors towards astrocyte differentiation. **(Chang et al., 2007)** Bim may control survival and differentiation of adult-born neural precursor cells. **(Bunk et al., 2010)** There is also widespread distribution of Bid-expressing cells in the developing murine nervous system, and the caspase-cleaved tBid has been detected during this process. **(Krajewska et al., 2002)** The contributions of these family members have been more extensively reviewed elsewhere. **(Solá et al., 2012)**

Survivin

Survivin is a multi-functional IAP that is widely expressed during embryonic development, as well as during M phase in proliferating cells, and it has emerged as one the most tumor-specific genes in the human genome. **(Jaiswal, Goel, & Mittal, 2015)** However, most terminally differentiated cells do not express survivin during interphase. While survivin inhibits apoptosis, it can also facilitate NF-κB activation and cell cycle progression by associating with proteins that regulate all three of these processes. **(Wang et al., 2010, Tracey et al., 2005)**

In addition to its regulation of caspase activity discussed above, survivin plays a critical role in regulating cell cycle progression and apoptosis in proliferating cells. **(Skoufias et al., 2000)** In normally proliferating cells, survivin is upregulated prior to M phase, where it facilitates the propter segregation of chromosomes by localizing to kinetochores during early mitosis. At the kinetochore, survivin associates with INCEP and Borealin as a component of the Chromosome Passenger Complex (CPC). **(Skoufias et al., 2000)** Even as survivin facilitates M phase progression, it simultaneously bridges the fidelity of this process with the induction of cell death by binding to cell cycle and apoptotic regulators. For example, survivin phosphorylation by the cdc2-B1 complex during M phase enables it to restrict caspase 9-mediated apoptosis. Survivin also co-localizes to centrosomes with caspase 3 and p21 to restrict cell death, and loss of survivin function during M phase promotes apoptosis, polyploidy, and multi-nucleation. **(Skoufias et al., 2000, F. Li et al., 1999, F. Li et al., 1998)**

While survivin is rarely expressed during interphase in proliferating cells, survivin can be upregulated in the G_1 phase of differentiating cells. In the G1 phase of HepG2 cells, survivin has been shown to promote progression into S phase by translocating into the nucleus and promoting CDK4, and potentially CDK6, activity, though a direct interaction with CDK6 was not tested. **(Suzuki et al., 2000)** This interaction was dependent on Fas receptor stimulation and cell

proliferation, further highlighting the importance of this cross-talk. In the nucleus, survivin can displace the CKI p16 from CDK4 and allow activation of the D-CDK4 complex, which phosphorylates the retinoblastoma protein (pRb). This promotes progression into S phase, possibly under conditions of elevated CKIs. **(Suzuki et al., 2000)** In addition, survivin's interaction with CDK4 promotes the formation of a procaspase 3/p21 complex at the mitochondria that restricts cell death in response to Fas stimulation. **(Suzuki et al., 2000)** It would be interesting to see if survivin can displace the other INK4 proteins, such as p15, p18, or p19, from CDK4/6, as well.

Enzymatically-induced DNA Damage and Differentiation

It has been suggested that controlled DNA damage may be an important phenomenon in regulating differentiation, and the associated changes in gene expression, of a variety of cell types. **(Larsen et al., 2010)** This can be mediated by CAD during the terminal differentiation of myoblasts. T cell receptor (TCR) rearrangements, and class-switch recombination, both see regulated DNA damage promoting differentiation. **(Blom et al., 1999, Daniel & Nussenzweig, 2013)** Hormone receptors have also been shown to modify gene expression patterns through DNA damage. Estrogen Receptor α (ERα), Retinoic Acid Receptor (RAR), Androgen Receptor (AR), and even Activating Protein-1 all stimulate transcription through recruitment of a TOPOIIβ/PARP1/DNA-PK (DNA-dependent protein kinase)complex that generates dsDNA breaks to modify nucleosome structure. **(Ju et al., 2006)** ERα, RAR, and AR are all know to control differentiation processes in different cell types. **(Mérot et al., 2005, Luca, 1991, Culig, 2016)**

During myoblast differentiation, caspase 3 cleavage of ICAD frees CAD to translocate to the nucleus to induce DNA strand-breaks, as is commonly seen during apoptosis. **(Larsen et al.,**

2010) In this instance, however, phospho-H2AX foci were observed and the breaks were transient in nature, indicating a functional DDR. CAD also selectively localizes to the p21 promoter during these events, and generates a strand-break to induce transcription of this CKI. **(Larsen et al., 2010)** p21 induction likely results in cell cycle arrest through CDK inhibition while the cell repairs the damage. It may also lead to p21-mediated inhibition of procaspase 3 processing, as described above. If both of these events were to take place simultaneously, it would provide an efficient shut off mechanism that restricts lethal levels of caspase 3 activity after this protease cleaves the substrates that are important to terminal differentiation, such as ICAD in this instance. In line with the role of CAD in mediating differentiation, loss of the DNA damage response protein that repairs CAD-induced DNA damage in myoblasts, XRCC1, leads to unrepaired damage and failed differentiation. **(Al-Khalaf et al., 2016**)

The widespread use of DNA damage to regulate cell fate necessitates an examination of the potential nucleases that can mediate this type of damage. The apoptotic nuclease CAD is an established mediator of differentiation-inducing DNA damage in myoblasts, and Endo G is also involved in both class-switch recombination in maturing B cells, and DNA rearrangements in MLL break-cluster region. **(Zan et al., 2011, Gole et al., 2015)** MIF, a cytokine and nuclease that can mediate DNA damage downstream of PARP1 signaling, is widely expressed during mouse embryonic development. **(Wang et al., 2016, Kobayashi et al., 1999, Fingerle-Rowson & Petrenko, 2007**) MIF expression coincides with tissue specification and organogenesis, and its ability to inhibit p53 activity, mediate DNA damage, and regulate DNA damage responses may allow it to coordinate the differentiation program. **(Fingerle-Rowson & Petrenko, 2007)**

