MECHANISMS AND REGULATION OF APOPTOTIC AND FERROPTOTIC PROGRAMMED CELL DEATH

by

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ABSTRACT

Programmed cell death is critical to the development and maintenance of multicellular organisms, and is also significant in the pathogenesis and treatment of various diseases. Several biochemically and morphologically unique forms of cell death have been defined in mammalian cells. This thesis examines the regulation and mechanism of two distinct cell death processes - apoptosis and ferroptosis.

Apoptosis is dependent on the activation of a family of serine proteases, known as caspases. The cell intrinsic pathway, one of two critical caspase activation pathways in mammals, is characterized by mitochondrial outer membrane permeabilization. This leads to release of cytochrome C into the cytosol, where it binds to the adaptor protein Apaf-1, and triggers formation of the apoptosome, a caspase activation complex. The cellular apoptosis susceptibility protein (CAS) can facilitate apoptosome assembly by stimulating nucleotide exchange on Apaf-1 following cytochrome C binding. Here, I describe a signaling cascade that induces upregulation of CAS during TRAIL-induced apoptosis. I find that upon activation, caspase-8 mediates degradation of cIAP1, an E3 ligase that targets CAS for ubiquitin-dependent proteasomal degradation. Additionally, using *in silico* analysis, I observe that CAS is overexpressed in a subset of human breast tumors, and provide evidence to suggest that this could sensitize these tumor cells to apoptotic insults.

In the second part of this dissertation, I describe our efforts in characterizing a novel cell death process induced by amino acid starvation in the presence of serum. We determine this process to be ferroptosis, a recently described form of non-apoptotic cell death characterized by an iron-dependent accumulation of reactive oxygen species. I subsequently explore the mechanism underlying this form of cell death, and find that ferroptosis is accompanied by gross morphological changes in the mitochondrial architecture of cells. Genetically or pharmacologically perturbing mitochondrial fission inhibits ferroptosis. Additionally, using live cell imaging, I observe that ferroptotic stimuli induce robust mitochondrial membrane hyperpolarization. Finally, I demonstrate that the presence of mitochondria with an intact membrane potential contributes to the execution of ferroptosis, downstream of lipid peroxidation.

BIOGRAPHICAL SKETCH

Prashant Monian was born in Mumbai, India. He spent most of his childhood in Dubai, U.A.E, and in 2003, relocated with his family to Chennai, India. Following graduation from high school in 2005, he moved to the United States and enrolled at the University of Georgia (Athens, Georgia). His first exposure to research was in the laboratory of Dr. Brian S. Cummings, where he spent two years studying the effects of cellular stress on phospholipids, and completed an honors thesis on the same. He graduated from the University of Georgia in 2009 with a double major in Cell Biology and Microbiology. In July 2009, Prashant moved to New York City, where he began the first year of his PhD at the Gerstner Sloan Kettering Graduate School of Biomedical Sciences. Following three rotations, in 2010, he joined the laboratory of Dr. Xuejun Jiang at Memorial Sloan Kettering Cancer Center.

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LIST OF ABBREVIATIONS

Apaf-1: Apoptotic protease activating factor-1

CAS: Cellular apoptosis susceptibility protein

DISC: Death-inducing signaling complex

FADD: FAS-associated death domain

GPX4: Gluathione peroxidase 4

GSH: Glutathione

IAP: Inhibitor of apoptosis protein

MEF: Mouse embryonic fibroblasts

MLKL: Mixed lineage kinase domain-like protein

MOMP: Mitochondrial outer membrane permeabilization

PARP: poly-ADP ribose polymerase

RIP1: Receptor interacting protein 1

RIP3: Receptor interacting protein 3

TRAIL: TNF-related apoptosis inducing ligand

TRADD: TNF receptor-associated death domain

TNF: Tumor necrosis factor

ROS: Reactive oxygen species

Z-VAD-FMK: benzyloxycarbonyl-VAD-fluoromethyl ketone

CHAPTER 1

INTRODUCTION

1.1 Programmed Cell Death: A Historical Perspective

Somewhat ironically, the process of cell death is essential to the development and maintenance of eukaryotic life. Cell death provides a counter-balance to cell proliferation, and is thus critical in shaping the structure and function of multicellular organs, by allowing for precise control of cell number. Historically, the death of cells was viewed as a purely “accidental” phenomenon induced by extreme environmental stress. However, as scientists began studying the process of development in various model organisms, evidence contradicting this idea began to appear. In 1842, Carl Vogt described the death of individual notochordal and cartilaginous cells as a normal part of amphibian embryogenesis (Vogt, 1842). Many years later, Lockshin and Williams demonstrated that the coordinated degradation of muscles cells during silkworm metamorphosis was orchestrated by a specific signaling system (Lockshin and Williams, 1964). They coined the term “programmed cell death” to describe the idea that a mechanism to kill specific cells at particular times is somehow programmed into the developmental plan of the organism. Pioneering work in the nematode *C. elegans* revealed that a small family of genes (including Egl-1, Ced-9, Ced-4 and Ced-3) mediates the precise and controlled execution of the same 131 (out of 1090) somatic cells during the maturation of each worm (Ellis et al., 1991). Collectively, these studies and others established the concept that cell death is

genetically encoded within the dying cell itself, and can be executed in response to a specific signal(s).

Since then, programmed cell death has been implicated in a wide variety of processes in adult organisms as well. Epithelial cells in the intestine undergo constant cycles of death and renewal (Barker, 2014). Lymphocytes undergo rapid expansion when participating in host defense, but then contract to resting state numbers thereafter (Rathmell and Thompson, 2002). Not surprisingly, cell death can also have deleterious effects and is involved in various human pathologies including autoimmune diseases, neurological disorders, ageing and cancer (Thompson, 1995).

Historically, cell death has been divided into three types based on morphological features: type I (apoptosis), type II (autophagy), and type III (necrosis). Apoptosis and autophagy were considered to be “programmed”, while necrosis was believed to be unregulated (Schweichel and Merker, 1973). However, these traditional lines of classification have increasingly blurred with the discovery of additional forms of regulated cell death (Galluzzi et al., 2015). Furthermore, it is now well appreciated that necrosis itself can be a regulated process (Berghe et al., 2014). Ultimately, studying the signaling pathways controlling the different cell death forms will help understand how they are interconnected at the molecular level, and refine our understanding of their respective physiological relevance.

1.2 Apoptosis

For decades, programmed cell death has been synonymous with apoptosis. First described in 1972, apoptosis is now recognized as a biochemically and morphologically unique form of cell death, characterized by cell shrinkage, chromatin condensation, membrane blebbing and fragmentation of the cell into apoptotic bodies (Kerr et al., 1972). Plasma-membrane integrity is maintained throughout this process, and the apoptotic bodies are eventually eliminated by phagocytosis, thus preventing an inflammatory reaction.

Apoptosis was eventually linked to the programmed cell death pathway observed in *C. elegans* development (Vaux et al., 1992). Subsequently, mammalian orthologs of the *C.elegans* death proteins were identified and shown to possess similar functions: *Ced-9* was linked to the Bcl-2 family, *Ced-4* to Apaf-1 and *Ced-3* to the caspase family (Vaux and Korsmeyer, 1999). A similar set of apoptotic regulators were also identified in the fruit fly *Drosophila melanogaster*, establishing that the core machinery of this pathway is conserved across metazoans (Fuchs and Steller, 2011). It should however be noted that there are clear differences in the relative importance and function of individual proteins in the pathway between mammals and invertebrates (Kornbluth and White, 2005).

In mammals, apoptosis appears to be the most common form of cell death during development. It is required for many diverse events including the elimination of vestigial structures, formation of digits and luminal structures, and

negative selection of lymphocytes, among many others (Jacobson et al., 1997). Thus, it is not surprising that deletion of the core apoptotic proteins including Apaf-1, caspase-9 and caspase-3 leads to perinatal lethality (Kuida et al., 1996; Kuida et al., 1998; Yoshida et al., 1998).

Aberrations in apoptosis have been implicated in various human pathologies. Evasion of apoptosis has been recognized as a hallmark of cancer, since it provides tumors cells the opportunity to proliferate unchecked (Hanahan and Weinberg, 2011). In fact, one of the most common alterations in tumors is inactivation or loss of the tumor suppressor p53, which is instrumental in directing cells to die in response to toxic accumulation of DNA damage or other chromosomal abnormalities (Levine, 1997). Tumor cells also upregulate potent anti-apoptotic genes such as members of the Bcl-2 and IAP family of proteins, which confer resistance to various apoptotic signals (Yip and Reed, 2008; Gyrd-Hansen and Meier, 2010). Consequently, promoting apoptosis is a key strategy in developing chemotherapeutic agents. Conversely, excessive apoptosis is detrimental as well, particularly when the dying cells are not easily replaced. For example, the loss of neurons in neurodegenerative diseases such as Alzheimer's, Parkinson's and Huntington's diseases stems from over activation of apoptotic pathways (Mattson, 2000). In these scenarios, inhibiting apoptosis is likely to provide therapeutic benefit (Waldmeier, 2003).

The successful execution of apoptosis is dependent on the activity of a family of cysteine-dependent aspartate-specific proteases, termed caspases. The role of caspases in apoptosis first became apparent when it was discovered that the *C. elegans Ced-3* protein was homologous to the mammalian interleukin-1ß-converting enzyme, a cysteine protease now known as caspase-1 (Yuan et al., 1993). Ironically, this founding member of the caspase family does not appear to possess any direct apoptotic function, but rather plays a role in inflammation and an alternate form of cell death termed pyroptosis (Franchi et al., 2009). To date, eleven human and ten murine caspases have been identified, and can be functionally divided into apoptotic (caspase-2, -3, -6, -7, -8, -9,-10) and inflammatory (caspase-1, -4, -5, -11, and -12) subfamilies (Li and Yuan, 2008). However this is likely an oversimplification, since caspases clearly play a role in other processes like cellular differentiation that cannot be classified as inflammatory or apoptotic (Shalini et al., 2015). Additionally, certain caspases appear to be involved in both processes, as well as in non-apoptotic forms of programmed cell death (Galluzzi et al., 2016).

An alternate way to classify caspases is based on their structure and mode of activation. All caspases are synthesized as catalytically inactive zymogens and must undergo proteolytic processing in order to be activated during apoptosis (Riedl and Shi, 2004). Initiator caspases (caspase-1, -2, -4, -5, -8, -9, -10, -11, -12) possess long prodomains that contain either a death effector domain (DED) or a caspase-activation and recruitment domain (CARD). These

domains mediate the recruitment of the initiator caspases to specific adaptor proteins, which is believed to increase the local concentration of the caspase, and thus promote proximity induced homodimerization and proteolytic processing (Thornberry and Lazebnik, 1998; Degterev et al., 2003). In contrast, executioner caspases (caspase-3, -7, -8) contain a shorter prodomain and exist as dimers even in their inactive state. Their activation is mediated through direct cleavage by upstream initiator caspases, which induces a conformational change that favors formation of the catalytic site (Pop and Salvesen, 2009). Active executioner caspases cleave a variety of cellular substrates resulting in the inactivation of apoptotic inhibitors, disassembly of cellular structures, and shutdown of various metabolic and homeostatic functions, thus preparing the cell for its demise.

As is to be expected, the regulation of apoptosis in mammals is more complex relative to lower organisms. Research over the decades has identified two primary mammalian apoptotic pathways: the cell intrinsic or mitochondrial-mediated pathway, and the cell-extrinsic or death receptor-mediated pathway (Figure 1.1). In the following sections, I describe the signaling cascades underlying these pathways in detail.

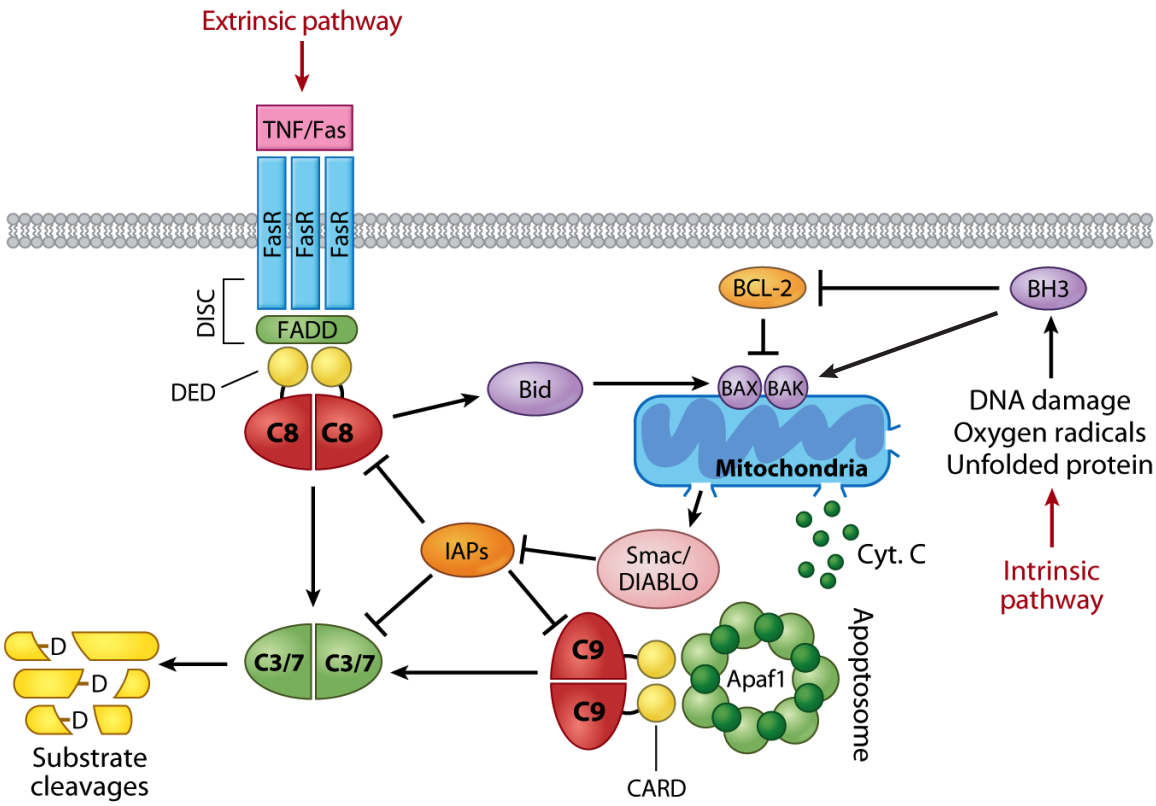

Figure 1.1. Intrinsic and extrinsic pathways of apoptosis in mammalian cells. In the intrinsic pathway, cellular damage signals act through BH3-only proteins to activate Bax and Bak, leading to mitochondrial outer membrane permeabilization (MOMP). This results in the release of cytochrome C (Cyt. C) and subsequent formation of the apoptosome, a scaffold for activating caspase-9 (C9). Caspase-9 goes on to activate the effector caspases-3 and -7 (C3/7). MOMP also leads to the release of Smac/DIABO which antagonize the IAP family of caspase inhibitors. In the extrinsic pathway, extracellular ligands (such as Fas or TNF-α) engage their cognate cell surface receptor, leading to the recruitment of adaptor molecules such as FAS-associated death domain protein (FADD), and formation of a death-inducing signaling complex (DISC). Caspase-8 (C8) is activated by association with the DISC and goes on to activate effector caspases-3 and -7 directly. Caspase-8 can also cleave Bid, which leads to MOMP by Bax and Bak, thus engaging the intrinsic pathway as well. Figure is modified from Crawford ED, Wells JA. (2011) Caspase substrates and cellular remodeling. Annu Rev Biochem. 80, 1055-87.

Intrinsic apoptosis pathway

The cell intrinsic pathway is characterized by its dependence on the mitochondria as the sentinel of death (Jiang and Wang, 2004). Upon sensing signals like DNA-damage or endoplasmic reticulum (ER)-stress, mitochondria undergo outer membrane permeabilization, allowing for the release of cytochrome C from the intermembrane space (Liu et al., 1996). In the cytosol, cytochrome C binds to the essential mediator Apaf-1, triggering the formation of the heptameric protein complex, the apoptosome (Zou et al., 1997). The apoptosome recruits the initiator caspase, caspase-9, leading to its dimerization and subsequent activation (Zou et al., 1999). Active caspase-9 then directly cleaves and activates the executioner caspases-3 and -7, which target a variety of substrates, ultimately leading to cellular demise (Li et al., 1997).

The process of mitochondrial outer membrane permeabilization (MOMP) is intricately regulated by the Bcl-2 family of proteins, which includes both pro- and anti-apoptotic members. The pro-apoptotic Bcl-2 family members can be further divided into two subfamilies: effectors and BH3-only proteins. The effectors (Bax, Bak and Bok) contain four Bcl-2 homology (BH1-4) domains. Upon activation, Bax and Bak undergo conformational changes leading to homo-oligomerization and insertion into the mitochondrial outer membrane. Bok appears to be a non-canonical effector, activated specifically in response to ER-stress (Llambi et al., 2016). The BH3-only proteins contain a single BH3 domain and include Bid, Bim, Bad, Puma, Noxa, Bik, Mule, Bmf and Hrk. Upon activation

by death signals, they stimulate MOMP either by directly inducing oligomerization of Bax and Bak (direct activators), or by inhibiting the anti-apoptotic Bcl-2 repertoire (sensitizers). The anti-apoptotic members of the family (Bcl-2, Bcl-xL, Bcl-w, Mcl-1 and A1) also contain four BH domains, and mediate their effect by directly inhibiting the pro-apoptotic family members (Youle and Strasser, 2008; Chipuk et al., 2010). Thus, the Bcl-2 family of proteins acts as an internal "conscience" of the cell, by sensing intracellular stress and relaying these signals to the mitochondria to determine cell fate.