As DNA damage appears to be a core component of differentiation signaling, and as several apoptotic nucleases can mediate this type of damage downstream of programmed cell

death signaling, the upstream regulation surrounding the recruitment of alternate nucleases to mediate DNA damage needs to be outlined in more detail. As mentioned above, PARP1-mediated upregulation of Ca^{2+} can lead to the activation of calpains, and the translocation of AIF, Endo G, and/or MIF into the nucleus. This may also activate DNAS1L3, a Ca2+-dependent, ER-released DNase that translocates into the nucleus to facilitate internucleosomal DNA fragmentation (INDF) in HT-29 cells. **(Errami et al., 2013)** Importantly, DNAS1L3-mediated INDF downstream of TNF-α required both Ca^{2+} and co-expression of CAD, which is interesting as CAD is a Mg^{2+}-dependent nuclease that can cooperate with a Ca^{2+}-dependent nuclease to mediate INDF. How distinct nucleases can work synergistically to facilitate DNA fragmentation, as well as how cells differentially respond to the different types of DNA damage need to be outline in more detail. As cells must restrict lethal levels of DNA damage when apoptotic nucleases facilitate cell differentiation, the bifurcation of DNA fragmentation into large-scale chromatin fragmentation (LSCF) and internucleosomal cleavage of the DNA may enable the necessary cleavage events, while also restricting death. **(Oberhammer et al., 1993)**

Large-scale Chromatin Fragmentation and Internucleosomal DNA Fragmentation

DNA fragmentation is one on the most important events in apoptosis, and the associated DDR may be central to coordinating differentiation. Large-scale chromatin fragmentation (LSCF) precedes, and can even occur in the absence of, internucleosomal DNA cleavage during apoptosis. **(Oberhammer et al., 1993, Nagata et al., 2003)** LSCF is an event that is characterized by the excision of 50-300kB chromosomal loop structures from nuclear scaffold proteins (e.g. TOPO II). CAD and AIF can both mediate this type of DNA damage, but as AIF lack nuclease activity, it may coordinate with other nucleases, such as MIF or Endo G to facilitate LSCF. **(Oberhammer et al., 1993, Adachi et al., 1989, Earnshaw et al., 1985)** For

instance, after facilitating the nuclear translocation of MIF, AIF interacts with MIF in the nucleus to alter MIF substrate specificity, and can enhance MIF's association with both ssDNA and dsDNA. **(Wang et al., 2016)** Notably, Endo G has been shown to bind to TOPOIIα and to H2BS14 during apoptotic cell death, and may mediate chromatin fragmentation through these associations. **(Vařecha et al., 2011)**

LSCF is observed in a variety of cell types, such as prostate carcinoma cells, colon adenocarcinoma cells, and fibroblasts, though LSCF does seem subject to cell type-specific regulation. **(Oberhammer et al., 1993, Errami et al., 2013)** For example, fibroblasts, specifically, undergo CAD-dependent LSCF when treated with TNFα, without subsequent INDF. **(Yakovlev et al., 2000, Boulares et al., 2001)** The cell's ability to restrict CAD activity to LSCF without INDF may also be important in regulating differentiation as LSCF-restricted CAD activity may instead facilitate the chromatin modifications that are necessary for differentiation to occur. Distinctions in the downstream signaling consequences of LSCF vs. INDF, and whether these events coordinate with NF-κB signaling, need to be outlined in detail.

The γ-H2AX Ring

The γ-H2AX ring was discovered through confocal microscopy by S. Solier et al., and she and her collaborators have since been characterizing the phenomenon. The γ-H2AX ring describes a ring-shaped staining pattern for γ-H2AX that forms within the nuclear envelope. **(Solier & Pommier, 2009)** It initiates at the nuclear periphery as an early apoptotic response, and it is predominantly distributed to peripheral heterochromatin regions. **(Solier & Pommier, 2009)** The γ-H2AX Ring also sees co-localization of a variety of DDR proteins including Chk2, ATM, DNA-PK, Hsp90α, and phosphorylated H2BS14; however, it lacks traditional DDR proteins such as 53BP1 and mediator of DNA damage checkpoint protein 1 (MDC1). **(Solier et**

al., 2009) Caspase 3 has been shown cleave MDC1 early during apoptosis, and abrogate is DDR function, which may explain its absence in the complex, while DNA-PK_{CS} is cleaved during late apoptosis. **(Solier & Pommier, 2011, Song et al., 1996)** The γ-H2AX ring has been shown to be a ubiquitous event seen in numerous primary cell, and cancer cell lines, and it is observed downstream of various death receptor activations, as well as following treatment with several chemotherapeutics. **(Solier & Pommier, 2009, Solier & Pommier, 2014)** Importantly, the formation of the γ-H2AX ring can be delayed for several days after DNA damage.

Cell Cycle- and DNA Damage-mediated Control of Differentiation

Programmed cell death signaling is rarely viewed as a cell cycle-dependent process, and yet several important cell cycle considerations must be made when examining apoptotic signaling events during differentiation. Many apoptotic stimuli lead to DNA damage, which results in the activation of the phase-specific G_1/S, intra-S, or G_2/M DNA damage checkpoints. **(Shaltiel, et al., 2015)** These checkpoints coordinate cell cycle arrest with DDR signaling to promote repair, and dysregulated DDRs can lead to death. Notably, survivable apoptotic stimuli can promote progression through cell cycle, as demonstrated following IR-induced DNA damage, and induction of death can be delayed for several days following the apoptotic stimuli. **(Liu et al., 2017, Solier & Pommier, 2014)** However, if the DNA damage provides the impetus to differentiate, progression through cell cycle makes sense, as proliferation is a frequent requirement of differentiation. Induction of death, potentially following several passages through cell cycle, may be the result of dysregulated resolution of this program.

Cell differentiation frequently requires that a cell proliferate prior to differentiating. **(Ruijtenberg & Heuvel, 2016)** While the precise reasons for this proliferative requirement require further investigation, it has been shown that progression through mitosis can help

maintain silencing of repressed genes. **(Ritland Politz et al., 2013, Zullo et al., 2012)** During mitosis, lamina-associated domains likely interact in lamina B during anaphase to direct silencing in early G_1 following cytokinesis, which may direct them to the nuclear periphery, a frequent localization of heterochromatin. Interestingly, a lengthened G_1 phase is often observed during differentiation. **(Calder et al., 2013)** As many cells undergo multiple passages through cell cycle during the differentiation process alongside progressive specialization, distinctions in the control of progression though G_1 during the initial, and subsequent, passages through cell cycle must be emphasized and compared with the cell cycle regulation that restricts continued cycling during terminal differentiation. Notably, proliferative signaling often antagonizes differentiation signaling, and much of this G_1 regulation centers on the interplay among the CDKs, the CKIs, and the pocket proteins (i.e. pRb, p107, and p130) that regulate progression through the G_1 phase of cell cycle, and cycling cells are known to alter this interplay. **(Ruijtenberg & van den Heuvel, 2016, Lezaja & Altmeyer, 2018)**

Quiescent cells that are signaled to divide must pass through the G_1 Restriction Point (R Point) to be committed to division. After the R Point, a cell will progress all of the way through mitosis even in the absence of further mitogenic signaling; however, continued cycling after the initial passage through cell cycle, seems to require that a cell have mitogenic signaling in the preceding G_2 phase of cell cycle, with ras-mediated induction of cyclin D1 being of critical importance. **(Hitomi & Stacey, 1999, Yang, Hitomi, & Stacey, 2006)** This may allow a cell to gauge the proliferative environment after S phase to determine if continued cycling is necessary. Following mitosis, two distinct sub-populations of cells are present: one population have elevated CDK2 activity, low p21 levels, and a shortened G_1, while the other population have low levels of CDK2 activity, elevated p21 levels, and a lengthened G_0/G_1. **(Lezaja & Altmeyer,**

2018) The former is primed to continue proliferating, while the latter enters a transient quiescent state and may be more susceptible to cell cycle exit and terminal differentiation.

S phase signaling activity can also influence whether or not a cell will continue cycling after mitosis. The unperturbed passage through S phase can lead to the accumulation of DNA lesions that can be transmitted to daughter cells, and replication stress can further promote the formation of these lesions. **(Lezaja & Altmeyer, 2018)** These lesions result in the formation of 53BP1 foci in G_1, and can lead to an accumulation of cells in the G_1 phase of the next cycle. G_1 arrest is heavily p53-dependent, and p53 inhibition, or mutation, can lead to S phase progression, potentially with unrepaired 53BP1 lesions. This can lead to an accumulation of mutations through multiple passages through cell cycle. As controlled DNA damage has been suggested to be an important phenomenon in regulating differentiation, and as 53BP1 has an established role in mediating the class-switch recombination, the mechanisms by which 53BP1 lesions escape repair during the intra-S and G_2/M DNA damage checkpoints need to be outlined in detail. **(Ward et al., 2004)** It is possible that in addition to restricting continued cell cycling, the generation, and epigenetic modification, of the DNA in the preceding passage facilitates differentiation. Such modifications could function as a form of mitotic imprinting that mediates the chromatin modifications that are necessary for differentiation to occur in the subsequent G_1 phase. The DDR that mediates repair of these lesions in G_1 may serve as an upstream signal that initiates the activations of PCD pathways, as well as the downstream nucleases that mediate differentiation-inducing DNA damage. Interestingly, elevated chromatin plasticity is seen during early G_1 compared to the rest of interphase in various cell types. **(Thomson et al., 2004, Walter et al., 2003)** As differentiation can entail large-scale changes in chromatin territories, examining

how the detection of 53BP1-foci, or the formation of the γ-H2AX ring influences this plasticity should prove insightful.

A Differentiation Program

Virtually all of the mediators of programmed cell death discussed above have putative roles in mediating cell differentiation across diverse cell types. These proteins often control differentiation by performing their canonical cell death functions, and by activating various components of PCD signaling networks. This can include the ligation of death receptors, the activation of caspases, a reduction in mitochondrial membrane potential, the liberation of proteins from the IMS, external presentation of phosphatidylserine, and the activation of various nucleases, as well as downstream DDR proteins. **(Fernando & Megeney, 2007, Solá, Morgado, & Rodrigues, 2013, Eijnde et al., 2001, Larsen et al., 2010)** Fittingly, these networks can cross-talk with proliferative signaling networks, frequently via DDRs, at each phase of cell cycle to allow PCD signaling to control cell cycle progression.

Cell Differentiation - A Multi-passage Program

The process of cell differentiation is a DNA-damage-directed, cell cycle-dependent specialization program that functions at the crossroads of life and death. This program can entail multiple passages through cell cycle, and it initiates with cell type-specific differentiation-inducing signals that leads to entry into G_1. **(Basson, 2012)** The accumulation of D-type cyclins promotes G_1 restriction point progression and leads to S phase entry and DNA synthesis that may entail the formation of 53BP1 lesions. These lesions can escape G_2/M checkpoint repair via checkpoint adaptation, and may accumulate across multiple cell cycle passages. **(Shaltiel, et al., 2015)** Following replication, the ras-mediated induction of cyclin D1 in the G_2 phase can

promote continued cycling after mitosis by shortening the length of the subsequent G_1 phase, and attenuating the expression of the CKI p21. **(Yang, Hitomi, & Stacey, 2006, Overton et al., 2014)** This can promote another passage through cell cycle where the accumulation of DNA damage, and induction of D1 in the G_2 phase of cell cycle, will once again regulate continued proliferation. **(Barr et al., 2017)** The discontinuation of cycling after the final passage can be regulated by p53-dependent DDRs and by G_1 CDK/CKI regulation, where inherited 53BP1 lesions, or other types of DNA damage, can lead to cell cycle arrest via CKI regulation. **(Lezaja & Altmeyer, 2018, Barr et al., 2017)** This may lead to entry into a transient, or potentially prolonged, quiescent state that varies depending on the quiescence-inducing signal. **(Coller, Sang, & Roberts, 2006)** This may also promote activation of components of PCD signaling networks, as well as downstream cell death nucleases, that mediate differentiation-inducing DNA damage. Successful repair of the DNA can entail cell cycle exit, or continued proliferation, whereas failed repair can lead to cell death.