Extrinsic apoptosis pathway

The cell extrinsic pathway is triggered by transmembrane "death receptors", primarily of the tumor necrosis factor (TNF) superfamily (Ashkenazi and Dixit, 1998). Well-characterized apoptosis inducing members of this family include TNF-α, CD95/FAS ligand, and TRAIL. Binding of their respective extracellular ligands triggers the clustering of these death receptors on the cell surface, and promotes association of their death domains on the cytoplasmic side. This in turn leads to recruitment of other cytoplasmic adaptor proteins with their own death domains including FADD (FAS-associated death domain) and TRADD (TNF receptor-associated death domain), which recruit the initiator caspases-8 and -10 to form the death-inducing signaling complex (DISC). At the DISC, caspases-8 and -10 are activated through autocatalytic processing, and can then directly cleave and activate the executioner caspases -3 and -7 (Wilson et al., 2009; Ashkenazi and Salvesen, 2014). Activation of caspase-8 can be

inhibited by the cellular FLICE inhibitory protein (c-FLIP), a decoy protein that is structurally similar to caspase-8 but lacks a functional protease. c-FLIP thus competes with and displaces caspase-8 from binding FADD at the DISC (Irmler et al., 1997; Scaffidi et al., 1999a).

In parallel with activating caspase-3, caspase-8 can also cleave the BH3-only protein Bid, inducing its translocation to the mitochondria, where it can induce MOMP through mechanisms discussed earlier, and thus activate the cell intrinsic pathway as well (Li et al., 1998). Intriguingly, in some cell types (type I cells, e.g. lymphocytes), induction of MOMP by Bid is dispensable for apoptosis induced by death ligands. In contrast, type II cells (eg. hepatocytes) rely on MOMP for successful execution of the extrinsic apoptosis pathway. Thus, overexpression of anti-apoptotic Bcl-2 family members is sufficient to inhibit death-receptor mediated apoptosis in type II cells (Scaffidi et al., 1998; Scaffidi et al., 1999b). The reason for this dichotomy is linked to expression of the X-linked inhibitor of apoptosis protein (XIAP) (Jost et al., 2009), a direct inhibitor of both caspase-3/7 and caspase-9 (Deveraux et al., 1997; Srinivasula et al., 2001). XIAP activity in-turn is inhibited by SMAC/DIABLO and Omi/HtrA2, proteins that are released from the mitochondria following MOMP (Du et al., 2000; Verhagen et al., 2000; Suzuki et al., 2001). Thus MOMP mediated XIAP-antagonism is necessary for death receptor-mediated apoptosis in type-II cells.

Remarkably, in some cell lines, if caspase-8 activity is inhibited pharmacologically or genetically, activation of certain death receptors (including FAS and TNFR1) can promote a programmed necrosis pathway termed necroptosis instead (Vandenabeele et al., 2010). Under these circumstances, death receptor stimulation leads to the formation of the necrosome, a protein complex containing the kinases RIP1 and RIP3 (Degterev et al., 2008; He et al., 2009; Zhang et al., 2009; Li et al., 2012). The formation of the necrosome is regulated by trans-phosphorylation of RIP1 and RIP3 as well as ubiquitin-editing of RIP1 by various E3 ligases and deubiquitinases (Cho et al., 2009; Vandenabeele et al., 2010). At the necrosome, RIP3 phosphorylates the psuedokinase MLKL, promoting its oligomerization and translocation to the plasma membrane. Once at the plasma membrane, MLKL oligomers disrupt membrane integrity and induce necrotic cell death (Murphy et al., 2013; Cai et al., 2014; Wang et al., 2014; Quarato et al., 2016). It is believed that active caspase-8 inhibits necroptosis, likely through cleavage of key substrates involved in the pathway, including RIP1, RIP3 and the deubiquitinase CYLD (Lin et al., 1999; Feng et al., 2007; O'Donnell et al., 2011; Oberst et al., 2011).

Several of the death ligands also promote signaling pathways linked to cell proliferation including the NF-κB, c-Jun and MAPK pathways (Ashkenazi and Salvesen, 2014). The death receptor is thus a key signaling node that can mediate apoptotic, necroptotic, and survival pathways depending on cellular context and co-stimulatory signals.

In summary, apoptosis is a critical programmed cell death process with wide ranging physiological implications. While the core apoptotic machinery has been well characterized, several novel regulators of the pathways continue to be identified. Furthermore, understanding the functional interplay between apoptosis and other cell death pathways remains an exciting area of research.

1.3. Non-apoptotic programmed cell death.

Historically, necrosis or "type III" cell death was considered to be an accidental process, caused by extreme physio-chemical stress. The first hints that necrosis can be regulated came from studying caspase-8 independent cell death in response to death ligands like TNFR and FAS. The kinase RIP1 was subsequently identified as a key effector of this new cell death process, now termed necroptosis (Holler et al., 2000). Small molecule screening later led to the discovery of necrostatin-1, a chemical inhibitor of necroptosis (Degterev et al., 2005). Further experiments revealed RIP1 to be the primary cellular target of necrostatin-1, lending further credibility to the idea that necrosis can be a programmed or regulated process (Degterev et al., 2008).

Concurrent with the characterization of necroptosis, several other forms of regulated, non-apoptotic cell death have been described. This includes anoikis (Frisch and Francis, 1994), parthanatos (Yu et al., 2002), entosis (Overholtzer et al., 2007), NETosis (Steinberg and Grinstein, 2007), ETosis (Wartha and Henriques-Normark, 2008), pyroptosis (Bergsbaken et al., 2009), ferroptosis

(Dixon et al., 2012), and autosis (Liu et al., 2013). It is important to note that while these cell death processes are triggered by distinct stimuli, the associated signaling pathways are highly interconnected (Berghe et al., 2014; Galluzzi et al., 2014). Thus, the term “necrosis” can be considered to encompass several different modes of cell death that share common morphological features such as cell rounding, swelling, and bioenergetic failure. Elucidating the molecular events leading from the initial trigger to cell death in each of these pathways should ultimately help develop therapeutic methods of modulating necrosis *in vivo*. In the following section, I describe one such form of programmed necrosis that is a focus of my dissertation.

1.4. Ferroptosis

While genetic and biochemical approaches have been frequently employed in cell death studies, small molecules have been an invaluable tool in identifying and characterizing programmed cell death pathways as well. Notable examples include the use of staurosporine (a broad spectrum kinase inhibitor) in the early study of apoptosis (Bertrand et al., 1994), the identification of necrostatin-1, which confirmed the programmed nature of TNF-induced necrosis (Degterev et al., 2005), and the subsequent use of necrosulfonamide to identify MLKL as an essential downstream mediator of this death process (Sun et al., 2012).

In the 2000's, the Stockwell lab used chemical genetic screens to isolate several compounds that displayed synthetic lethality with oncogenic RAS. These include erastin (to date, the best characterized one) (Dolma et al., 2003), and the structurally distinct compounds RSL3 and RSL5 (Yang and Stockwell, 2008). Erastin-induced cell death did not show any of the morphological or biochemical features associated with apoptosis such as chromatin condensation, nuclear fragmentation, MOMP, caspase activation, or PARP cleavage, clearly indicating this is a non-apoptotic cell death process (Yagoda et al., 2007). Subsequent studies revealed that antioxidants such as Vitamin E, and iron-chelators such as deferoxamine could inhibit cell death induced by these compounds (Yang and Stockwell, 2008), indicating that this cell death process is dependent on the formation of reactive oxygen species (ROS) and the presence of labile iron. The name ferroptosis was thus coined to describe this form of iron-dependent oxidative cell death (Dixon et al., 2012).

Mechanism of ferroptotic cell death

An early study identified the mitochondrial voltage-dependent anion channels VDAC2 and VDAC3 as the direct cellular targets of erastin using an affinity purification approach (Yagoda et al., 2007). However, subsequent work has demonstrated that the ability of erastin to induce cell death is likely dependent on in its inhibition of the cystine/glutamate antiporter, termed system x_c^- (Dixon et al., 2012; Dixon et al., 2014). System x_c^- is a cell surface amino acid transporter, which imports extracellular cystine in exchange for intracellular

glutamate (Sato et al., 1999). In the cell, cystine is reduced to the amino acid cysteine, a precursor for the synthesis of glutathione (GSH), a key cellular antioxidant (Lu, 2013). Thus, erastin treatment leads to cellular cysteine starvation and concomitant GSH depletion, thereby exposing the cells to excessive ROS accumulation.

RSL3 was shown to induce ferroptosis independent of system x_c^--inhibition or GSH depletion (Dixon et al., 2012), suggesting the existence of a key modulator further downstream in the pathway. Affinity purification revealed that RSL3 targets glutathione peroxidase 4 (GPX4) (Yang et al., 2014). GPXs are a family of GSH-dependent antioxidant enzymes that catalyze the reduction of hydrogen peroxides or organic hydroperoxides to water or the corresponding alcohols (Brigelius-Flohé and Maiorino, 2013). Erastin and RSL3 thus represent two distinct classes of ferroptosis inducers based on mechanism of action: while class 1 compounds like erastin induce depletion of cystine and GSH leading to GPX4 inactivation, class II compounds like RSL3 directly inhibit GPX4 without affecting cellular cystine or GSH levels (Cao and Dixon, 2016) (Figure 1.2).

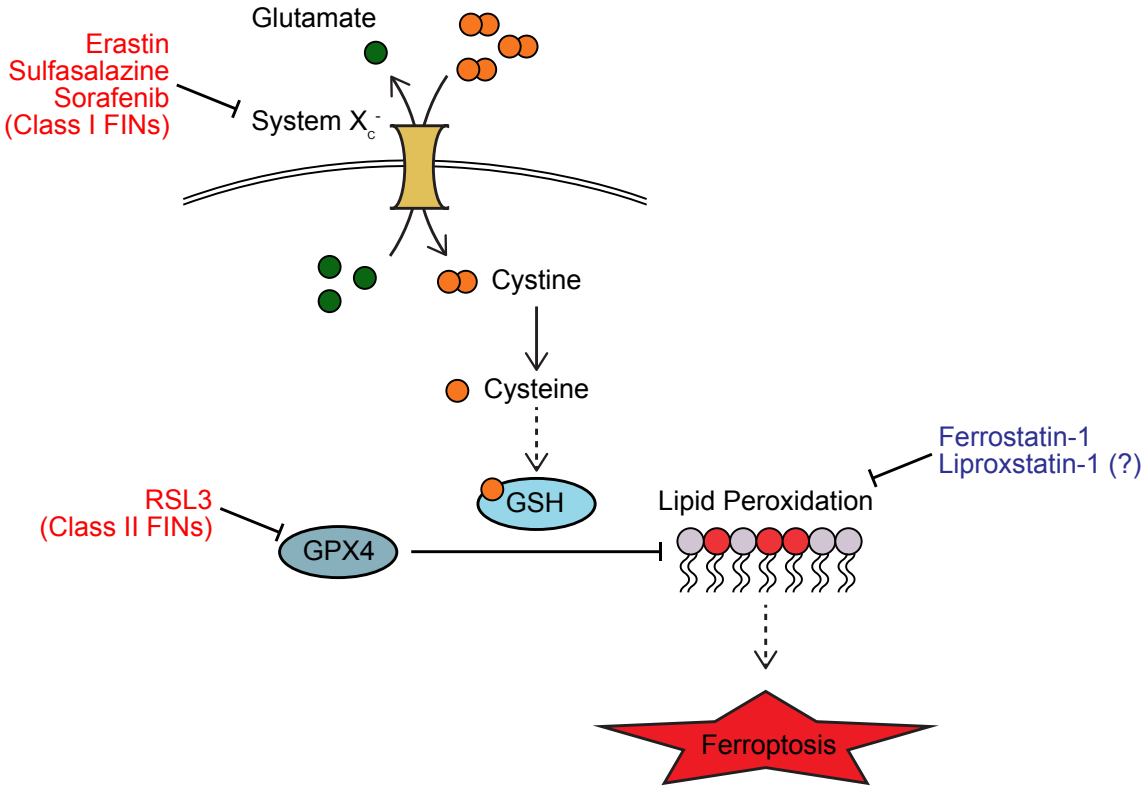

Figure 1.2. Overview of the ferroptosis pathway.
System x_c^- exchanges one molecule of intracellular glutamate for one molecule of extracellular cystine. Inside the cell, cystine is reduced to the amino acid cysteine, which is a key ingredient in the synthesis of GSH. GPX4 is a key antioxidant enzyme that reduces lipid peroxides in a GSH-dependent manner. Class I ferroptosis inducing compounds (FINs) inhibit system x_c^- leading to cystine starvation and GSH depletion, while class II FINs inhibit GPX4 directly. Both lead to lethal accumulation of lipid ROS in an iron-dependent manner, which ultimately induces ferroptosis. Inhibitors of lipid peroxidation such as ferrostatin-1 can inhibit ferroptosis. The precise mechanism of liproxstatin-1, another ferroptosis inhibitor, is unknown, but structural studies suggest it may also function as a lipophilic antioxidant.

Consistent with a central role for cystine, GSH, and GPX4 in mediating ferroptosis, other stimulants targeting this pathway have been described to induce ferroptosis as well. These include buthionine-(S,R)-sulfoximine (BSO) which directly inhibits GSH synthesis (Yang et al., 2014), as well as the clinically approved drugs sulfasalazine (Dixon et al., 2012) and sorafenib (Dixon et al., 2014), which inhibit system x_c^-. Additionally, the master tumor suppressor p53 has been shown to sensitize cells to ferroptosis by repressing expression of SLC7A11 (an essential component of the system x_c^- transporter), thus inhibiting cystine uptake (Jiang et al., 2015).

How does system x_c^- inhibition or GPX4 depletion lead to cell death? GPX4 is unique in its ability to reduce oxidized phospholipids and cholesterol hydroperoxides (Ursini et al., 1982). Consistently, ferroptosis is accompanied by the accumulation of lipid-based ROS derived from oxidation of poly-unsaturated fatty acids (Dixon et al., 2012; Dixon et al., 2014). Notably, small molecules that neutralize lipid ROS can protect cells from ferroptosis, highlighting the importance of lipid peroxidation in this process. Such ferroptosis inhibitors include ferrostatin-1 (Skouta et al., 2014) and possibly, liproxstatin-1 (Friedmann Angeli et al., 2014). These inhibitors appear to be specific to ferroptosis, as they do not inhibit death induced by classic apoptotic and necroptotic insults.

The precise cellular site of lipid ROS generation during ferroptosis is yet to be defined. Furthermore, the molecular events that follow lipid peroxidation are

also under investigation. Possible explanations include irreversible damage to the plasma membrane, or alternatively, inactivation of essential cellular proteins by reactive lipid intermediates (Cao and Dixon, 2016). Finally, although the requirement for intracellular iron in ferroptosis has been thoroughly established, how precisely iron contributes to cell death remains unclear. One proposed model is that iron is involved in the generation of lipid ROS, either as a direct electron donor through Fenton chemistry, or via the action of iron-dependent oxidases (Dixon and Stockwell, 2014; Yang and Stockwell, 2016). However, a redox-independent role for iron has not been ruled out either.

Physiological relevance of ferroptosis

The role of ferroptosis in development is perhaps best understood in the context of GPX4, whose activity appears to be essential to prevent accumulation of lipid ROS in the cell. Inducible knockout of GPX4 in cell culture was found to be sufficient to induce ferroptosis, while constitutive knockout in mice leads to embryonic lethality (Yant et al., 2003; Seiler et al., 2008). Similarly, conditional knockout of GPX4 in adult animals induces ferroptosis in various tissues; neurons (Yoo et al., 2012), renal tubule cells (Friedmann Angeli et al., 2014), and T-cells (Matsushita et al., 2015) have been shown to be especially susceptible. Thus, unlike apoptosis, which is essential for various developmental processes, current evidence suggests ferroptosis needs to be actively suppressed to ensure normal development.

Several studies using ferroptosis inhibitors such as ferrostatin-1, improved ferrostatin analogs, and liproxstatin-1 have pointed towards a role for ferroptosis in various pathological cell death scenarios. These include Huntington's disease, acute ischemia-reperfusion induced injury of the kidney, and periventricular leukomalacia (Linkermann et al., 2014; Skouta et al., 2014). Work from our own lab has implicated ferroptosis in heart injury induced by ischemia-reperfusion as well (Gao et al., 2015). While it is difficult to accurately estimate the specific contribution of ferroptosis to cell loss in these scenarios, these studies do suggest that inhibiting ferroptosis *in vivo* could be a promising therapeutic approach to treat such conditions.

While increased ferroptosis is highly detrimental to normal tissue, inducing ferroptosis poses to be a promising approach in cancer therapy. The ability of ferroptosis inducers to specifically kill RAS-mutant cells has been called into question, given that in a panel of 117 cancer cell lines, there was no significant correlation between RAS mutation status and erastin sensitivity (Yang et al., 2014). Yet, the same study found that cells from certain types of cancers including diffuse large B cell lymphoma and renal cell carcinoma are intrinsically more sensitive to ferroptosis. Further, it is worth noting that the ferroptosis-promoting ability of p53 significantly contributes to its tumor-suppressive function *in vivo* (Jiang et al., 2015),

In summary, ferroptosis has emerged as an intriguing form of non-apoptotic programmed cell death, characterized by the requirement of iron and the generation of lipid ROS. Current efforts are directed at further understanding the basic mechanism of ferroptosis, as well as developing both inducers and inhibitors of this process that could ultimately be used to provide therapeutic benefit in the clinic.

1.5. Thesis Objectives

The aim of this dissertation is to better understand the mechanisms underlying both apoptotic and ferroptotic cell death. In Chapter 2, I investigate the regulation of apoptosis post-MOMP. Previous work has demonstrated that the cellular apoptosis susceptibility (CAS) protein stimulates apoptosome formation following cytochrome C release. I characterize the mechanism by which CAS levels are regulated following an apoptotic insult, and also briefly explore the effect of oncogenic transformation on CAS. In Chapter 3, I interrogate the molecular mechanism underlying ferroptosis. My interest in this project was triggered by a serendipitous cell death process observed in our lab, which we eventually determine to be ferroptosis. I subsequently elucidate a functional role for mitochondria in ferroptosis. Finally, in Chapter 4, I discuss the implications and unanswered questions stemming from work presented in this dissertation, and propose directions for future research to better understand how cells make the difficult transition from life to death.