As outlined above, G_1/S, intra-S, and G_2/M, checkpoint signaling events, which are all inherently cell cycle-dependent, cross-talk with PCD, proliferative, and NF-κB signaling networks. In fact, by delaying the repair of 53BP1 lesions until the subsequent G_1 phase of cell cycle, it has been demonstrated that upstream checkpoint signaling can influence downstream checkpoint responses. In line with this, a distinct G_2/M checkpoint response is mounted following IR-induced DNA damage that depends on the cell cycle phase in which the damage was induced. **(Xu, et al., 2002)** DNA damage induced during S-phase or G_1 leads to a prolonged G_2/M checkpoint response, while damage induced in the G_2 phase sees an abbreviated response. As cycling cells may have a "G_2 Restriction Point" that regulates continued proliferation, a closer examination of how upstream DNA damage checkpoint signaling events can be

differentially regulated, as well as influence the response to differentiation-inducing DNA damage, should provide welcome insight into the multi-passage control of this program. **(Yang, Hitomi, & Stacey, 2006)**

Regulation of Differentiation

Regulation: Multi-phasic PCD and NF-κB Signaling

The cross-talk between proliferative and PCD signaling networks, as well as the spatiotemporal regulation of PCD signaling alongside NF-κB also need to be outlined in more detail. This entails better defining "early" vs "late" cleavage events to understand the sequence of protein activations that mediate programmed cell death or differentiation. Fortunately, the cellular response to either the ligation of death receptors, or DNA damage, is frequently multi-phasic. As outlined above, TNFR1 ligation leads to early activation of Complex I that facilitates anti-apoptotic NF-κB transcriptional responses; this is subsequently followed by the assembly of the cytoplasmic Complex IIa, IIb, or the necrosome that promotes cell death. Preceding Fas ligation, procaspase 8-mediated processing of c-FLIP to p22-FLIP can promote early activation of NF-κB via NEMO. This can be followed by formation of the membrane-restricted DISC, and possibly subsequently, the DEF DISC that can lead to procaspase 8 processing and cell death. Nuclear signaling events can similarly activate NF-κB as an "early" response, with "late" induction of death. Following DNA damage, PIDD is processed to PIDD-C, **(Janssens et al., 2005, Tinel et al., 2007)** which promotes NF-κB signaling as an early response before PIDD-CC assembles with RAIDD into a caspase 2 activation platform. Critically, the RAIDD-Piddosome is subject to mitotic regulation, and as caspase 2 is the best conserved of all known caspases and has the ability to cleave ICAD, Bid, and PARP1, it may be facilitating this multi-passage

differentiation program. **(Fava et al., 2012, Dahal et al., 2007, Guo et al., 2002, Gu et al., 1995)**

As several events, such as TNFR1 ligation or DNA damage, that initiate PCD signaling also co-activate NF-κB, and as NF-κB signaling can restrict cell death by inducing anti-apoptotic regulators that can also direct cell specification, correlating NF-κB induction, or discontinuation, with the activation of PCD networks and downstream DNA damage during differentiation should provide insight into the regulation of this system. Critically, the presence of NF-κB signaling should restrict cell death, so if differentiation-inducing caspase activations don't lead to cell death, coordinating the inactivation of NF-κB alongside restricted caspase activity may be important for differentiation. Importantly, these signaling networks cross-talk during every phase of cell cycle, and distinctions in their regulation can be tracked through multiple cell cycle passages. Mapping out these cross-connections in a few well-defined differentiation system should provide insight into global control mechanisms that are important for differentiation. Monocyte differentiation may provide a good model as monocytes can differentiate in macrophages via caspase-dependent mechanisms, or into dendritic cells via caspase-independent mechanisms. **(Sordet et al., 2002)**

Regulation: Selective Substrate Release from the IMS

One of the most critical regulatory intersections in programmed cell death centers on the release of proteins from the mitochondrial IMS. Fittingly, the evidence suggests that selective substrate release from the mitochondrial IMS is also a critical regulatory intersection in cell differentiation. Low-level cytochrome c release is seen in several differentiating cells types, as well as cancer cell types, and this can promote the caspase activity that is critical to differentiation. **(Garrido et al., 2006, Sordet et al., 2002, Liu et al., 2017)** It can also facilitate

sustained Ca^{2+} release via binding to inositol 1,4,5-trisphosphate receptor (IP3R) at the ER. **(Garrido et al., 2006)** Low-level cytochrome c release may also restrict apoptosome formation, especially in the presence of survivin, which is commonly upregulated in both differentiating cells and cancer cells. Aside from cytochrome c, the selective release of AIF, Endo G, and Smac may also regulate differentiation.

Bid, which mediates cross-talk between intrinsic and extrinsic apoptosis, is an established mediator of selective substrate release from the IMS. **(Hu et al., 2017, Deng et al., 2003)** In macrophages, cleavage of Bid to tBid can regulate the release of AIF and Endo G downstream of caspase 8 without the release of cytochrome c. This can be coordinated with ROS-mediated Akt inactivation. **(Hu et al., 2017)** Following TNF-α treatment of HeLa cells, JNK activity mediates cleavage of Bid to jBid and facilitates the selective release of Smac from the IMS, without impacting cytochrome c release. **(Deng et al., 2003)** This cleavage is caspase 8-independent, and acts upstream of caspase 8 in TNF-induced apoptosis. Smac release can remove IAP inhibition of caspases, though this can also be restricted by survivin. The selective release of Smac upstream of caspase-mediated release of cytochrome c may coordinate NF-κB inactivation with the activation of caspases that promote differentiation. Critically, Smac binding to XIAP does not lead to the degradation of this IAP, it relieves XIAP-mediated caspase inhibition, and Smac release can also disrupt the XIAP's association with the TAB1/TAK1 complex that promotes IKK activation. **(Fu et al., 2003, Jin et al., 2009, Lu et al., 2007)** As distinct BIR domains of XIAP mediate binding to different caspases, the Smac-XIAP complex may fine-tune downstream caspase activations. Alternatively, or possibly subsequently, caspase-mediated cleavage of XIAP downstream of Fas leads to the generation of an N-terminal BIR1-2 fragment, and a C-terminal BIR3-RING domain fragment. **(Deveraux et al., 1999)** The BIR1-2 fragment can inhibit caspase

3 and 7 and restrict Fas-mediated death, while the C-terminal fragment can inhibit caspase 9, but is no longer able to promote NF-κB. **(Deveraux et al., 1999, Levkau et al., 2001)** Importantly, co-expression of the C-terminal fragment alongside Bax potently restricts mitochondrial intrinsic apoptosis. Though Bid lacks a transmembrane domain, it can notably be post-translationally N-terminally myristolated following its cleavage, which facilitates mitochondrial membrane association. **(Roset, Ortet, & Gil-Gomez, 2007, Zha et al., 2000)** Identifying other mechanisms by which Bcl-2 proteins can facilitate selective substrate release requires further investigation.

Regulation: PAPR1 Coordinates Chromatin Modifications with Metabolic Reprogramming

Metabolic reprogramming is a common characteristic of many differentiating stem cells, including iPSCs, as well as cancer cells (Warburg Effect). **(Zhang, et al., 2012)** This reprogramming is cell-type specific, but it frequently sees elevated glycolytic activity, potentially under aerobic conditions, which can not only generate ATP, but can also redirect glucose in to Pentose Phosphate Pathway (PPP) to meet the biosynthetic requirements of rapidly dividing cells. **(Zhang, et al., 2012)** This reprogramming frequently uncouples glycolysis and TCA, and can allow TCA intermediates to be redirected for biosynthesis, as well. Upon terminal differentiation, a shift from anaerobic-to-aerobic metabolism frequently occurs, which sees preferential use of the ETC for energy. PARP1 is a mediator of this metabolic switch. **(Murata et al., 2018)**

PARP1 is an established regulator of cell differentiation in a variety of cell types. It can facilitate somatic cell reprogramming, promote ES cell differentiation, modify differentiation-specific transcriptional responses, and reportedly sensitize chromatin to nucleases, potentially the ones that PARP1 activates. **(Hottiger, 2015)** However, PARP1 activity can also deplete cellular NAD+/ATP stores by inhibiting glycolysis and reducing mitochondrial oxygen consumption.

(Andrabi et al., 2014) While PAPR1 enzymatic activity consumes NAD+, which is a critical glycolytic and TCA coenzyme, this doesn't directly deplete cellular NAD+ levels. As depicted in the lower left region (LLR) of Figure 1, in MNNG-treated cortical neurons, PARylation of the first glycolytic enzyme hexokinase I, or the cancer-associated isoform hexokinase II, inhibits the activity of these proteins. **(Andrabi et al., 2014)** Hexokinase activity promotes retention of imported glucose within the cell, and it is also critical for redirecting glucose into the PPP. The PPP is important for generating NADPH, a coenzyme that can both facilitate biosynthetic reactions, and also regulate cellular redox homeostasis by recycling cellular redox systems. **(Circu & Aw, 2010)** Importantly, PARP1-mediated inhibition of HK led to a decrease in cellular ATP levels, which is paralleled by reductions in both NADPH and reduced glutathione (GSH), and this can occur within 15min following MNNG treatment. Notably, AIF regulates Complex I of the ETC, and its liberation downstream of PARP1 may also help coordinate these events. **(Polster, 2013)** As it was demonstrated that NAD+ depletion occurs after ATP depletion, approximately an hour after MNNG treatment in this instance, PARP1 utilization of NAD+ does not directly cause the observed glycolytic and TCA defects. Critically, NAD+/ATP levels remained depressed for at least 6 hours following treatment. **(Andrabi et al., 2014)**

The PARP1-mediated regulation of NAD+/ATP levels has profound implications on cell fate. While ATP levels are rapidly depleted, which likely impairs intracellular phosphorylation events, maintenance of NAD+ levels for approximately an hour indicates that NAD+-dependent signaling can continue for a brief period after shutdown of energy metabolism. This can critically regulate the cellular acetylome. In elaboration, acetyl-CoA is a frequent coenzyme of lysine acetylases, and this coenzyme is generated by pyruvate dehydrogenase in an NAD+-dependent manner. **(Pietrocola et al., 2015)** Pyruvate dehydrogenase is inhibited by phosphorylation, an

event that is restricted in an ATP-depleted environment, and this may promote the generation of acetyl-CoA for acetlyase activity. However, some proteins, such as the class III histone deacetylase Sirtuin-1 (SIRT1), also utilize NAD+ for deacetlyase reactions, and balancing acetylation/deacetylation events during this brief period of time is likely critical to controlling cell death or differentiation. **(Yi & Luo, 2010)** For example, SIRT1 can deacetylate p53 to block its nuclear translocation, and redirect p53 to the mitochondria where it can interact with Bcl-2 family members to promote cytochrome c release, and potentially regulate cell respiration. **(Yi & Luo, 2010, Fields et al., 2008)** However, shutdown of PPP activity alongside ATP/NADPH depletion may also restrict dATP generation via ribonucleotide reductases, and diminished levels of both ATP and dATP can restrict apoptosome formation despite the release of cytochrome c. **(Sengupta & Holmgren, 2014, Zou et al., 1999)** Critically, ATP-depletion likely restricts ubiquitylation of lysine residues, as well, as the first step in the process of ubiquitylation is ATP-dependent. This may restrict ubiquitylation/acetylation antagonism during this time. Interestingly, in a study of MEFs, MNNG treatment only led to cell death if the cells were utilizing glycolysis, which may impair this regulation, and oligonucleosomal DNA fragmentation was not observed. **(Moubarak et al., 2007)** It is possible that cell death signaling needs to coordinate with temporary inactivation of energy metabolism to restrict death during differentiation, at least in some instances, and this may serve to reset energy metabolism.