CHAPTER 2

REGULATION OF THE CELLULAR APOPTOSIS SUSCEPTIBILITY PROTEIN (CAS) DURING APOPTOSIS

2.1. Introduction

Induction of MOMP by the Bcl-2 family of proteins is a signature event during mitochondria-mediated apoptosis, and generally has several cellular consequences. MOMP is associated with release of several factors from the mitochondrial intermembrane space that can induce caspase activation (eg. cytochrome C) as well as caspase-independent cell death (eg. endonuclease G, apoptosis inducing factor-AIF) (Susin et al., 1999; Li et al., 2001). Additionally, loss of outer membrane integrity leads to an eventual decline in membrane potential and loss of key mitochondrial activities including ATP synthesis and mitochondrial protein import, while also contributing to increased reactive oxygen species (ROS) production (Waterhouse et al., 2001; Ricci et al., 2004). Given these dramatic consequences, MOMP has been postulated to be a “point-of-no-return” for cell death, i.e. following MOMP, cells are committed to die regardless of caspase activation (Green and Kroemer, 2004). However, while this may be true in some cases, several lines of evidence contradict this claim. For instance, cells lacking Apaf-1 or caspase-9 (essential components for cytochrome-c dependent caspase activation) undergo MOMP but are highly resistant to various apoptotic insults *in vitro*, and loss of these proteins dramatically enhances tumor growth *in vivo* (Cecconi et al., 1998; Hakem et al., 1998; Kuida et al., 1998; Yoshida et al., 1998; Soengas et al., 1999; Jia et al., 2001). Neurons undergo

cytochrome C release upon nerve growth factor (NGF)-withdrawal, but can be protected from cell death by pharmacological or genetic inhibition of caspases, and completely recover following NGF re-stimulation (Martinou et al., 1999; Deshmukh et al., 2000). Such recovery from late stage apoptosis upon removal of the apoptotic insult has also been observed in a variety of other primary and immortalized cell lines (Tang et al., 2012). Unsurprisingly, inhibition of caspase-3 activity has been identified as a key means to survive MOMP, as this allows cells to restore their mitochondrial network from the remaining pool of healthy mitochondria (Colell et al., 2007; Tait et al., 2010).

The ability to survive MOMP has several important physiological implications. Firstly, it provides a mechanism to protect cells against “accidental” MOMP. This is particularly relevant in the context of long-lived, post-mitotic cells like cardiomyocytes and neurons. Both these cell types express low levels of Apaf-1 upon differentiation and have the ability to survive MOMP due to decreased apoptosome formation (Potts et al., 2003; Sanchis et al., 2003; Wright et al., 2004; Potts et al., 2005). Furthermore, caspase-3 and -9 are involved in several non-apoptotic processes (Tang et al., 2015) including differentiation of various cell types (Ishizaki et al., 1998; Weil et al., 1999; Fernando et al., 2002; Fernando et al., 2005; Murray et al., 2008), development and maintenance of neuronal function (Campbell and Holt, 2003; Huesmann and Clayton, 2006; Li et al., 2010), and proliferation and maturation of immune cells (Woo et al., 2003; Santambrogio et al., 2005). The low levels of caspase-3 activation observed in these scenarios is not lethal, but rather leads to changes in cell shape or

function, presumably resulting from cleavage of specific substrates. In the context of oncogenesis, tumor cells often evolve mechanisms of inhibiting caspase-3 activation downstream of MOMP, including down regulation or loss of Apaf-1 (Soengas et al., 2001; Wolf et al., 2001) or caspase-3 (Devarajan et al., 2002), and overexpression of inhibitors of apoptosis proteins (IAPs) (Tamm et al., 2000; Ferreira et al., 2001). Overcoming chemotherapy or radiation induced MOMP by limiting caspase-3 activation can facilitate tumor cell survival and has obvious clinical implications. Low levels of caspase-3 activation can also directly promote tumorigenesis through genomic instability in the absence of cell death (Ichim et al., 2015; Liu et al., 2015). Finally, it is worth noting that even in cases where MOMP is sufficient to trigger cell death, caspase-3 activity is essential in preventing an immune response *in vivo* (Rongvaux et al., 2014; White et al., 2014). Collectively, these findings underscore the importance of understanding how caspase activation is regulated post-MOMP.

Mechanisms to regulate caspase activity post-MOMP include direct inhibition of caspase-3/7 and caspase-9 by XIAP (Deveraux et al., 1997; Srinivasula et al., 2001), as well as XIAP-antagonism by SMAC/DIABLO and Omi/HtrA2 which are released from the mitochondria following MOMP (Du et al., 2000; Verhagen et al., 2000; Suzuki et al., 2001). Caspase-9 is also subject to inhibitory phosphorylation by various kinases including Erk1/2, Akt, CDK1 (Cardone et al., 1998; Allan et al., 2003; Allan and Clarke, 2007).

Regulating apoptosome formation is another critical means by which caspase-3 activity can be controlled following MOMP. After binding cytochrome C, Apaf-1 undergoes nucleotide exchange, where bound dADP is exchanged for dATP. This is a necessary step for apoptosome formation, and in the absence of exogenous dATP, cytochrome-C bound Apaf-1 forms nonfunctional aggregates (Kim et al., 2005). Previous work in our laboratory demonstrated that the cellular apoptosis susceptibility protein (CAS), functioning together with PHAPI and Hsp70, stimulates apoptosome formation by enhancing nucleotide exchange on Apaf-1 following cytochrome C-binding (Kim et al., 2008) (Figure 2.1). CAS was first identified in a screen for factors that inhibit cell death induced by various naturally occurring toxins, consistent with its ability to promote apoptosome formation (Brinkmann et al., 1995).

In this chapter, I characterize a novel signaling pathway that regulates the expression of CAS during apoptosis. Using TRAIL as an apoptotic stimulus, I demonstrate that active caspase-8 induces a feed-forward cascade that leads to upregulation of CAS and thus amplifies the commitment to cell death following MOMP. I conclusively identify cIAP1 as an E3 ligase that targets CAS for ubiquitin-mediated proteasomal degradation. Furthermore, I explore the role of CAS in cancer cell growth and apoptosis. This work has been published in the *Journal of Biological Chemistry* (Monian and Jiang, 2016).

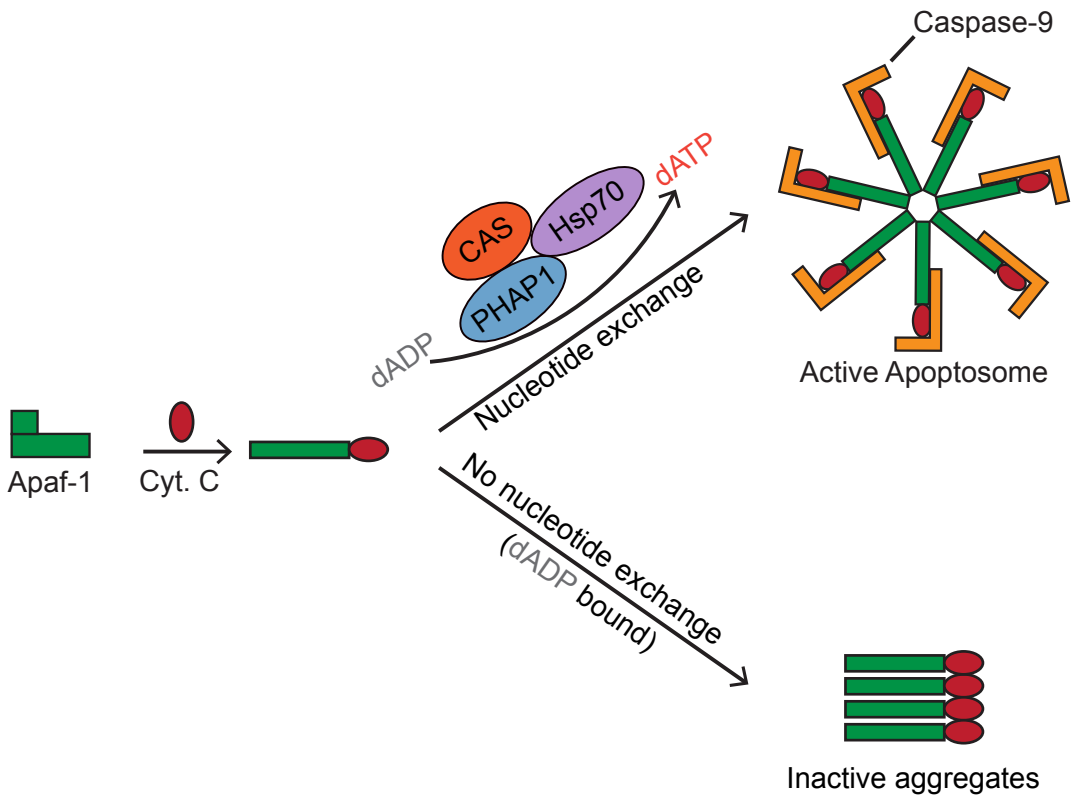

Figure 2.1. Model for apoptosome formation.
Apaf-1 is present as inactive monomer in resting cells. Upon binding cytochrome c, Apaf-1 undergoes a conformational change and subsequent nucleotide exchange, leading to efficient apoptosome formation and caspase activation. However, in the absence of exogenous dATP, Apaf-1 adapts a permanently inactive conformation. CAS, PHAP1 and Hsp70 stimulate nucleotide exchange on Apaf-1 and thus drive apoptosome formation.

2.2. Results

CAS promotes TRAIL-induced apoptosis

Previous work by our lab demonstrated that knockdown of CAS rendered cells resistant to specific apoptotic stimuli including UV-irradiation and stimulation with TNF-α and IFN-γ (Kim et al., 2008). Intriguingly, these same stimuli also cause an upregulation of CAS at the protein level. Meanwhile, apoptosis induced by staurosporine remained unaffected by knockdown of CAS, and staurosporine failed to induced upregulation of CAS. This striking correlation suggests that upregulation of CAS plays a functional role in mediating the cellular response to select apoptotic stimuli. I sought to define the regulatory pathway that controls the regulation of CAS. In search of an ideal experimental system to study this pathway, I found that stimulation of MCF-10A cells with TRAIL (TNF related apoptosis-inducing ligand) induces a rapid and robust upregulation of CAS (Figure 2.2). TRAIL is a member of the TNF superfamily of cytokines, and has been investigated extensively as a therapeutic agent, particularly in the context of autoimmunity and cancer (Cretney et al., 2006; Johnstone et al., 2008).

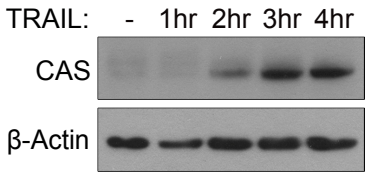

Figure 2.2. TRAIL induces robust upregulation of CAS
MCF10A cells were treated with 30 ng/ml TRAIL and harvested for western blot analysis at the indicated time points.

To confirm that CAS plays a role in TRAIL-induced apoptosis, I used siRNA to silence CAS expression. Relative to control siRNA, I found that knockdown of CAS significantly inhibited TRAIL induced caspase-3/7 activation in multiple cell lines tested (Figure 2.3A and B). Consistently, overall cell viability following TRAIL-treatment was greater in CAS-siRNA transfected cells (Figure 2.3C and D), confirming that CAS facilitates TRAIL-induced apoptosis by stimulating caspase activation.

TRAIL induced upregulation of CAS is caspase-8 dependent, but MOMP-independent

TRAIL induces apoptosis via the cell-extrinsic pathway, through activation of the initiator caspase-8, followed by the executioner caspases-3/7. I hypothesized that activation of one or more of these caspases plays an essential role in upregulating CAS upon TRAIL treatment. Indeed, I found that co-treatment with a pan-caspase inhibitor (ZVAD-FMK) completely blocked TRAIL-induced CAS upregulation (Figure 2.4A). Furthermore, specific inhibition (Figure 2.4A) or shRNA-mediated knockdown of caspase-8 (Figure 2.4B) inhibited increase in CAS-levels upon TRAIL-treatment. However, use of a caspase-3 specific inhibitor failed to inhibit CAS upregulation (Figure 2.4B). All three inhibitors completely abolished downstream activation of caspase-3, confirming their respective efficacy. These results demonstrate that TRAIL-induced CAS upregulation is caspase-8 dependent, but caspase-3 independent.

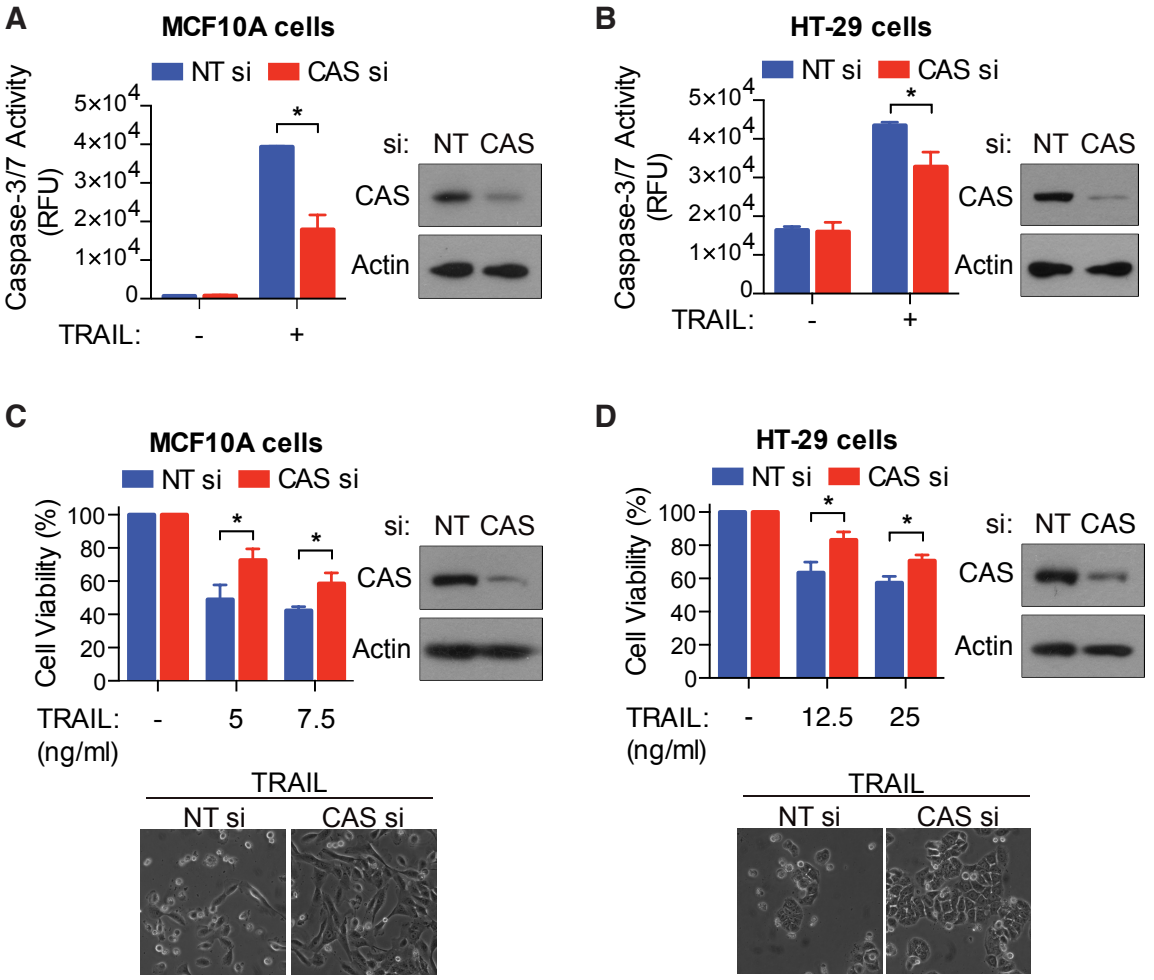

Figure 2.3. CAS promotes TRAIL induced apoptosis.
MCF10A cells transfected with non-target (NT) or CAS siRNA were treated with TRAIL (15 ng/ml for 4 hrs) for measurement of caspase-3/7 activity (**A**) or for 8 hrs at indicated concentration for measurement of cell viability (**c**). HT-29 cells transfected with NT or CAS siRNA were treated with TRAIL (12.5 ng/ml for 6 hrs) for measurement of caspase-3/7 activity (**B**) or for 12 hrs at indicated concentration for measurement of cell viability (**D**). Representative phase-contrast images displaying cell morphology and western blots confirming knockdown of CAS are shown. All values are mean ± SEM from at least three independent experiments (*P<0.05).

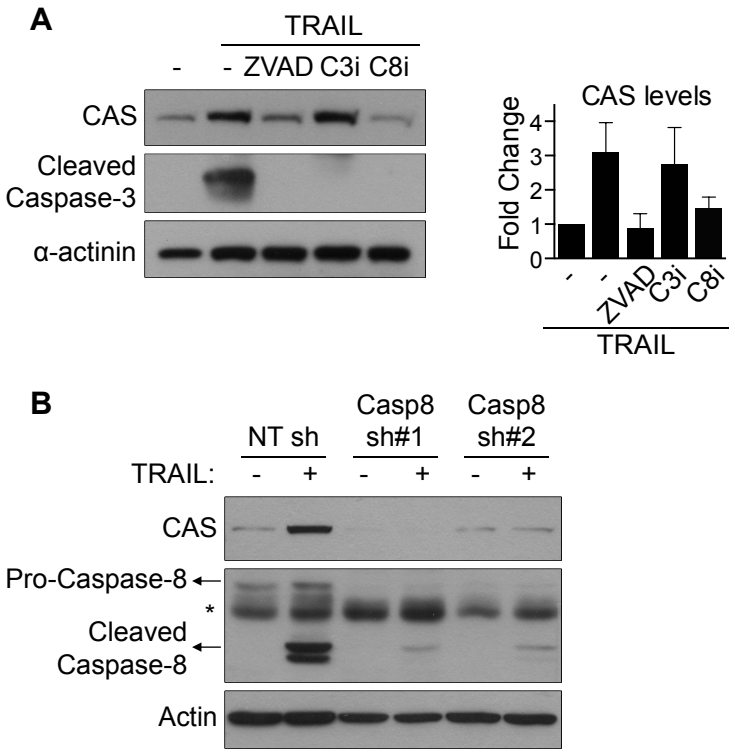

Figure 2.4. TRAIL induced CAS upregulation is caspase-8 dependent but caspase-3 independent.
(A) MCF10A cells were pre-treated for 30 min with 20 μM of a pan-caspase inhibitor (ZVAD-FMK), a caspase-3 inhibitor (C3i; DEVD-FMK) or a caspase-8 inhibitor (C8i; IETD-FMK) followed by 40 ng/ml TRAIL for 3 hrs and subsequently harvested for western blot analysis. Adjacent panel shows quantification of CAS levels (mean±SD) relative to untreated cells from three independent experiments (**B**) MCF10A cells stably expressing control shRNA or one of two different shRNA against caspase-8 were treated with 30 ng/ml TRAIL for 3 hrs and expression of the indicated proteins was analyzed by western blot. * Indicates a non-specific band

Caspase-8 can in turn activate caspase-3 by two distinct mechanisms: direct cleavage of caspase-3, or through cleavage of the BH3-only protein, BID, leading to MOMP and cytochrome C release. To determine if MOMP is required for upregulation of CAS, I generated an MCF10A cell line stably overexpressing Bcl-xL, an anti-apoptotic Bcl-2 family protein that antagonizes MOMP by maintaining Bax in the cytosol (Edlich et al., 2011). To monitor the onset of MOMP, I used a fluorescent SMAC-cherry reporter construct, which localizes to mitochondria in healthy cells, but is released into the cytosol following TRAIL stimulation and subsequent MOMP. Bcl-xL overexpressing MCF10A cells were resistant to TRAIL-induced MOMP (Figure 2.5A), and caspase-3 activation. However overexpression of Bcl-xL failed to inhibit CAS upregulation (Figure 2.5B), indicating that CAS upregulation occurs upstream of MOMP. Additionally, this also demonstrates that MCF10A are “type II” cells, i.e. they rely on the mitochondrial pathway to execute cell-extrinsic apoptotic signals.