Internal Ribosome Entry Site-mediated Translation

Cell differentiation and apoptotic stimuli, such as serum starvation, ER stress, or hypoxia, see a shutdown of cap-dependent translation with a switch to Internal Ribosome Entry Site (IRES) translation. **(Komar & Hatzoglou, 2011, Gerlitz, Jagus, & Elroy-Stein, 2002, Lewis & Holcik, 2005, Stein et al., 1998)** IRES translation allows ribosomes to bypass stable secondary

structures in the 5'-UTR that might otherwise impede translation, possibly via ATP-independent mechanisms, and caspase-mediated cleavage of several eukaryotic initiation factors, such as eIF4GI, eIF4GII, eIF2α, and Death-associated Protein 5 (DAP5), can facilitate this shift. **(Hellen & Sarnow, 2001, Agalarov et al., 2014, Fischer et al., 2003)** IRES translation is regulated by IRES-interacting trans-acting factors (ITAFs). **(Ray, Grover, & Das, 2006)** Critically, it has been suggested that IRES translation is important for mRNAs that regulate proliferation, differentiation, and apoptosis, and it can enable continued protein synthesis of select cellular RNAs under conditions of stress. For example, XIAP, cIAP1, Bcl-2, c-myc, apaf-1, and p53, as well as several growth factors, can all be regulated through IRES translation. **(Lewis & Holcik, 2005, Hellen & Sarnow, 2001, Marash et al., 2008, Ray, Grover, & Das, 2006)**

IRES translation can dramatically alter the regulation of the synthesized proteins. For example, p53 has two distinct IRES sites, one of which resides in the open reading frame of the gene. **(Ray, Grover, & Das, 2006)** Consequently, IRES-mediated translation of p53 can generate a full-length p53 (FL-p53), as well a ΔN-p53 isoform which has been shown to exert dominant-negative regulation of the full-length variant. This ΔN-p53 isoform lacks the AD1 subdomain of the p53 transactivation domain, and has altered transactivation potential. **(Raj & D Attardi, 2016)** Notably, it also lacks a variety of N-terminal phosphorylation sites, such as S15, which is phosphorylated by ATM, ATR, and Chk1/2, as well as S20, which is phosphorylated by Chk1/2, and these residues normally regulate p53 stability. **(Ou, et al., 2005)** Critically, IRES-mediated translation is subject to cell cycle control, with the ΔN-p53 isoform expressed during the G_1/S transition, while FL-p53 IRES translation is upregulated during the G_2/M transition, during which cap-dependent translation is known to be downregulated. **(Ray, Grover, & Das,**

2006) It will be important to expand on the role of IRES-mediated translation in the control of death and differentiation.

Regulation: Stabilization of the Intracellular Environment?

Though more speculative, there are several events that are associated with cell death, and potentially differentiation, that may lead to coordinated, large-scale changes in the intracellular environment. Caspases have been shown to cleave a variety of targets, such as nucleoporins and lamin-associated proteins, that disrupt nuclear transport and impair chromatin binding during apoptosis, and as mention above, recruitment of RCC1 following DNA damage can impair the GTP gradient that facilitates nuclear exchanges. **(Fischer et al., 2003, Wong et al., 2009)** Caspase 3 also facilitates the accumulation of various proteins into the nucleus, either by promoting their nuclear import, or inhibiting their export, as with Mst-1 and PKC, respectively. Additionally, caspase 3 cleaves ATM and MDC1 to restrict DDR signaling; however, as DNA-PK is primarily associated NHEJ, which is the predominant repair mechanism during G_1, this may restrict inappropriate DDR signaling during apoptosis/differentiation. **(Smith et al., 1999, Solier & Pommier, 2011, Davis, Chen, & Chen, 2014)**

In addition to impaired nuclear transport, major cytoplasmic alterations are also observed. Depletion of intracellular ATP and metabolic reprogramming may restrict ub-mediated targeting of proteins to the proteasome, as well as the ATP-dependent activity of this proteolytic complex. **(Peth, Nathan, & Goldberg, 2013)** This may occur alongside a switch to IRES-mediated translation. In addition, macromolecular synthesis is impaired and various cell-surface receptors can be cleaved, as well. **(Fischer et al., 2003)** For example, Met, the hepatocyte growth factor receptor, can be cleaved by caspases, as can TNFR1 and EGFR. **(Lefebvre et al., 2013, Ethell, Bossy-Wetzel, & Bredesen, 2001, Bae et al., 2001)** As proliferation antagonizes differentiation,

these likely transient events may restrict countervailing signals that could impair the differentiation process. **(Ruijtenberg & Heuvel, 2016)** It would be interesting to monitor the cleavage of cell-surface receptors, or other mechanisms of their inhibition, during differentiation.

Connecting Cancer

The cross-talk between the above-outlined signaling networks have broad implications in the treatment and understanding of cancer. Cancer is a complex disease that is often characterized based on hallmarks that are commonly to virtually all malignancies. **(Hanahan & Weinberg, 2000, Hanahan & Weinberg, 2011)** During malignant transformation, cancer cells often hyperactive proliferative signaling, alter PCD signaling, modify energy metabolism, and fail to properly differentiate, and this is often under conditions of constitutive inflammation. Cancer cells frequently need to contend with genomic instability, as well, as various DDR pathways are commonly dysregulated in cancer cells. As outlined above, all of these systems cross-talk to regulate cell differentiation.

Cancer as a Failure of Differentiation

I propose that the underlying cause of cancer is a failure to properly differentiate in the absence of death. Cancer cells can be seen attempting to progress down a differentiation program, but due to cancer-causing mutations that dysregulate this program, they are unable to terminally differentiate, discontinue cycling, or die. Critically, the hyper-proliferative phenotype seen in cancers may alternate between proliferation with the intent to differentiate and clonal expansion of a cell population. This dysregulation stems from the characteristic mutations that facilitate cancer formation, such as oncogenic ras mutations that promote continuous cycling. p53 mutations can simultaneously impair pro-apoptotic and pro-differentiation processes, impair

cell cycle exit, and dysregulate energy metabolism via TIGAR and other mechanisms. **(Solá, Aranha, & Rodrigues, 2012, Hanahan & Weinberg, 2011, Bensaad et al., 2006)** NF-kB signaling, which is constitutively active in many tumors, not only mediates the differentiation program, it also restricts apoptosis, and likely allows for the constitutive caspase activity that has been noted in a variety of tumors via upregulation of IAPs. This also explain why the activation of PCD signaling components in cancer can so often promote proliferation and survival. **(Mérino et al., 2007, Rivas et al., 2008)** In identifying which PCD signaling components are utilized to facilitate the differentiation of different cell types, cell-type-specific treatments can be developed for transformed cells that take advantage of the commonly observed mutations in different cancer subtypes. Targeting the interdependent checkpoint signaling that regulates proliferation, differentiation, and death may provide cancer-specific responses that can be exploited with combined radiation and chemotherapy.

PCD Signaling in Cancer

Apoptotic signaling has been shown to facilitate the growth and division of cancer cells in multiple contexts, and transient apoptotic stimuli is able to promote both progression through cell cycle and oncogenic transformation in primary cells. **(Liu et al., 2017, Tang et al., 2012)** DNA damage, which can act both upstream and downstream of PCD signaling to promote differentiation, helps sustain tumorigenicity and stemness in cancer cells. **(Liu et al., 2017)** Mechanistically, cancer cells have been shown to self-inflict DNA damage via low-level cytochrome c release from the mitochondria. This activates downstream caspases, and leads to the translocation of apoptotic nucleases (*i.e.* Endo G and CAD) into the nucleus, which may induce 53BP1-foci. This can lead to downstream activation of NF-κB signaling via STAT3, which can promote genetic instability and oncogenesis. **(Liu et al., 2017, Ichim et al., 2015)**

STAT3, unsurprisingly, has also been shown to promote the maintenance of a stem cell state, the self-renewal of stem cells, and somatic cell reprogramming. **(Liu et al., 2017, Matsuda et al., 1999, Niwa, et al., 1998, Tang & Tian, 2013)** Importantly, 53BP1 does not associate with DDR mediators downstream of γ-H2AX ring formation, so this is a distinct response. As 53BP1-foci can be transmitted through cell divisions and alter downstream DDRs that can mediate the discontinuation of cycling and terminal differentiation, illuminating the interrelated regulation of these DDRs is of critical importance.

Also connecting PCD and proliferative signaling with cancer initiation is that many primary cell types, upon treatment with apoptotic stimuli, are able to survive late-stage apoptotic events if the stimulus is removed. **(Tang et al., 2012)** These events include nuclear condensation, mitochondrial fragmentation, membrane blebbing, and DNA damage, all of which can be reversed upon removal of the stimuli. Caspase 3 activation, AIF and Endo G nuclear translocation, and even PARP1 cleavage were also survivable events. **(Tang et al., 2012)** The addition and removal of the apoptotic stimuli often led to the proliferation of these cells, some of which harbored chromosome aberrations. If these defects impair the resolution of the differentiation program, and cell death is not induced, malignant transformation may ensue.

Concluding Remarks

Outlined above is the foundation of a system that sees proliferative signaling, differentiation signaling, inflammatory signaling, Ca^{2+} signaling, DNA damage signaling, and many other pathways, working in concert to maintain the fidelity of the differentiation program. These networks are intricately co-regulated to ensure the proper sequence of protein activations take place to control life and death decisions, and yet these networks are also flexible, with different components being activate during the differentiation of different cells types. Critically,

as PCD networks regulate this program, dysregulation of this system can lead to cell death, which may safeguard against cancer, and other pathologies. These pathways need to be examined more closely, and yet with a wider focus, as the assumption that downstream induction of death is the intended outcome of many of these proteins' activities has led to fragmentary studies in which the regulation of too few components were analyzed. An examination of how common cancer-associated mutations can dysregulate this system and enable cell survival should facilitate better targeting of the disease.

It will also be important to examine mitochondrial fission/fusion dynamics during cell differentiation. Stem cell mitochondria often exhibit a fragmented morphology; however, as proliferating cells approach the G_1/S transition, the mitochondria coalesce into an electrically continuous tubular network with elevated ATP output. **(Prieto & Torres, 2017, Mitra et al., 2009)** As Bcl-2 family members can regulate mitochondrial fission/fusion dynamics, and as metabolic reprogramming during differentiation often sees elevated oxidative phosphorylation, a closer look the regulation of these events may prove insight into the control of differentiation. **(Shamas-Din et al., 2013, Zhang et al., 2012)**

To better define this system, future work should be aimed at elucidating the molecular mechanisms that regulate programmed cell death and differentiation in a cell type- and cell cycle-specific manner. As multiple DNA damage events can mediate differentiation, and phase-specific DNA damage checkpoints can be activated to mount distinct DDRs, defining the roles of cell death regulators in facilitating progression through these checkpoints in the presence, or absence, of proliferative signaling should be a key point of focus. For example, the combined activation of Fas alongside proliferative signaling promotes the differentiation of HepG2 cells via survivin's interaction with CDK4. As this interaction promotes S phase entry, it may be a

differentiation-specific mechanism to bypass G_1/S checkpoint arrest. It will also be important identify other differentiation-specific mechanisms of cell cycle progression, and expand on the role of G_2 proliferative signaling in the control of cell cycling.