To dissect the mechanism for CAS upregulation, I tested if TRAIL induces an increase in transcription of CAS. qRT-PCR experiments revealed that CAS mRNA levels are not increased upon TRAIL treatment (Figure 2.6A). Additionally, TRAIL treatment led to comparable upregulation of a GFP-CAS fusion protein expressed under the control of a naive retroviral promoter (Figure 2.6B), indicating that CAS upregulation is post-transcriptional.

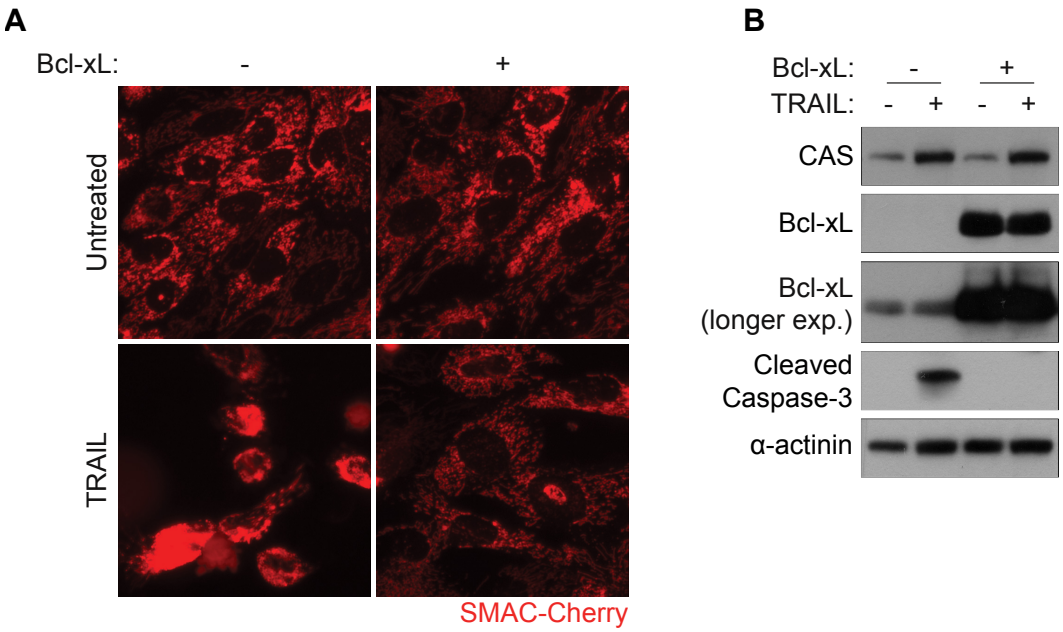

Figure 2.5. TRAIL-induced CAS upregulation occurs independent of MOMP.
(A) Wild type (WT) and Bcl-xL overexpressing MCF10A cells stably expressing SMAC-cherry were treated with 40ng/ml TRAIL for 2.5 hrs, and imaged for the onset of MOMP. **(B)** WT and Bcl-xL overexpressing MCF10A cells were treated with 40 ng/ml TRAIL for 3 hrs and harvested for western blot analysis with the indicated antibodies.

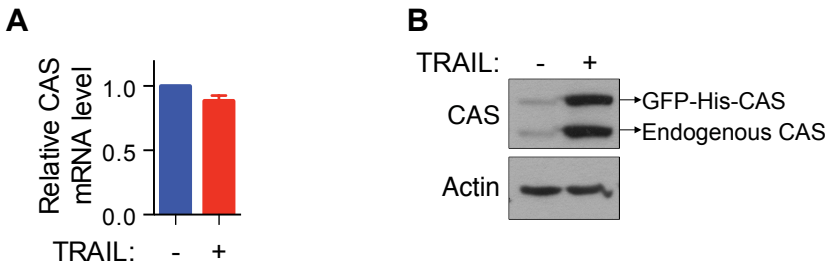

Figure 2.6. TRAIL-induced CAS upregulation is post-transcriptional.
(A) qRT-PCR was used to detect expression of CAS mRNA in MCF10A cells treated with 30 ng/ml TRAIL for 1hr. GAPDH was used as an internal control. Values are mean ± SEM from three independent experiments. **(B)** Western blot of MCF10A cells stably expressing a GFP-His-CAS fusion protein following stimulation with TRAIL (30 ng/ml, 3 hrs).

Common pathways of post-translational protein regulation include the ubiquitin-proteasome system and the lsyosome-autophagy system (Ciechanover, 2005). To test if either or both of these proteolytic pathways play a role in regulating CAS levels, I tested the effects of MG132 (a proteasome inhibitor) and Bafilomycin A1 (a lysosome inhibitor) on CAS expression. MG132 treatment was sufficient to elevate basal levels of CAS while Bafilomycin A1 failed to do so (Figure 2.7A), suggesting that CAS is prone to ubiquitin-mediated proteasomal turnover under steady state conditions. Such a model would predict CAS ubiquitination to be decreased in response to TRAIL. Indeed, TRAIL-stimulation significantly reduced the accumulation of ubiquitinated CAS, but not global ubiquitinated proteins (Figure 2.7B). Consistent with the essential role of caspase-8 activation in promoting CAS upregulation, addition of the pan-caspase inhibitor ZVAD-FMK abolished the decrease in CAS ubiquitination in response to TRAIL.

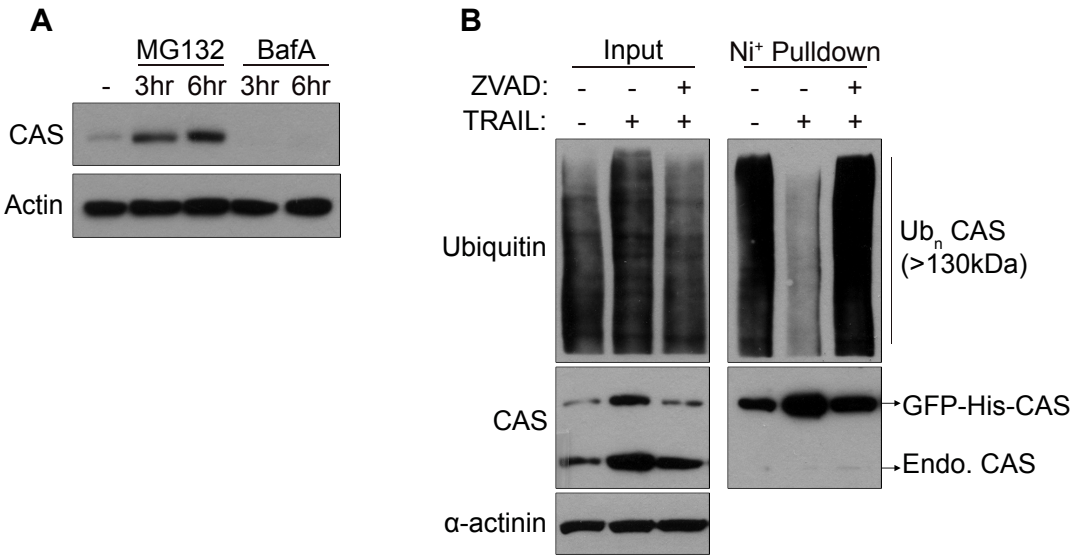

Figure 2.7. TRAIL-induced CAS upregulation occurs through inhibition of its proteasomal degradation.
(A) MCF10A cells were treated with the proteasome inhibitor MG132 (75 μM) or the lysosomal inhibitor Bafilomycin A1 (BafA, 20 nM) for the indicated time and then harvested for western blot analysis. **(B)** Lysates from MCF10A cells stably expressing GFP-His-CAS were subjected to pulldown with nickel beads under denaturing conditions, and subsequently probed with the indicated antibodies. Where indicated, cells were treated with 10 μM ZVAD for 30 min prior to addition of TRAIL (30 ng/ml, 4 hrs).

Degradation of cIAP1 mediates CAS accumulation during TRAIL-induced apoptosis

I hypothesized that TRAIL-treatment could lead to decreased CAS ubiquitination through two possible mechanisms - inhibition of an E3 ubiquitin ligase that targets CAS for degradation, or activation of a deubiquitinase (DUB) that removes polyubiquitin chains from CAS. To further dissect this signaling pathway, I sought to identify the protein(s) involved in this process. I undertook a candidate-based approach, focusing on proteins that meet two key criteria: 1) the candidate must possess E3 ligase and/or DUB catalytic activity, and 2) its expression or activity must be regulated by caspase-8 and/or TRAIL signaling. A thorough literature search revealed cIAP1 as a promising candidate. cIAP1 is a member of the IAP family of proteins, and possesses significant anti-apoptotic activity, but cannot directly inhibit the proteolytic activity of caspases (Eckelman and Salvesen, 2006). Rather, cIAP1 promotes cell-survival by regulating mitogenic signaling pathways induced by TNF and associated ligands through its potent E3 ligase activity (Bertrand et al., 2008; Mahoney et al., 2008). Consistent with previously published data (Guicciardi et al., 2011), I found that TRAIL stimulation leads to a significant decrease in cIAP1 levels in a caspase-8 dependent manner (Figure 2.8A), thereby satisfying both criteria in our model. To test the relevance of cIAP1 in regulating CAS expression, I transfected siRNA targeting cIAP1 into MCF10A cells. Silencing cIAP1 expression led to a modest increase in basal levels of CAS relative to control siRNA (Figure 2.8B). Additionally, co-immunoprecipitation in 293T cells revealed that cIAP1 and CAS

can interact *in vivo* (Figure 2.8C). Meanwhile, silencing other putative candidates including XIAP (another IAP family member with E3 ligase activity that is degraded upon TRAIL treatment (Guicciardi et al., 2011)) or CYLD (a DUB known to be targeted by active caspase-8 (O'Donnell et al., 2011)) had no effect on basal CAS expression or TRAIL-induced CAS upregulation, respectively (Figure 2.8D and E).

I found that overexpression of WT- but not E3 dead- cIAP1 in 293T cells promoted CAS degradation in a dose-dependent manner (Figure 2.9A). Consistently, WT-cIAP1 overexpression significantly increased polyubiquitination of CAS *in vivo*, while E3-dead cIAP1 had no such effect (Figure 2.9B), indicating that cIAP1 regulates CAS levels through its E3 ligase activity. To confirm that CAS is a direct substrate for cIAP1, I performed an *in vitro* ubiquitination assay, using cIAP1 purified from bacteria and CAS purified from HEK293T cells. I found that WT- but not E3-dead cIAP1 was sufficient to catalyze *in vitro* polyubiquitination of CAS in the presence of ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2), and ATP (Figure 2.9C). Collectively, these results demonstrate that cIAP1 directly ubiquitinates and targets CAS for proteasomal degradation. TRAIL-induced caspase-8 activation leads to a marked decrease in cIAP1 levels, thereby mitigating CAS ubiquitination and allowing for its accumulation.

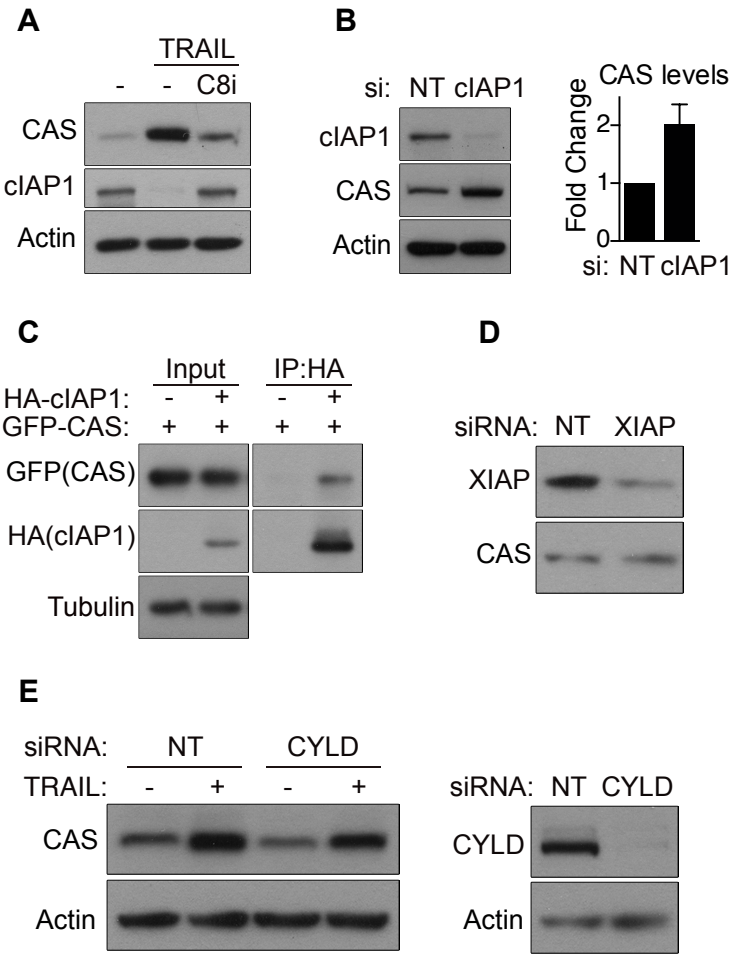

Figure 2.8. TRAIL induces CAS upregulation through degradation of cIAP1
(A) TRAIL induces caspase-8 dependent degradation of cIAP1. MCF10A cells were treated with TRAIL (30 ng/ml, 4 hrs) and harvested for western blot analysis. Where indicated, caspase-8 inhibitor (C8i, 20 μM) was added 30 min prior to stimulation with TRAIL. (**B**) cIAP1 regulates basal CAS expression. MCF10A cells were transfected with non-target (NT) or cIAP1 siRNA and harvested for western blot analysis 72 hrs later. Quantification of CAS levels (mean±SD) relative to controls cells from three independent experiments is shown on the right. **(C)** cIAP1 interacts with CAS *in vivo*. Lysates from 293T cells expressing the indicated plasmids were subjected to immunoprecipitation with HA-agarose beads. Bound proteins were washed and probed by western blotting with indicated antibodies. **(D)** MCF10A cells were transfected with NT or XIAP siRNA and harvested for western blot analysis 72 hrs later. **(E)** MCF10A cells transfected with NT or CYLD siRNA were stimulated with TRAIL (30 ng/ml) for 2 hrs and harvested for western blot analysis. Adjacent panel shows knockdown of CYLD.

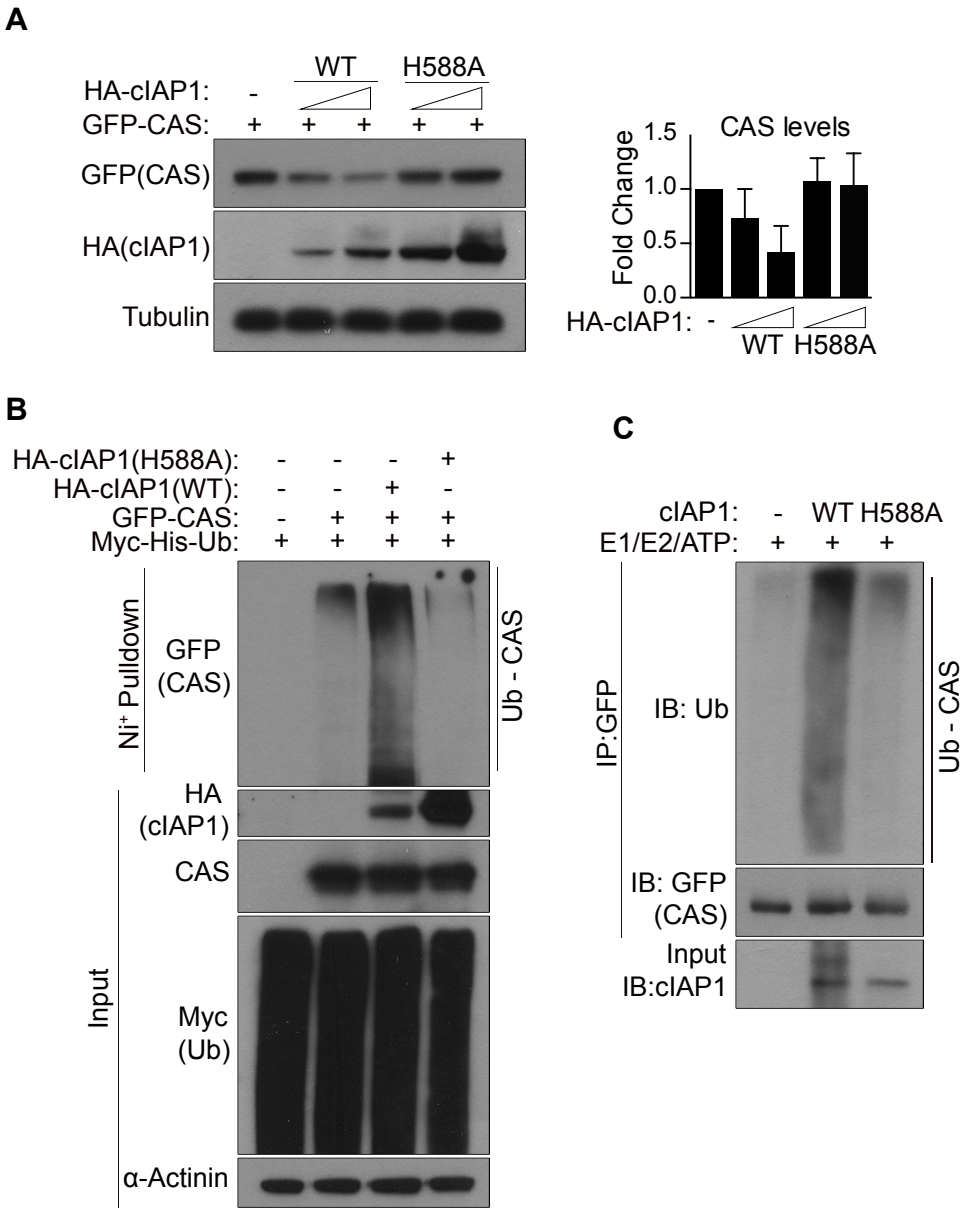

Figure 2.9. cIAP1 directly ubiquitinates and targets CAS for degradation.
(A) cIAP1 mediates the degradation of CAS *in* vivo. 293T cells were transfected with GFP-CAS and WT- or E3-dead (H588A) cIAP1 and subjected to western blot analysis 24 hours later. Total amount of DNA transfected was balanced using empty vector. Quantification of CAS levels (mean±SD) relative to control cells from three independent experiments is shown on the right. (**B**) cIAP1 ubiquitinates CAS *in* vivo. 293T cells transfected with the indicated plasmids were treated with 25 μM MG132 for 4 hrs to allow for accumulation of ubiquitinated proteins. Whole cell lysates were then subjected to a pulldown with nicked beads under denaturing conditions and bound proteins were probed with indicated antibodies. Note that in (A) and (B), expression of E3-dead cIAP1 is significantly higher than WT due to lack of autoubiquitination-induced degradation. **(C)** CAS is a direct substrate of cIAP1. *In vitro* ubiquitination of GFP-CAS purified from 293T cells was performed using recombinant cIAP1, as described in experimental methods. Note that WT-cIAP1 shows a smearing pattern due to autoubiquitination activity.

Aberrant expression of CAS in human tumors

The pro-apoptotic function of CAS would imply that it is a potential tumor suppressor and we would thus expect to find deletions or loss-of-function mutations in tumor samples. However, multiple studies have reported that CAS is actually amplified in various human tumors (Böni et al., 1999; Wellmann et al., 2001; Bar-Shira et al., 2002; Brustmann, 2004), thereby implicating CAS as a potential oncogene. Consistent with early studies that pointed towards an essential role for CAS in cell division (Ogryzko et al., 1997; Bera et al., 2001), I found that sustained knockdown of CAS leads to a clear inhibition of cell growth in MCF10A cells (Figure 2.10).

CAS expression has been documented to be higher in rapidly proliferating tissue like testis and fetal liver (Brinkmann et al., 1995). I thus reasoned that elevation of CAS levels is necessary to support the increased proliferative capacity of tumor cells, and sought to understand the cause and consequences of such aberrant CAS expression specifically in the context of breast cancer. Analysis of the breast carcinoma dataset from The Cancer Genome Atlas (TCGA) project revealed alterations in CAS in about 16% of tumors, with mRNA upregulation being the most frequent (Figure 2.11A). Given the role of CAS in cell proliferation, I hypothesized that oncogenic transformation could drive enhanced expression of CAS in tumors. To test this idea, I introduced various oncogenes, including Erbb2, Myc, $PI3K^{H1047R}$, and $KRAS^{G12V}$ into untransformed MCF10A cells. This led to a clear increase in expression of CAS at the protein

level, driven by an increase in the transcription of CAS mRNA (Figure 2.11B and C), indicating that multiple oncogenic signaling pathways are capable of driving CAS expression to support cell growth. I next examined the co-occurrence of CAS mRNA upregulation with alterations in the same set of oncogenes (Figure 2.11D). CAS mRNA upregulation was significantly correlated with alterations in Myc (P<0.001), but not with the other oncogenes tested

Increased CAS expression contributes to the heightened apoptotic sensitivity of Myc-overexpressing cells

Given the high prevalence of Myc amplification in breast cancer, and its tendency to co-occur with CAS mRNA upregulation, I focused on further exploring the functional significance of elevated CAS in c-Myc overexpressing cells (MCF10A-Myc). Since CAS can facilitate caspase-3 activation by driving apoptosome formation, I hypothesized that MCF10A-Myc cells (with higher basal CAS expression) would be more sensitive to TRAIL than their wild type counterparts. Indeed, in response to TRAIL, I observed increased caspase-3 activation and lower cell survival in MCF10A-Myc cells (Figure 2.12A & B). Importantly, knockdown of CAS reversed these trends (Figure 2.12C and D). Collectively, these results indicate that elevated basal levels of CAS can sensitize cells to TRAIL.

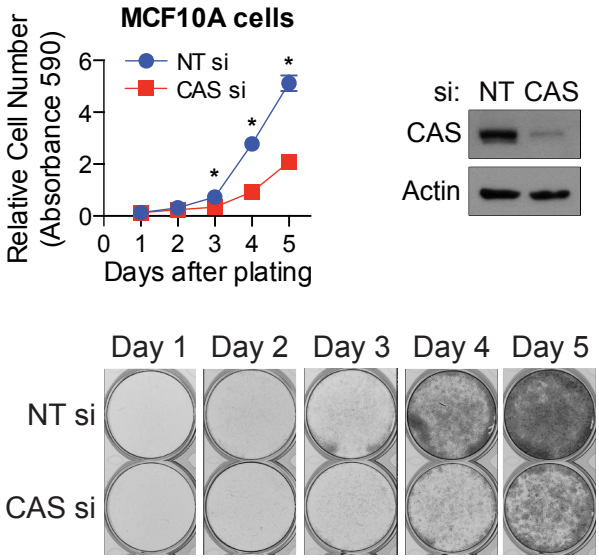

Figure 2.10. CAS plays an essential role in cell growth.
Knockdown of CAS dramatically inhibits cell proliferation. MCF10A cells were transfected with NT or CAS siRNA and seeded at equal density on day 0. At the indicated time points, cells were stained with crystal violet, and the relative number of cells was quantified. A representative image of stained cells and western blot confirming knockdown of CAS are shown. Data represent mean ± SEM from two independent experiments each performed in triplicate (*P<0.05).

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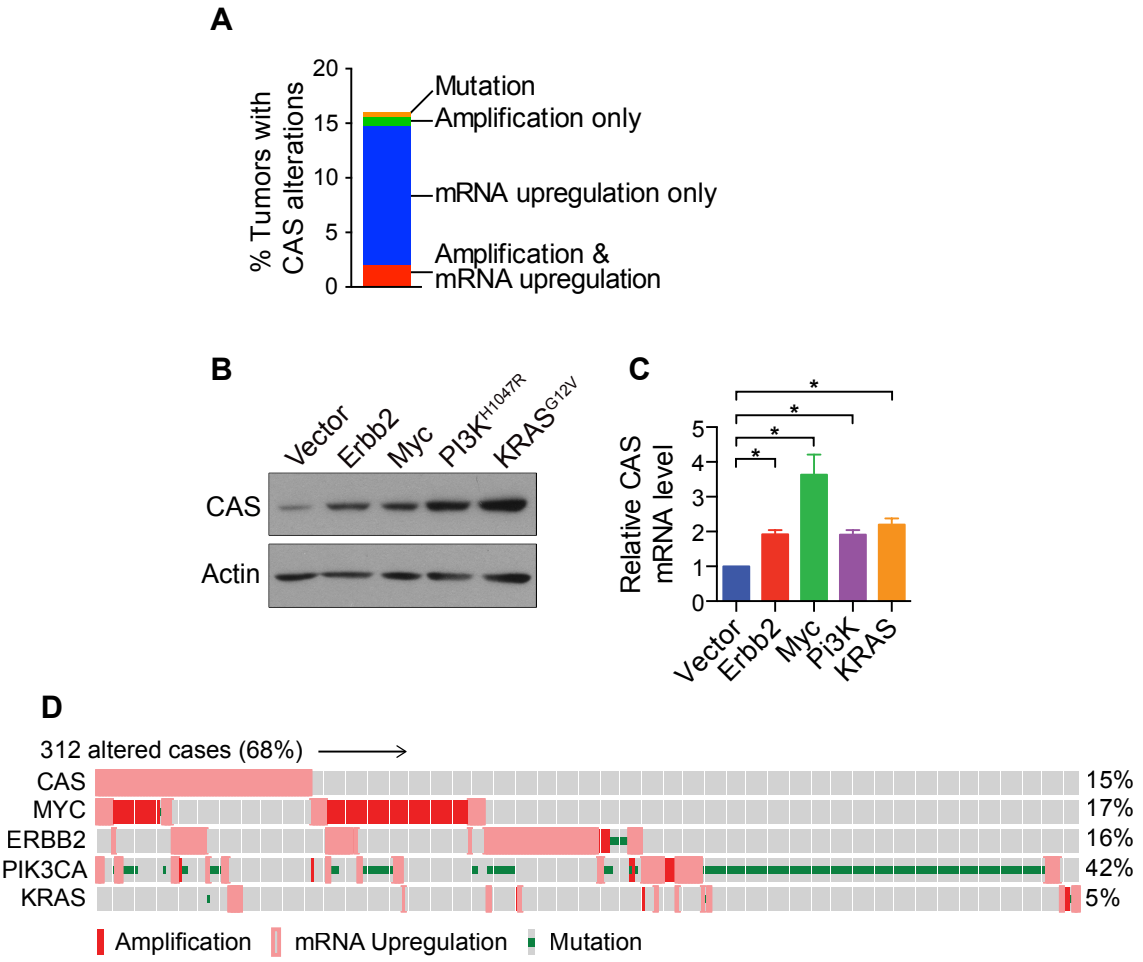


Figure 2.11. Overexpression of various oncogenes mediates transcriptional upregulation of CAS.

(A) CAS expression is upregulated in human breast tumors. Frequency of genomic alterations in CAS in the TCGA breast carcinoma data set was retrieved using the cBioPortal. **(B)** Oncogene overexpression induces transcriptional upregulation of CAS. Lysates from MCF10A cells stably expressing the indicated oncogenes were subjected to western blot analysis. **(C)** qRT-PCR was used to measure expression of CAS mRNA in the MCF10A cell lines expressing indicate oncogenes. Values are mean ± SEM from three independent experiments (*P<0.05). **(D)** Oncoprint from cBioPortal showing co-occurrence of CAS mRNA upregulation with alterations in select oncogenes. Increase in basal CAS expression significantly correlates with alterations in Myc but not other oncogenes tested (Fisher's exact test: CAS-Myc, P<0.001; CAS-Erbb2/PI3K/KRAS, ns). Only cases with alterations in any of the indicated genes are displayed.

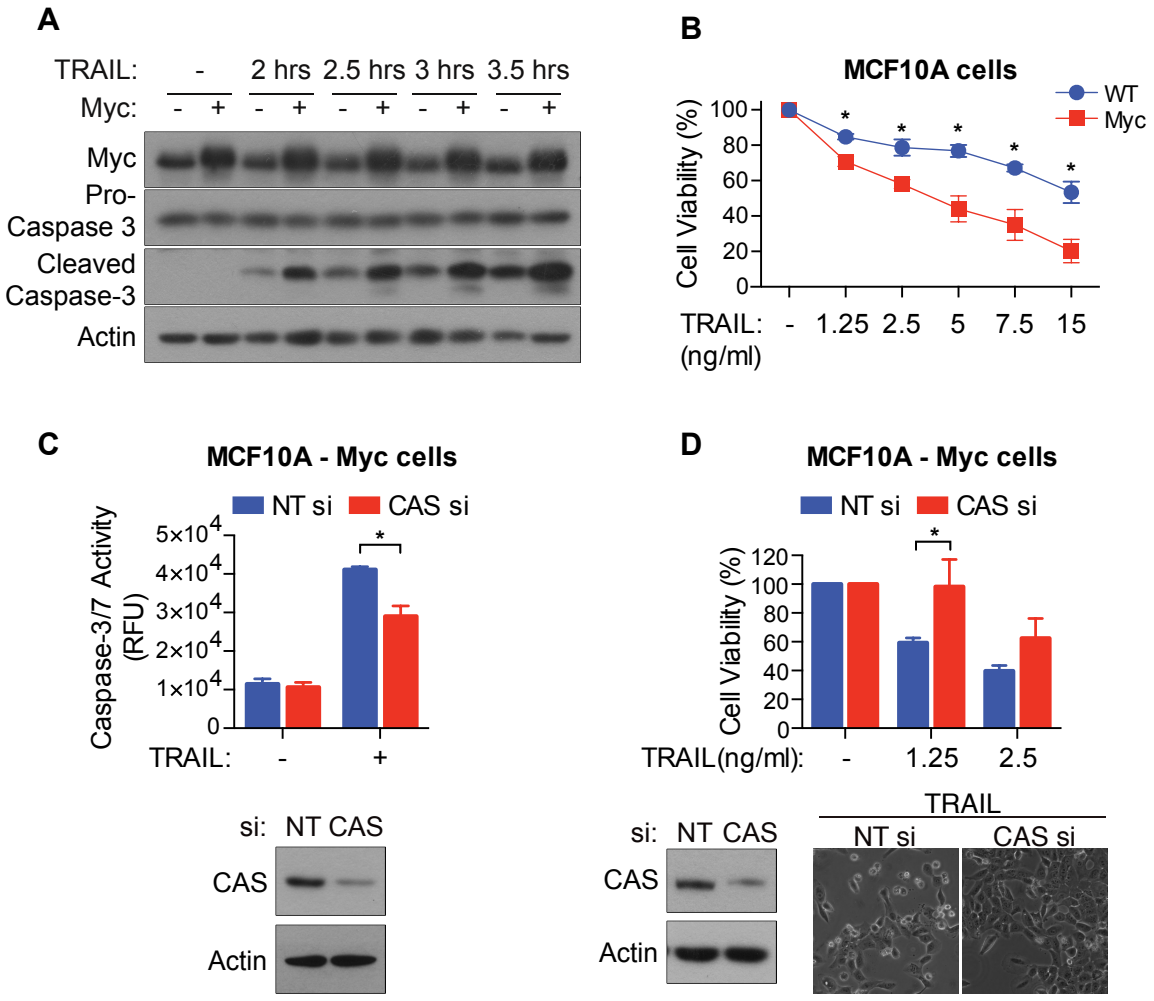


Figure 2.12. Elevated CAS expression contributes to increased TRAIL-sensitivity in Myc-overexpressing cells.

(A) Myc overexpressing cells display increased sensitivity to TRAIL. WT- and Myc- MCF10A cells were treated with 5 ng/ml TRAIL for the indicated time and harvested for western blot analysis to detect caspase-3 activation (**B**) WT- and Myc- MCF10A cells were treated with the indicated concentration of TRAIL for 8 hrs and assayed for cell viability. (**C**) MCF10A-Myc cells transfected with NT or CAS siRNA were treated with TRAIL (1.25 ng/ml for 4 hrs) for measurement of caspase-3/7 activity or (**D**) for 8 hours with the indicated concentrations for measurement of cell viability. Representative phase-contrast images displaying cell morphology and western blots confirming knockdown of CAS are shown. All values are mean ± SEM from three independent experiments (*P<0.05).

2.3. Discussion

Multiple studies have revealed that cells can survive incomplete MOMP provided downstream caspase-3 activity is limited (Colell et al., 2007; Tait et al., 2010). Thus, the decision to live or die following MOMP is largely determined by the extent of caspase-3 activation. However, our understanding of how caspase-3 activity is regulated following MOMP remains incomplete. Our lab had previously found that CAS can drive caspase activity by stimulating apoptosome formation (Kim et al., 2008). Here, I demonstrate that during TRAIL-induced apoptosis, CAS levels are rapidly upregulated, and that knockdown of CAS inhibits TRAIL-induced caspase-3 activity and cell death. Based on these results, I propose that upregulation of CAS amplifies the apoptotic signal, and strengthens the commitment to cell death following MOMP.

Mechanistically, the upregulation of CAS in response to TRAIL is caspase-8 dependent. Active caspase-8 induces degradation of cIAP1, an E3-ligase that ubiquitinates and promotes degradation of CAS. How caspase-8 targets cIAP1 for degradation remains an unanswered question. The most straightforward explanation is a direct cleavage of cIAP1 by caspase-8. However, I was unable to reconstitute this cleavage event *in vitro* using purified proteins, suggesting other factors may perhaps be necessary to mediate cleavage of cIAP1. Alternatively, caspase-8 could target a binding partner that stabilizes cIAP1, or potentially regulate cIAP1's auto-ubiquitination activity. Further work will be necessary to tease apart these possibilities. My experiments also reveal that caspase-8 induces upregulation of CAS independent of stimulating MOMP. Thus,

increased levels of CAS can feed-forward into the apoptotic cascade following the onset of MOMP (Figure 2.13).

In this study, I focused on examining the regulation of CAS exclusively in the context of TRAIL-induced apoptosis. However, it is worth noting that our lab has previously reported other extrinsic apoptotic stimuli such as TNF-α and IFN-γ can also induce CAS upregulation (Kim et al., 2008), and as with TRAIL, knockdown of CAS inhibits apoptosis induced by these stimuli. While these stimuli target distinct receptors leading to accumulation of different adaptor proteins, they all induce caspase-8 activation. Thus, it is likely that the caspase 8 - cIAP1 pathway that I have described here for TRAIL, mediates upregulation of CAS induced by these stimuli as well.

Contrary to its proto-tumor suppressive role in facilitating apoptosis, CAS is frequently amplified in human tumors. CAS levels are higher in proliferating fibroblasts and decrease upon growth arrest, highlighting its function in cell proliferation (Brinkmann et al., 1995). Furthermore, homozygous deletion of CAS in mice leads to embryonic lethality (Bera et al., 2001), and mutations in the yeast homologue CSE1, are lethal as well (Xiao et al., 1993). The precise molecular function of CAS in cell proliferation remains to be identified. Interestingly, overexpression of multiple oncogenes led to increased expression of CAS mRNA and protein. This indicates the existence of a regulatory network closely linking cell growth and CAS levels, which can be co-opted during oncogenic transformation.