Future work should also entail an examination of how distinct nucleases can cooperate to mediate differentiation-specific DNA damage, as well as the mechanisms by which they are recruited to the appropriate sites on the chromatin. This should be extended to include a comparison of the epigenetic changes observed during apoptosis with those observed during differentiation. **(Füllgrabe, Hajji, & Joseph, 2010)** It will also be important to identify overlapping targets of both caspases and calpains in the control of cell death and differentiation, as they have been shown to have redundant targets. **(Harwood, Yaqoob, & Allen, 2005)** Defining other mechanisms of differentiation-specific regulation of DNA damage checkpoints will be a focus of future study, as will be identifying the molecular mechanisms by which cancer-specific mutations allow this system to fail.

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Conflicts of Interest:

The author declares no conflicts of interest.

Figure 1: Regulation of Inflammation and Cell Death

(**ULR**) Extrinsic Apoptosis via Fas Signaling: Fas activation leads to procaspase 8 processing with the downstream cleavage of caspase 3, Bid, and several other proteins. These cleavages can promote mitochondrial IMS protein release, apoptosome formation, DNA fragmentation, and cell death. (**URR**) Signaling Downstream of TNFR1 Ligation: TNFR1 Complex I assembly sees the generation of ubiquitin chains that mediate recruitment of NF-κB-activating protein complexes, such as TAK1/TAB2/TAB3 and IKKα/IKKβ/NEMO. These complexes facilitate the inactivation of IκBα and downstream NF-κB transcriptional activity. Subsequently, a variety of cytosolic Death-inducing Signaling Complexes can form that promote cell death. (**LRR**) NF-κB Activation Downstream of DNA Damage: Following DNA damage, a PARP1 signalosome facilitates nuclear NEMO modifications to promote its export into the cytoplasm. Cytoplasmic NEMO can be further modified to facilitate IκBα degradation and NF-κB transcriptional activity. (**LLR**) DNA Damage-induced PARP1 Signaling: DNA damage leads to the activation of PARP1, which generates PAR polymers that can promote intracellular Ca^{2+} mobilization and the inactivation of energy metabolism. This promotes the release of a variety of IMS proteins that can translocate into the nucleus to facilitate chromatin fragmentation. PARP1 activity, if unchecked, may also deplete intracellular ATP levels.

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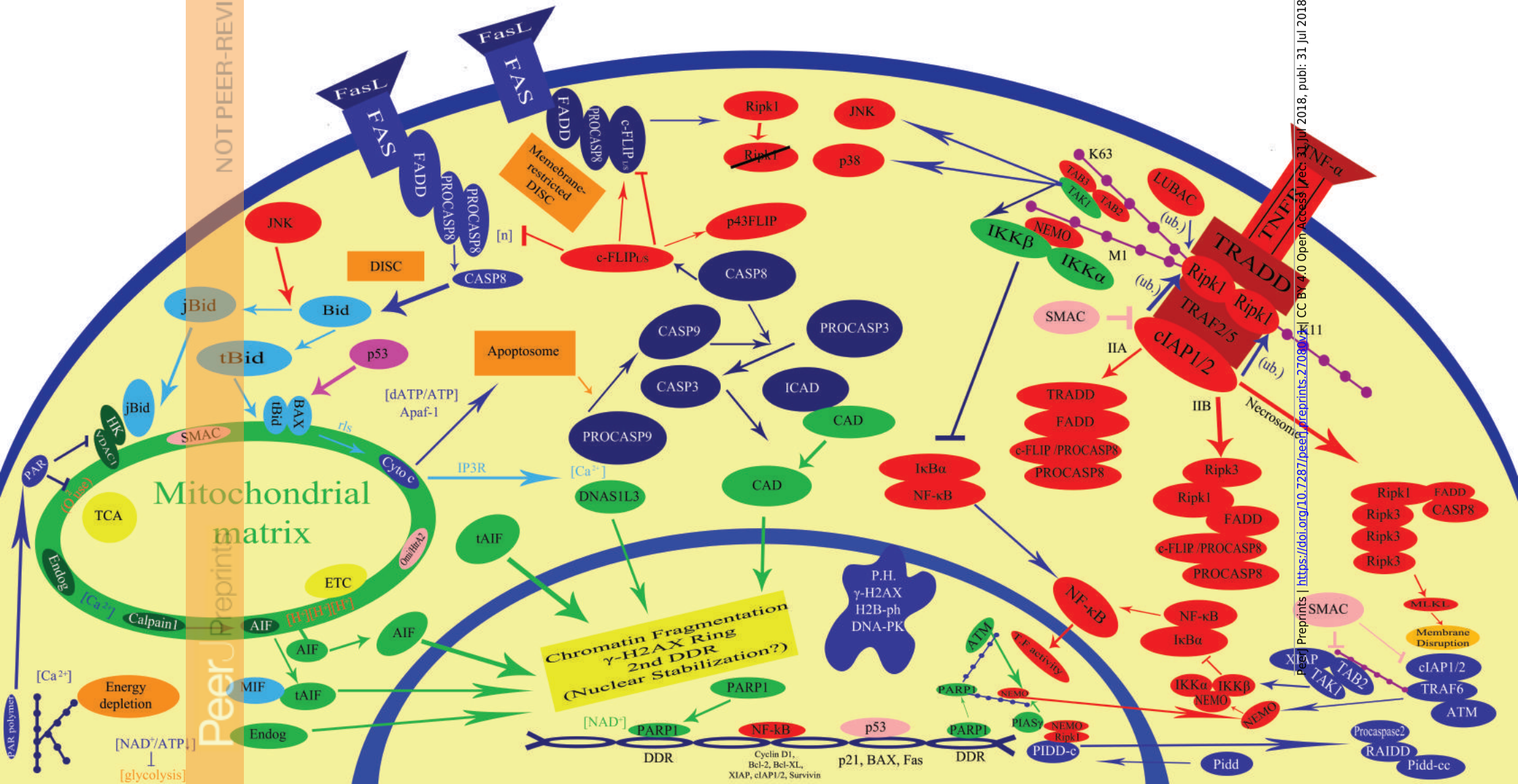
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