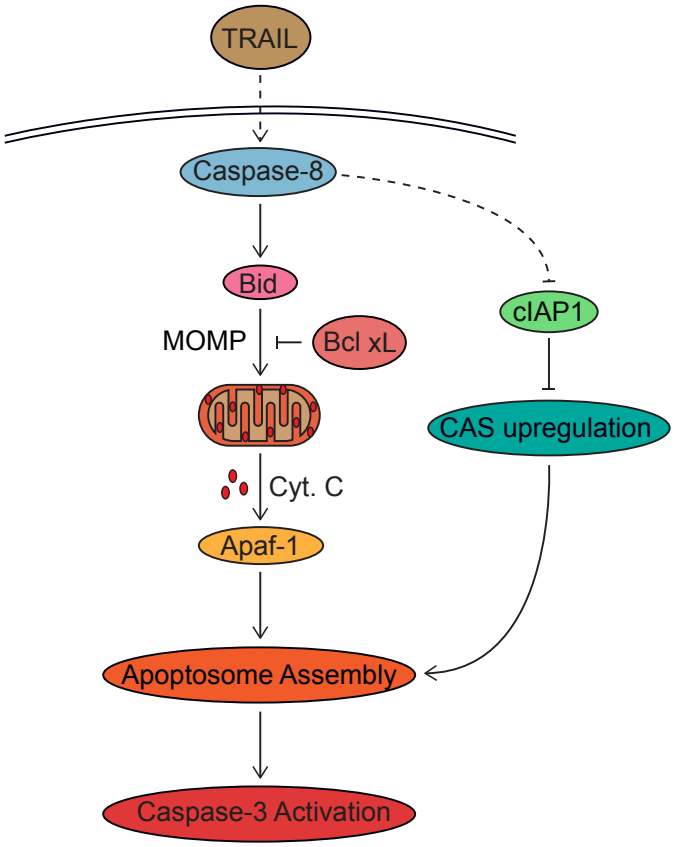


Figure 2.13. Model for TRAIL-induced CAS upregulation.
In resting cells, cIAP1 keeps CAS levels in check through ubiquitination-mediated proteasomal degradation. Upon stimulation by TRAIL, caspase-8 is activated and induces degradation of cIAP1. This allows for the accumulation of CAS, which feeds forward into the apoptotic pathway by stimulating apoptosome formation following MOMP.

The data presented here demonstrate that increased basal expression of CAS in oncogene-overexpressing cells is at least partly responsible for sensitizing these cells to TRAIL. This raises the intriguing possibility that the increased levels of CAS seen in several human tumors could represent an "Achilles' heel"; tumor cells upregulate CAS to support their enhanced proliferation, but this in turn could make them more vulnerable to certain apoptotic stimuli, providing a therapeutic window to treat such tumors. However, not all apoptotic stimuli induce CAS upregulation, and those that do not (e.g., staurosporine) are unaffected by knockdown of CAS (Kim et al., 2008). Thus, profiling CAS expression in patient tumor tissue could potentially be used as a biomarker to guide therapeutic choices in the clinic.

In summary, the data presented here characterize a novel signaling pathway wherein caspase-8 amplifies the TRAIL-induced apoptotic signal by mediating upregulation of CAS. Further, I highlight the increased transcription of CAS in response to oncogene overexpression, and the consequences of this upregulation. The regulation of CAS at both the transcriptional and post-translational levels is reflective of its multiple roles in proliferation and apoptosis. It is likely that such intricate regulation is key in maintaining proper function of this dynamic protein in these two contrasting cellular processes.

2.4. Experimental Procedures

Cell Culture

MCF-10A cells were cultured in DMEM/F12 supplemented with 5% horse serum, EGF (20 ng/ml), hydrocortisone (0.5 μg/ml), cholera toxin (100 ng/ml), insulin (10 μg/ml) and penicillin-streptomycin. 293T and HT-29 cells were cultured in DMEM-high glucose supplemented with 10% FBS, L-glutamine (2 mM) and penicillin-streptomycin. Lentiviral and retroviral constructs were co-transfected with the respective packaging vectors into 293T cells for virus production. Virus containing media was passed through a 0.45 μM PES filter and supplemented with polybrene before being used to transduce cells.

Reagents, Antibodies and plasmids

SuperKiller TRAIL (ALX-201-115-3010) and ZVAD-FMK (ALX-260-020) were from Enzo Life Sciences. Caspase-8 inhibitor (IETD-FMK, #550380), and caspase-3 inhibitor (DEVD-FMK, #550378) were from BD Biosciences. MG132 was from EMD Millipore (#474790). Bafilomycin A1 was from Sigma. For western blot analysis, the following antibodies were used: anti-CAS (Bethyl, #A300-473A), anti-Caspase-3 (Cell Signaling, #9662), anti-Caspase-8 (Cell Signaling, #9746), anti-cIAP1 (Cell Signaling, #7065 and #4952), anti-XIAP (Cell Signaling, #2045), anti-CYLD (Cell Signaling, #8462), anti β-Actin (Sigma, #A1978 and #5316), anti α-actinin (Santa Cruz, #sc-17829), anti-Ubiquitin (Millipore, #05-944), anti-GFP (Roche, 11814460001), anti-HA clone 16B12 (Covance, #MMS-101P). The SMAC-cherry reporter construct was generated by fusing the N-terminal 171 bp of human SMAC cDNA to the N-terminus of mCherry, on the

pBabe-puro vector. Bcl-xL was subcloned into the pQCXIP vector using the EcoRI and BamHI sites. N-terminal tagged GFP-His-CAS and GFP-CAS were generated by subcloning the respective fragments into the pQCXIP-GFP vector. cIAP1 cDNA was purchased from Addgene (WT-#8311, H588A-#8334), and subcloned into the pQCXIP-HA vector (N-terminal tagged) at the XhoI and EcoRI sites or the pET28A vector at the Nhe1 and Xho1 sites. pcDNA-Myc-His-Ubiquitin, pBabe-Erbb2, MSCV-mCherry-Myc, pBabe-PI3KH^{1047R}, and pBabe-KRAS^{G12V} were generously provided by Dr. Filippo Giancotti (MSKCC).

Co-immunoprecipitation

For co-immunoprecipitation assays, 293T cells grown in 10-cm plates were transfected with the indicated plasmids. 24 hours later, cells were lysed in plate with IP buffer (10 mM Hepes pH 7.5, 150 mM NaCl, 0.5% NP-40, 1 mM EDTA, 1 mM DTT, and 10% Glycerol) supplemented with protease inhibitors. Lysates were cleared by centrifugation at 14,000 rpm for 10 min at 4°C, and incubated with HA-agarose beads (Sigma, #A2095) overnight at 4°C. Beads were then washed 5 times in IP buffer, and eluted by boiling in 1X SDS sample buffer.

In-vivo ubiquitination assays

For assays in MCF10A cells, cells stably expressing GFP-His-CAS were lysed in 100 µL of RIPA buffer supplemented with 25 µM MG132, 10 mM *N*-ethyl-maleimide (Sigma), and protease inhibitors. Ni-NTA agarose beads (Qiagen) were then used to pull down His tagged protein under denaturing conditions. For assays in 293T cells, cells were transfected with Myc-His-Ubiquitin and indicated

plasmids in 6-cm plates. 24 hours later, cells were treated with 25 μM MG132 for 4 hrs. After washing with PBS, a small fraction of cell pellet was saved for input and lysed in RIPA buffer. Rest of the cell pellet was resuspended in lysis buffer (6 M Guanidine, 0.1 M NaH_2PO_4, 10 mM Tris pH 8.0, 10 mM β-me) freshly supplemented with 15 mM imidazole, 10 μM MG132, 10 mM *N*-ethyl-maleimide, protease inhibitors, and sonicated to reduce viscosity. Lysates were then incubated with pre-washed Ni-NTA agarose beads for 3 hours at room temperature. Beads were then washed once with buffer A (lysis buffer + 0.2% triton), once with buffer B (8 M urea, 0.1 M NaH_2PO_4, 10 mM Tris pH 8.0, 10 mM β-me, 0.2% triton), twice with buffer C (8 M urea, 0.1 M NaH_2PO_4, 10 mM Tris pH 6.3, 10 mM β-me, 0.2% triton), and twice with buffer D (8 M urea, 0.1 M NaH_2PO_4, 10 mM Tris pH 6.3, 10 mM β-me, 0.1% triton). All wash buffers were freshly supplemented with 15 mM imidazole. Finally, bound proteins were eluted by boiling in RIPA buffer containing 1X SDS loading buffer and 200 mM imidazole for 10 min. Samples were then subjected to western blot with the indicated antibodies.

In vitro ubiquitination assay

His-tagged cIAP1 proteins were cloned into the pET28A vector, expressed in BL21-DE3 cells (Novagen) and purified from bacterial lysates using Ni-NTA agarose beads. GFP-CAS was expressed in 293T cells and purified using GFP-Trap beads (ChromoTek). Briefly, cells were lysed in IP buffer and incubated with GFP-Trap beads for 1 hour, followed by sequentially washing three times in IP buffer and twice in ubiquitination reaction buffer (50 mM Tris pH 7.5, 5mM $MgCl_2$,

and 2 mM DTT). Beads were then resuspended in reaction buffer containing 75 nM E1, 600 nM E2, 5 mM ATP and 10 μg/uL ubiquitin in a final volume of 20 μL. Where indicated, 800 ng of WT- or H588A- cIAP1 were included, and reactions were incubated at 30°C for 2 hours with gentle agitation. Following the reaction, beads were washed 4 times in denaturing buffer (8M Urea, 1% SDS in PBS) to remove nonspecific binding. Following a final wash in IP buffer, bound proteins were eluted by boiling in 1X SDS sample buffer and subjected to immunoblotting with the indicated antibodies.

Knockdown by siRNA and shRNA

The caspase-8 shRNA sequences were sh#1:GACATGAACCTGCTGGATATT, and sh#2:GCCTTGATGTTATTCCAGAGA. Lentiviruses harboring the knockdown sequences were used to infect MCF10A cells. Cells when then selected with puromycin for at least 3 days before testing for knockdown. siGenome SMARTpool siRNA's (CAS, cIAP1 and XIAP), control siRNA (D-001210-05-20), and individual siRNA against CYLD (M-004413-02-0005) were purchased from Dharmacon. Cells were seeded in 6-well plates at a density of 80,000 cells/well (MCF10A) or 200,000 cells/well (HT-29) and 24 hours later, transfected with 25-30 nM CAS siRNA using Oligofectamine (Invitrogen) or Dharmafect 1 (Dharmacon), respectively. Cells were routinely assayed 64-72 hrs post-transfection. For cell growth assays, a higher concentration of CAS siRNA (75 nM) was used. Knockdown of cIAP1, XIAP and CYLD was achieved with 100 nM siRNA.

Cell viability and growth assays

Cell viability was measured using the CellTiter-Glo Luminescent Cell Viability Assay kit (Promega). Briefly, cells were plated in triplicate, at a density of 1500-3000 (MCF10A) or 6000 (HT-29) cells/well and allowed to adhere for 24-48 hrs. Following treatment with TRAIL, the percent of viable cells was quantified relative to untreated cells according to the manufacturer's directions. All cell viability data are values from at least three independent experiments. To measure cell growth, following transfection with siRNA, cells were split into 12-well plates at a density of 10,000 cells/well. At 24 hour intervals, cells were fixed in 10% formalin for 10 min. Following washing with PBS, cells were stained with 0.1% crystal violet solution for 20 min, washed 3 times with water, and allowed to air dry. The stain was extracted by incubating in a 10% acetic acid solution for 20 min with shaking. The relative number of cells was then quantified by measuring absorbance of the extracted stain at 590 nm.

Caspase assay

To quantify cellular caspase activity, cell lysates were collected following treatment, and 20μg total protein was incubated with 15 μM fluorogenic caspase-3 rhodamine-DEVD substrate (AnaSpec, #60304) in a final volume of 20 μL. Samples were then immediately loaded on 384-well plates and fluorescence was measured at 30°C at an interval of 2 min for 3 hours using a SpectraFluor Plus Spectrometry Reader (Tecan) with an excitation wavelength of 485 nm and an emission wavelength of 535 nm. Average relative fluorescence units (RFU) ± SEM of three independent experiments were plotted.

qRT-PCR

Total RNA was extracted using Aurum Total RNA mini kit (Bio-Rad) and reverse transcription was performed using iScript cDNA synthesis kit (Bio-Rad). Quantitative RT-PCR was performed with iQ SYBR Green Supermix (Bio-Rad) using a CFX connect Real-time System (Bio-Rad). The relative level of mRNA was calculated by the comparative Ct method using GAPDH as a control. The following primers were used:

CAS_Fw: TGCCTCGTTTTGTTACAGCC

CAS_Rev: GGTCTCTCACAAACTGAAGCC

GAPDH_Fw:TGCACCACCAACTGCTTAGC

GAPDH_Rev: GGCATGGACTGTGGTCATGAG

Genomic analysis

The TCGA data set used has been previously described (Network, 2012). All data was retrieved and analyzed using the cBioPortal for Cancer Genomics (http://cbioportal.org) (Cerami et al., 2012; Gao et al., 2013)

Statistical analysis

Unless otherwise noted, results were analyzed by a Student's t-test, performed using the GraphPad Prism 6 software. Significance was defined as $P<0.05$.

CHAPTER 3

THE ROLE OF MITOCHONDRIA IN FERROPTOTIC CELL DEATH

3.1. Introduction

Mammalian cells have evolved several complex mechanisms to constantly monitor and regulate their growth based on signals from their extracellular environment. While growth factors typically provide survival signals that stimulate metabolism and cell proliferation, nutrients such as glucose, lipids, and amino acids are also essential in maintaining cellular viability. Sustained absence of nutrients inhibits key metabolic processes and can lead to activation of cell death programs. For instance, glucose withdrawal results in impaired glycolytic flux and alters the cellular AMP:ATP ratio. This induces activation of the nutrient sensing kinase AMPK which triggers a p53-dependent apoptotic cascade (Jones et al., 2005; Okoshi et al., 2008). Similarly, growth factor deprivation results in disruption of mitochondrial homeostasis, prompting the release of cytochrome C, subsequently leading to apoptotic cell death (Heiden et al., 1999; Vander Heiden et al., 2001; Wei et al., 2001). Thus, nutrient availability and metabolism are intricately linked to cell death (Plas and Thompson, 2002; Mason and Rathmell, 2011).

However, apoptosis is not the only cellular outcome in response to nutrient starvation. In the absence of certain nutrients, cells can activate autophagy, a catabolic process that utilizes lysosomal activity to degrade intracellular proteins and organelles, thus providing an alternate source of nutrients to maintain

essential cellular processes (Yorimitsu and Klionsky, 2005). Interestingly, excessive starvation induced-autophagy has been reported to induce autosis, a form of cell death mediated by the Na^+,K^+-ATPase pump and dependent on the autophagy machinery (Liu et al., 2013). However, only a small fraction of cells appear to undergo autosis in culture, and thus the physiological relevance of autosis remains unclear.

Can deprivation of nutrients trigger others forms of cell death? In the first portion of this chapter, I describe the characterization of an unusual form of cell death induced by amino acid starvation in the presence of serum. We eventually determined this death to be ferroptosis, a newly described form of iron-dependent oxidative cell death. This study was carried out in close collaboration with Dr. Minghui Gao**,** a postdoctoral fellow in the lab. The work has been published in full in the journal *Molecular Cell* (Gao et al., 2015), and is included here in part.

The molecular mechanism mediating ferroptosis has not been firmly established. To date, depletion of cellular glutathione, inactivation of the lipid-detoxifying enzyme GPX4, and the subsequent formation of lipid-based reactive oxygen species (lipid ROS) are the best characterized molecular events during ferroptosis (Yang and Stockwell, 2016). However, the precise cellular compartment where lipid ROS are generated remains undefined. Furthermore, how oxidized lipids ultimately mediate cellular demise is not well understood.

In the second portion of this chapter, I explore the role of mitochondria in mediating ferroptosis. Morphologically, ferroptosis is characterized by shrunken mitochondria with increased membrane density and reduced cristae (Yagoda et al., 2007; Dixon et al., 2012; Friedmann Angeli et al., 2014). Notably, an shRNA screen for factors required for ferroptosis identified enzymes related to mitochondrial fatty acid metabolism (Dixon et al., 2012). However, despite such circumstantial pieces of evidence, a clear role for mitochondria in ferroptosis has neither been established nor disproved. Based on the data presented in the latter part of this chapter, I propose that mitochondria play an essential role in mediating ferroptosis induced by multiple triggers including the small molecule erastin and cystine starvation.

3.2. Results

Serum induces a non-apoptotic form of cell death under amino acid free conditions

In an effort to model cell death induced by nutrient/growth factor deprivation, Dr. Minghui Gao observed a striking phenomenon: when mouse embryonic fibroblasts (MEFs) were subjected to amino acid deprivation, the presence of serum potentiated robust cell death in virtually all cells within 12 hours (Figure 3.1A and B). Importantly, deprivation of both amino acids and serum only led to very modest cell death in the same time frame. This was intriguing since we expected the presence of numerous growth factors in serum to mitigate cell death induced by amino acid deprivation. Rather, our observations suggested that serum was actively promoting cell death in amino acid free conditions.

To further understand this phenomenon, we attempted to characterize the molecular nature of this cell death process. Cell death induced by combined deprivation of amino acids and serum was associated with caspase activation (Figure 3.1C). However, in the presence of serum, no caspase activation was observed. Consistently, serum-dependent cell death was not inhibited in *bax/bak* double knockout (DKO) MEFs, which are deficient for the mitochondrial apoptotic pathway (Figure 3.1D), or by the broad-spectrum caspase inhibitor ZVAD-FMK (Figure 3.1E), indicating that this is a non-apoptotic cell death process.

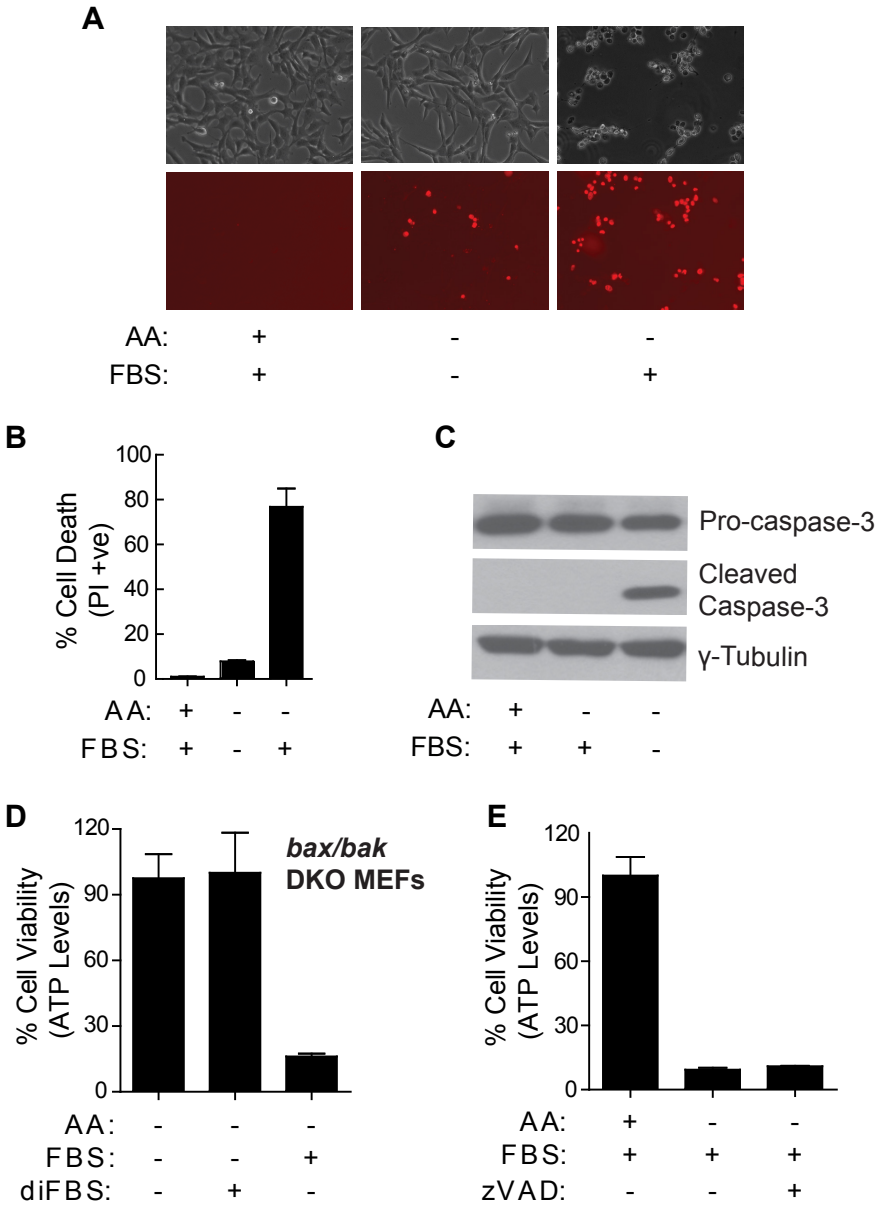


Figure 3.1. Serum induces potent non-apoptotic cell death upon amino acid starvation.

(A) Images showing cell death induced by serum upon amino acid deprivation. MEFs were treated as indicated for 12 hrs. Upper panel: phase-contrast, lower panel: propidium iodide (PI) staining. AA: amino acids, FBS: 10% (v/v) fetal bovine serum. **(B)** MEFs were treated as in (A) and cell death was quantified by PI staining coupled with flow cytometry. **(C)** MEFs were treated as indicated for 12 hr and caspase-3 activation was assessed by immunoblotting.**(D)** *Bax/Bak*-DKO MEFs were treated as indicated for 12 hrs and cell viability was determined using the CellTiter-Glo Kit. Note that dialyzed FBS (diFBS) does not induce cell death, and is thus an appropriate negative control. **(E)** MEFs were treated as indicated with or without the pan-caspase inhibitor zVAD-FMK for 12 hrs and cell viability was determined. All data represent mean ± SEM, n=3. Experiments in Figure 3.1 were carried out by Dr. Minghui Gao.

I performed live-cell imaging to compare the morphological changes associated with these two distinct forms of cell death. Cells undergoing serum-dependent cell death displayed classic morphological features associated with necrosis, including cell rounding, swelling, and eventual plasma membrane rupture (Figure 3.2A). Furthermore, imaging with the nuclear marker, H2B-mCherry, revealed that nuclear integrity is maintained throughout this process (Figure 3.2B). In contrast, consistent with caspase activation, cell death induced by combined amino acids and serum withdrawal displayed several hallmarks of apoptosis including membrane blebbing, chromosomal fragmentation and formation of apoptotic bodies (Figure 3.2B).

One of the best characterized forms of programmed necrosis is the TNF-induced necroptotic pathway involving the activation of the kinases RIP1 and RIP3 and subsequent activation of the pseudokinase MLKL (Linkermann and Green, 2014). I tested if this signaling pathway was involved in the unique serum-dependent cell death process we observed. RIP3 knockout MEFs, which were completely resistant to TNF-induced necrosis (Figure 3.3A and B), were susceptible to serum-dependent cell death (Figure 3.3C and D). Similarly, knockdown of MLKL was sufficient to block TNF-induced necrosis but failed to inhibit serum-dependent cell death (Figure 3.3E and F). These results indicate that serum-dependent necrosis occurs independent of the RIP3-MLKL necrosome complex.

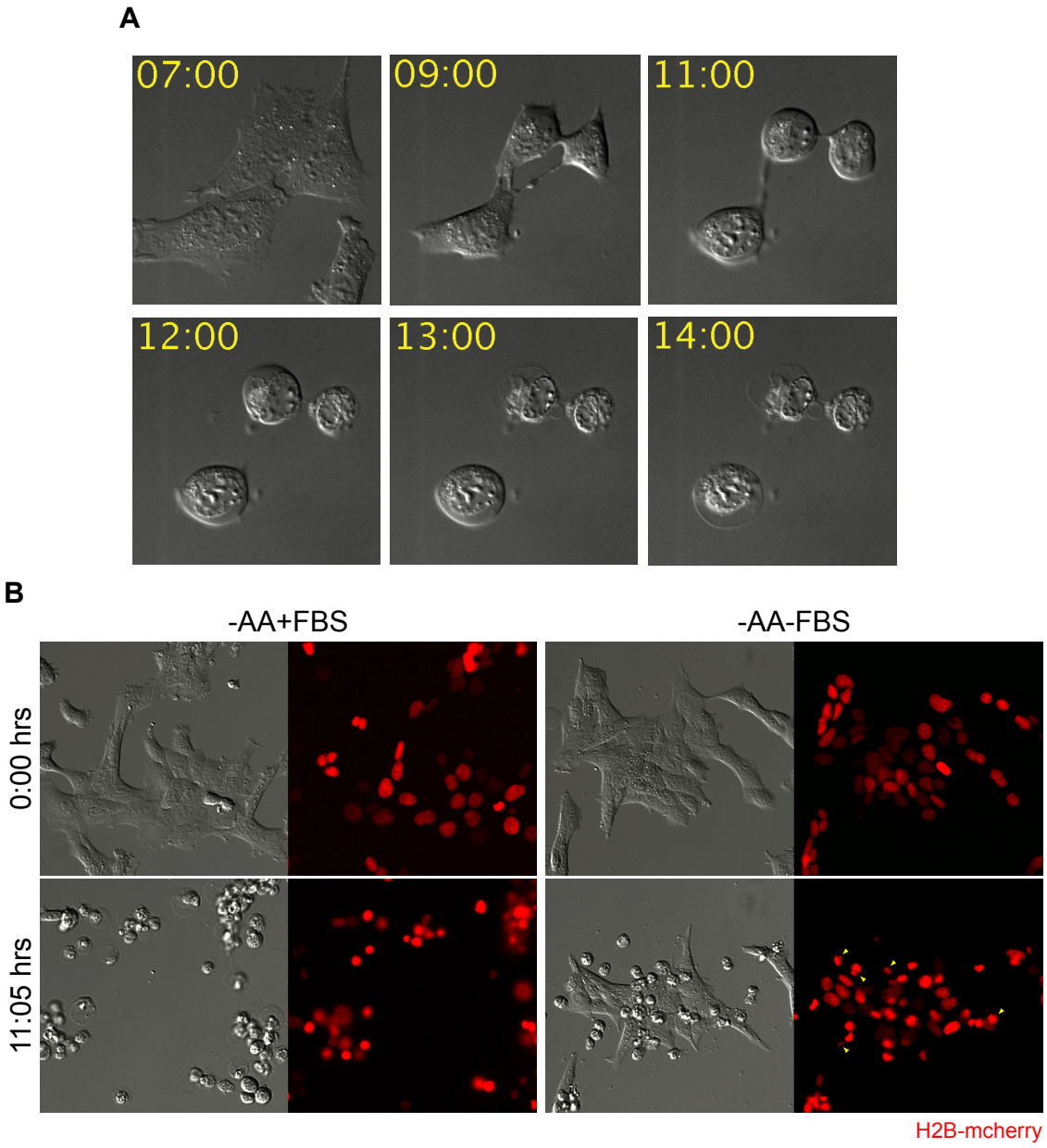


Figure 3.2. Serum-dependent cell death displays necrotic morphology.
(A) Representative stills from confocal time-lapse imaging of MEFs treated with amino acid starvation in the presence of FBS. Time (hr) after treatment is indicated. **(B)** Representative stills from time-lapse imaging of MEFs expressing H2b-mcherry, treated as indicated. Yellow arrowheads indicate fragmented nuclei characteristic of apoptosis.

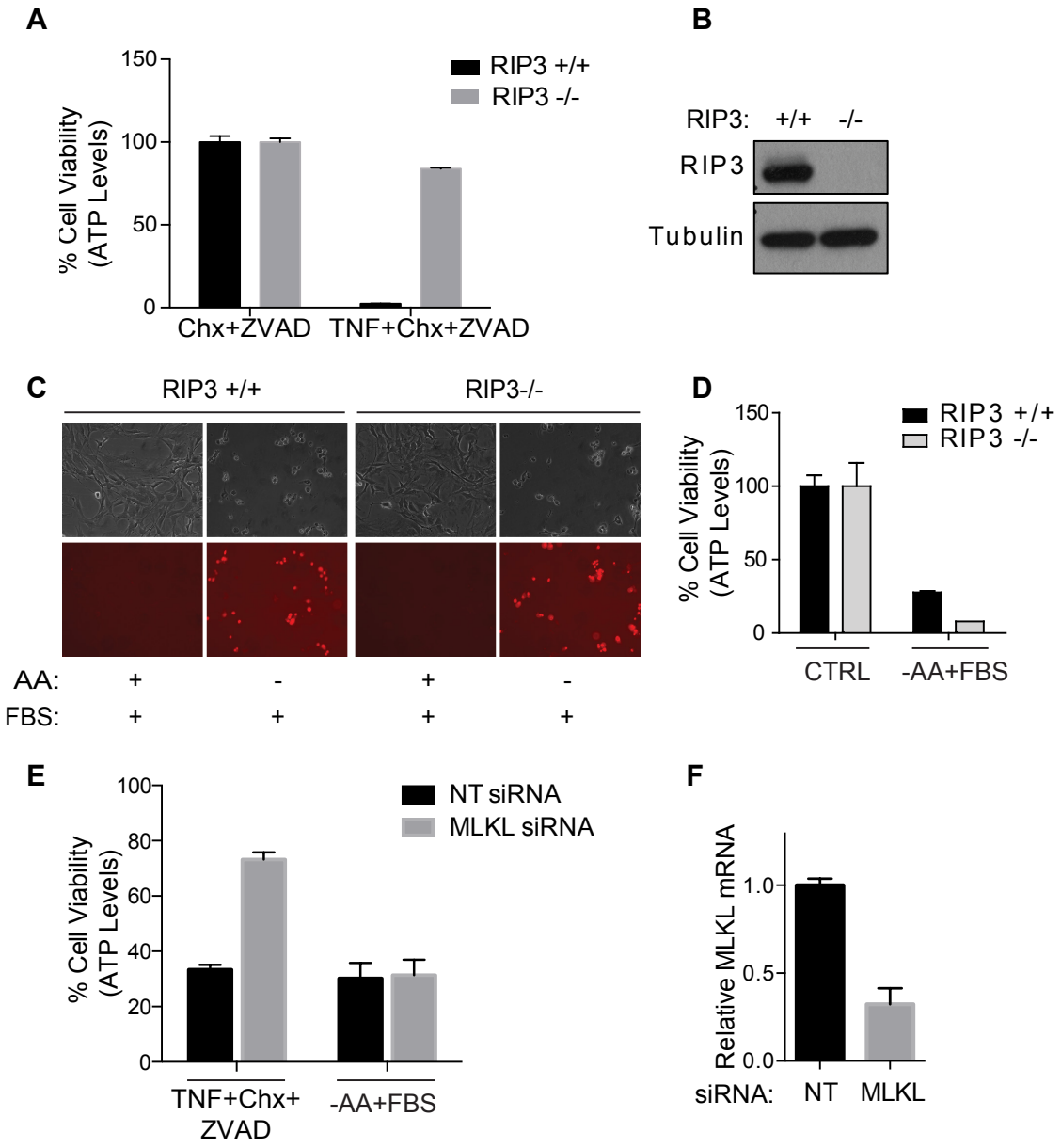


Figure 3.3. Serum-dependent cell death occurs independent of the RIP3-MLKL necrosome.

(A) *RIP3*$^{+/+}$ and *RIP3*$^{-/-}$ MEFs were stimulated to undergo TNFα-induced necrosis and cell viability was determined. **(B)** Western blot confirming knockout of *RIP3*. **(C)** *RIP3*$^{+/+}$ and *RIP3*$^{-/-}$ MEFs were treated as indicated and cell death was monitored by phase-contrast microscopy and PI staining**. (D)** Cells were treated as in (C) and cell viability was determined. Data are presented as mean ± SD from one representative experiment performed in triplicate. **(E)** MEFs transfected with non-target (NT) or MLKL siRNA were treated as indicated, and cell viability was determined. **(F)** RT-PCR confirming knockdown of MLKL by siRNA. Data are presented as mean ± SEM, n=2. Cycloheximide (Chx): 1μg/ml; zVAD: 20 μM, TNFα: 100 ng/ml.

Transferrin and glutamine are the death-inducing components in serum

We hypothesized that one or more factors present in serum was essential in driving necrotic cell death under amino acid free conditions. To identify these factors, Dr. Minghui Gao used an elegant biochemical approach, where he systematically fractionated serum and “chased” the killing activity of fractions that were able to induce cell death. Using this approach coupled with mass spectrometry analysis, Dr. Minghui Gao identified transferrin and glutamine as the two components of serum whose presence correlated well with killing activity. Consistently, we found that the combination of recombinant transferrin and L-glutamine was sufficient to recapitulate the killing activity of FBS death in amino acid free conditions (Figure 3.4).

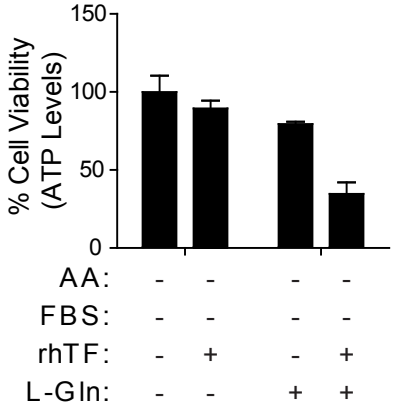


Figure 3.4. Glutamine in combination with transferrin recapitulates the cell death-inducing activity of serum in amino acid free conditions.
Bax/Bak-DKO MEFs were treated as indicated for 12 hr and cell viability was subsequently measured. L-Glutamine (L-Gln): 0.1 mM; Recombinant human holo-transferrin (rhTF). Experiments in Figure 3.4 were carried out by Dr. Minghui Gao.

Transferrin is an iron-carrier protein in serum that transports iron into the cell via receptor-mediated endocytosis (Andrews and Schmidt, 2007). Meanwhile, glutamine is the most abundant amino acid in serum, and its import into cells is primarily dependent on the receptors SLC38A1 and SLC1A5 (McGivan and Bungard, 2006). Once inside the cell, glutamine is used for the biosynthesis of various other nucleotides, amino acids and other macromolecule precursors, via the metabolic process of glutaminolysis (Figure 3.5A). In this process, glutamine is first converted into glutamate by glutaminases (GLS) (Curthoys and Watford, 1995), which can further be processed into α-ketoglutarate (α-KG) either by glutamate dehydrogenase (GLUD1)-mediated deamination or by transaminase-mediated transamination (Hensley et al., 2013). We found that pharmacological inhibition of glutamine uptake by L-g-glutamyl-p-nitroanilide (GPNA)(Esslinger et al., 2005) markedly blocked serum-dependent necrosis (Figure 5B). Additionally compound 968 (an inhibitor of GLS), and amino-oxyacetate (AOA - a pan inhibitor of transaminases) (Wang et al., 2010) also significantly inhibited cell death (Figure 3.5B). Collectively, these results indicate that import of glutamine into the cell, and subsequent metabolites derived from glutaminolysis are essential in mediating serum-dependent necrosis.

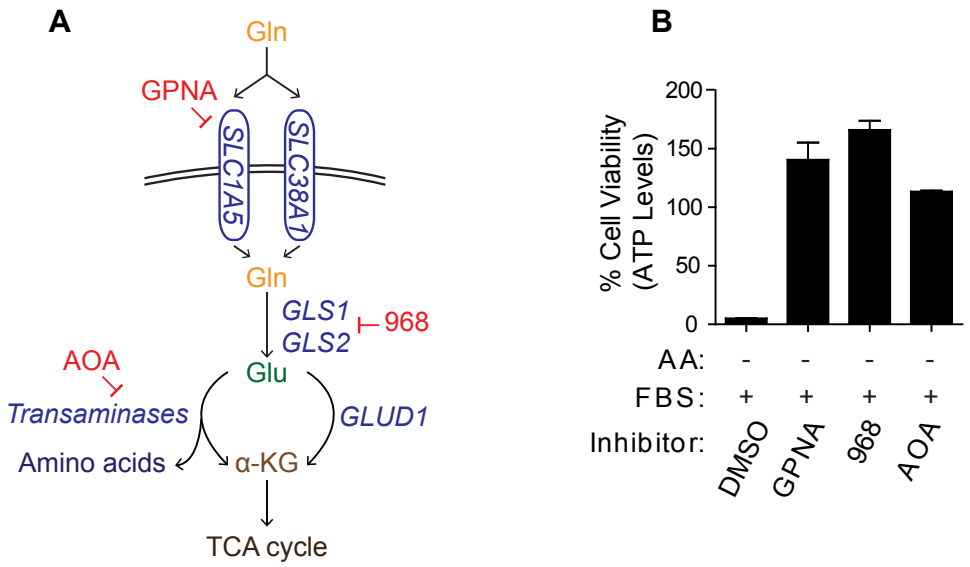


Figure 3.5. Glutaminolysis Mediates Serum-Dependent Necrosis.
(A) Schematic overview of the glutaminolysis pathway. Small molecule inhibitors used are shown in red. Glutamate (Glu); Glutaminase (GLS); glutamate dehydrogenase (GLUD1). **(B)** Pharmacological inhibition of multiple components in the glutaminolysis pathway abrogates serum-dependent necrosis. The following inhibitors were used: L-Gln transporter inhibitor (GPNA, 5 mM); GLS inhibitor (Compound 968, 20 μM); Pan-transaminases inhibitor (AOA, 0.5 mM). Figure 3.5B was performed by Dr. Minghui Gao.

Serum-dependent cell death is ferroptosis.

Stockwell and colleagues recently described a novel form of oxidative cell death induced by the small molecule erastin, termed ferroptosis (Dixon et al., 2012). Ferroptosis is characterized by the iron-dependent formation of lipid ROS. In the course of our studies, we noticed that serum-dependent cell death and ferroptosis share several striking similarities, including the requirement of iron import and the obligate accumulation of ROS. Erastin triggers ferroptosis by inhibiting cystine uptake and thus depleting glutathione, a key cellular antioxidant. In a similar vein, we found that deprivation of cysteine alone, in the presence of all other amino acids, was sufficient to induce serum-dependent necrosis (Figure 3.6A), leading us to test whether these two cell death processes were related.

Several experiments confirmed that serum-dependent necrosis was indeed ferroptosis. Firstly, ferrostatin-1 (Fer-1), an inhibitor of erastin-induced ferroptosis, also inhibited serum-dependent cell death triggered by full amino acid starvation or cystine starvation alone (Figure 3.6B). Secondly, both glutamine and transferrin (either recombinant or from dialyzed FBS) were required for erastin-induced ferroptosis (Figure 3.6C). Finally, the glutaminolysis inhibitors Compound 968 and AOA also inhibited erastin-induced ferroptosis, confirming that serum-dependent necrosis is indeed a form of ferroptosis (Figure 3.6D).

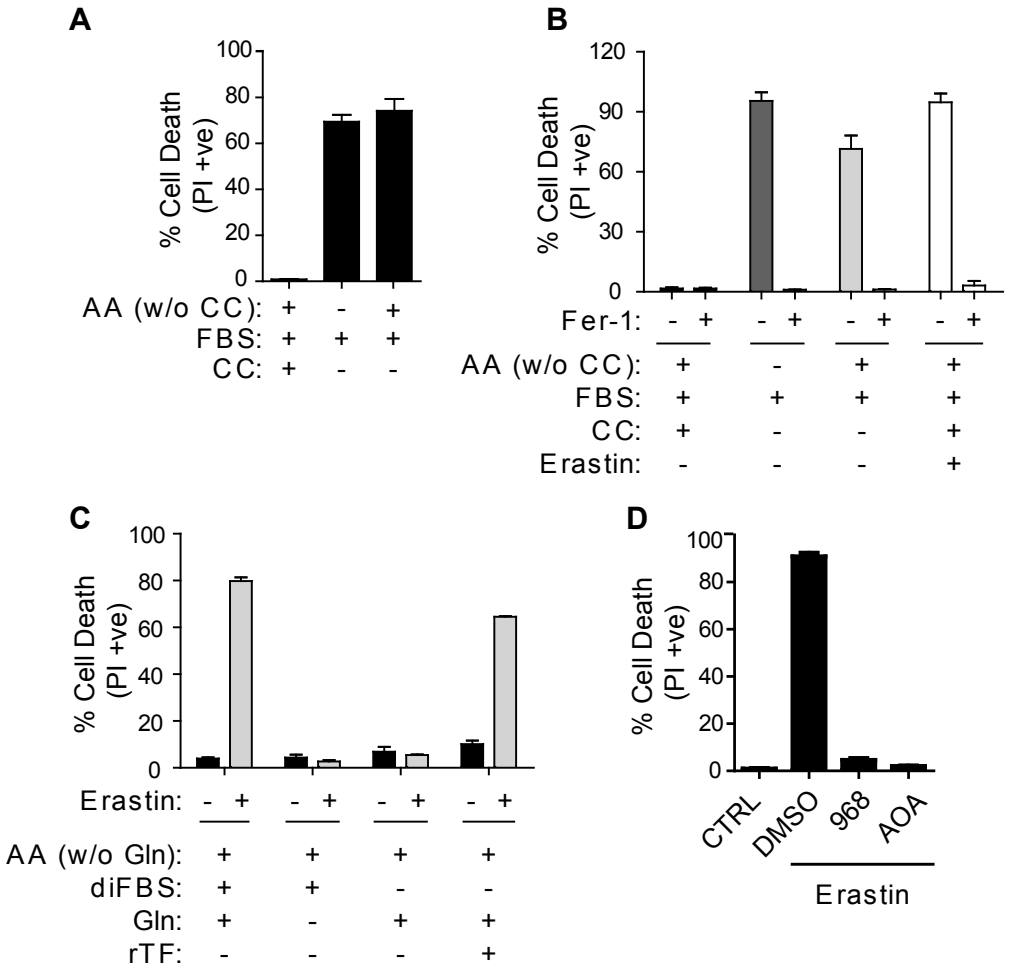


Figure 3.6. Serum-dependent cell death is ferroptosis.
(A) Cystine (CC) starvation alone is sufficient to induce serum-dependent cell death. MEFs were treated as indicated for 12 hrs and assayed for cell death. **(B)** Ferroptosis inhibitor Ferrostatin-1 (Fer-1) can inhibit serum-induced necrosis upon total AA starvation or cystine starvation. MEFs were treated as indicated for 12 hrs and assayed for cell death. **(C)** Erastin-induced ferroptosis requires both transferrin and glutamine. *Bax/Bak* DKO MEFs were treated as indicated for 12 hrs, and assayed for cell death. Note that dialyzed FBS (diFBS) contains transferrin but not glutamine, and can thus substitute for recombinant transferrin **(D)** Inhibition of glutaminse by Compound 968 (20 μM) or transaminases by the pan-transaminases inhibitor AOA (0.5 mM) blocks erastin-induced ferroptosis. MEFs were treated as indicated for 12 hrs, and assayed for cell death. Erastin, 1 μM. In all cases, cell death was measured by PI staining coupled with flow cytometry. Figure 3.6 was performed by Dr. Minghui Gao.

Mitochondria undergo dramatic morphological changes during serum-dependent ferroptosis

Despite significant progress in identifying ferroptotic stimuli *in vitro* and *in vivo*, our understanding of the executionary mechanism in ferroptotic cell death remains limited. I decided to explore the role of mitochondria in this process for several reasons. Firstly, mitochondria have been clearly implicated in several other forms of cell death including apoptosis, as well as several forms of programmed necrosis dependent on cyclophilin-D, the mitochondrial permeability transition pore (MPTP), and Poly(ADP-ribose) polymerase (PARP) (Bergsbaken et al., 2009). Secondly, several of the reactions in the glutaminolysis pathway (which we demonstrated to be essential for serum-dependent and erastin-induced ferroptosis) occur in the mitochondria (DeBerardinis et al., 2008). Finally, mitochondria are a chief reservoir of intracellular iron and play a key role in iron metabolism through the synthesis of heme and iron-sulfur clusters (Hentze et al., 2010).

I first examined mitochondrial morphology using a GFP fusion protein that localizes localizes to the mitochondrial matrix. The vast majority of cells grown in full media displayed normal morphology, characterized by an interconnected network of mitochondria with few to no fragmented structures. However, during serum-dependent ferroptosis, I observed that mitochondria underwent dramatic morphological changes, characterized by clear loss of a tubular network and increased fragmentation into small, condensed spheres (Figure 3.7).

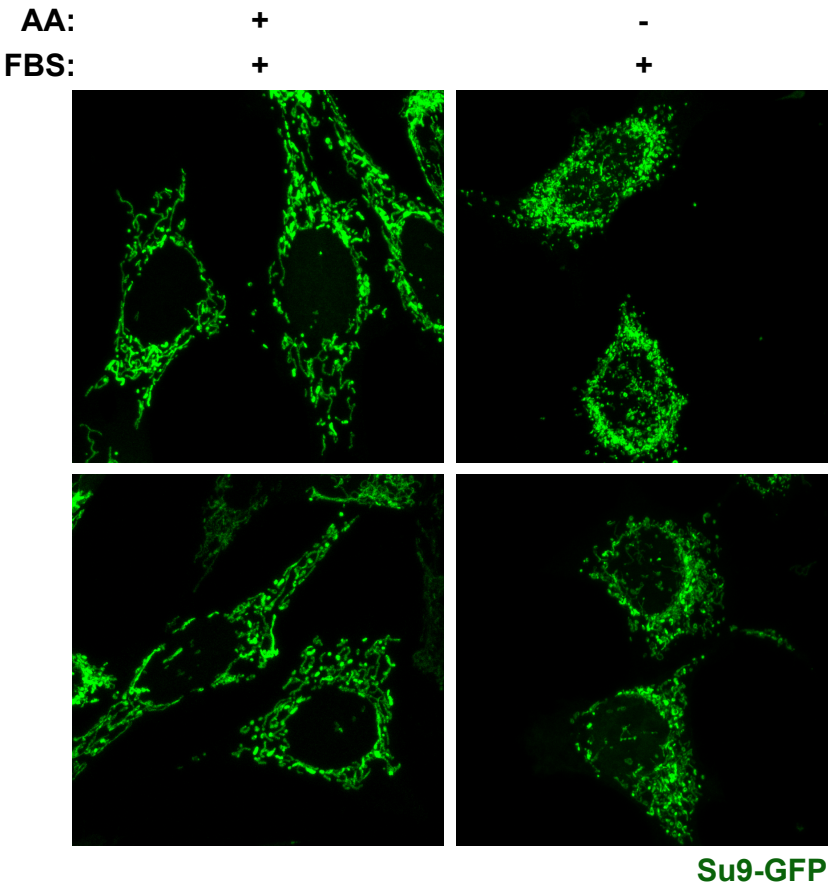


Figure 3.7. Serum-dependent cell death is characterized by dramatic changes in mitochondrial morphology.
MEFs expressing mitochondrial matrix-targeted GFP (Su9-GFP) were treated as indicated and imaged by confocal microscopy. Two representative fields of view are shown.

Mitochondria are highly dynamic organelles that undergo constant cycles of fusion and fission. By modulating the rates of fusion and fission, cells can establish, maintain, and alter the overall architecture of these organelles as needed (Detmer and Chan, 2007). Both fusion and fission of mitochondria are controlled by members of the dynamin related protein (DRP) family of large GTPases, and the master regulator of mitochondrial fission in mammals is Drp1 (Smirnova et al., 2001; Hoppins et al., 2007). Given the extensive mitochondrial fission observed during serum-dependent ferroptosis, I tested if inhibiting fission could prevent cell death as well. To do so, I used two independent shRNA against Drp1, which resulted in a highly elongated mitochondrial network as expected (Figure 3.8A). I found that Drp1-knockdown MEFs were significantly resistant to serum-dependent ferroptosis (Figure 3.8B and C). Notably, Drp1 knockdown also inhibited ferroptosis induced by erastin, ruling out the possibility that this is a stimulus specific phenomenon (Figure 3.8D). Furthermore, chemical inhibition of Drp1 by the small molecule Mdivi-1 (Cassidy-Stone et al., 2008), also inhibited erastin-induced ferroptosis in MEFs (Figure 3.8E). Collectively, these results point towards a fundamental role for mitochondria in regulating ferroptosis.

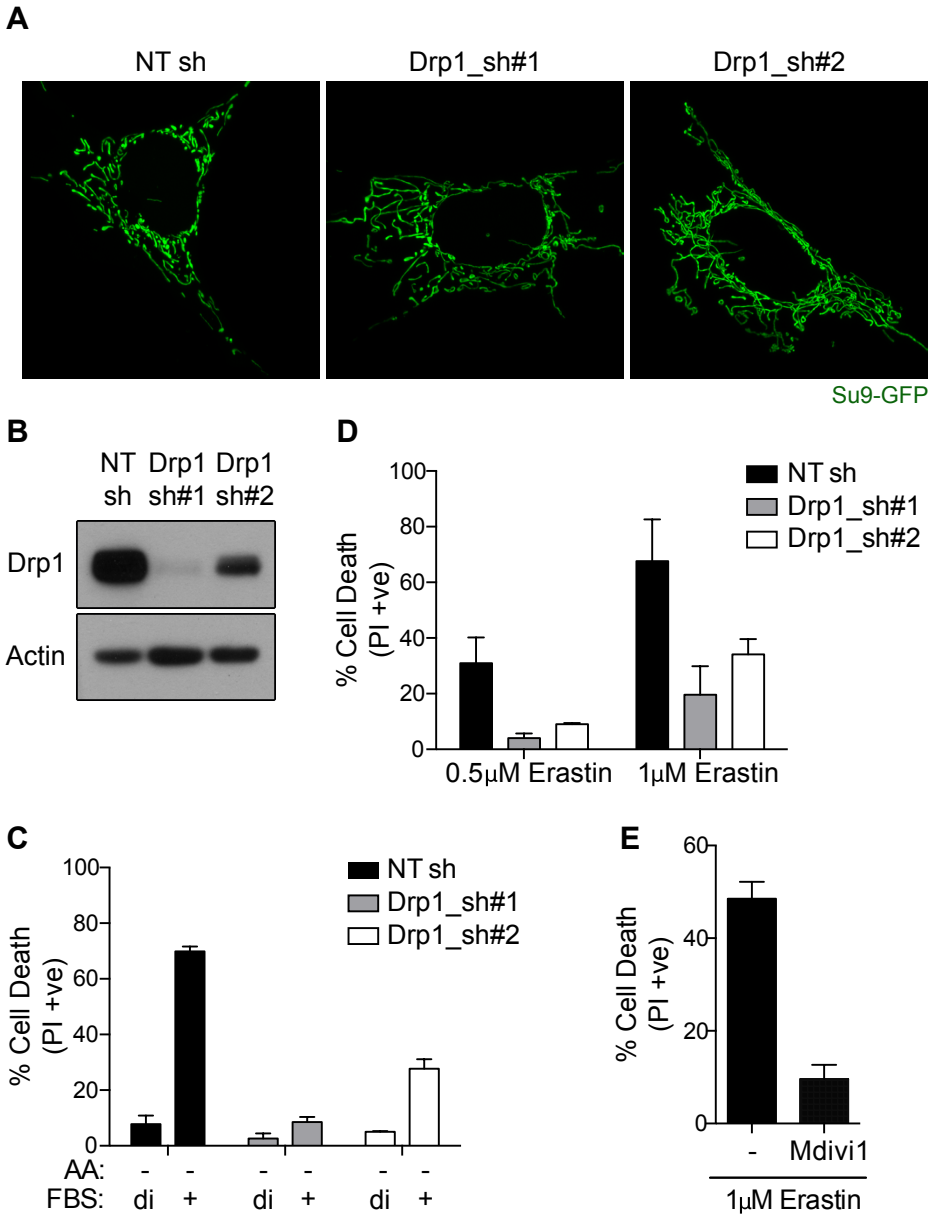


Figure 3.8. Inhibiting mitochondrial fission attenuates ferroptosis.
(A) Mitochondrial morphology of MEFs stably expressing Su9-GFP and indicated shRNAs (NT; non-target) was observed by confocal microscopy. **(B)** Western blot confirming knockdown of Drp1 by respective shRNA. **(C)** MEFs stably expressing indicated shRNA were treated as indicated for 10 hrs and assayed for cell death. Dialyzed FBS (di) was used as a control. Data are presented as mean ± SD from one representative experiment performed in triplicate. **(D)** MEF expressing indicated shRNA were treated with Erastin for 16 hrs and assayed for cell death. Data are mean ± SD, n=3 independent experiments. **(E)** MEFs were treated with Erastin and the Drp1 inhibitor Mdivi1 (100μM) for 16 hrs and assayed for cell death. Data are mean ± SD, n=2 independent experiments. In all cases, cell death was measured by PI staining coupled with flow cytometry.

Mitochondrial hyperpolarization during serum-dependent ferroptosis

To further characterize functional changes in mitochondria during ferroptosis, I performed time-lapse imaging in cells loaded with the membrane potential sensitive dye, TMRE, which is readily sequestered by actively respiring mitochondria. During serum-dependent ferroptosis, I found that cells undergo mitochondrial hyperpolarization shortly before cell-rounding and plasma membrane rupture, as observed by a sustained increase in TMRE fluorescence (Figure 3.9A). In stark contrast, death induced by combined starvation of amino acids and serum was characterized by a gradual reduction in membrane potential (Figure 3.9B), as is to be expected with apoptotic cell death (Ly et al., 2003). Notably, co-treatment with the ROS scavenger N-acetylcysteine (NAC), inhibited cell death and abolished mitochondrial hyperpolarization (Figure 3.10A and B), indicating that hyperpolarization occurs downstream of, and is dependent on, ROS production.

Figure 3.9. Serum-dependent ferroptosis is accompanied by mitochondrial hyperpolarization.
Representative stills from time-lapse imaging of mitochondrial membrane potential in MEFs undergoing **(A)** serum-dependent ferroptosis or **(B)** starvation induced apoptosis are shown. Cells were loaded with TMRE (200nM) prior to indicated treatment. Quantification of mean cellular fluorescence over time for two distinct cells is shown in adjacent panels.

Figure 3.10. Mitochondrial hyperpolarization occurs downstream of ROS production during ferroptosis.
Representative stills from time-lapse imaging of mitochondrial membrane potential in MEFs. **(A)** Serum-dependent ferroptosis is accompanied by mitochondrial hyperpolarization **(B)** The ROS scavenger N-acetylcysteine (NAC; 20μM) abolishes mitochondrial hyperpolarization.

Figure 3.9. (Continued)

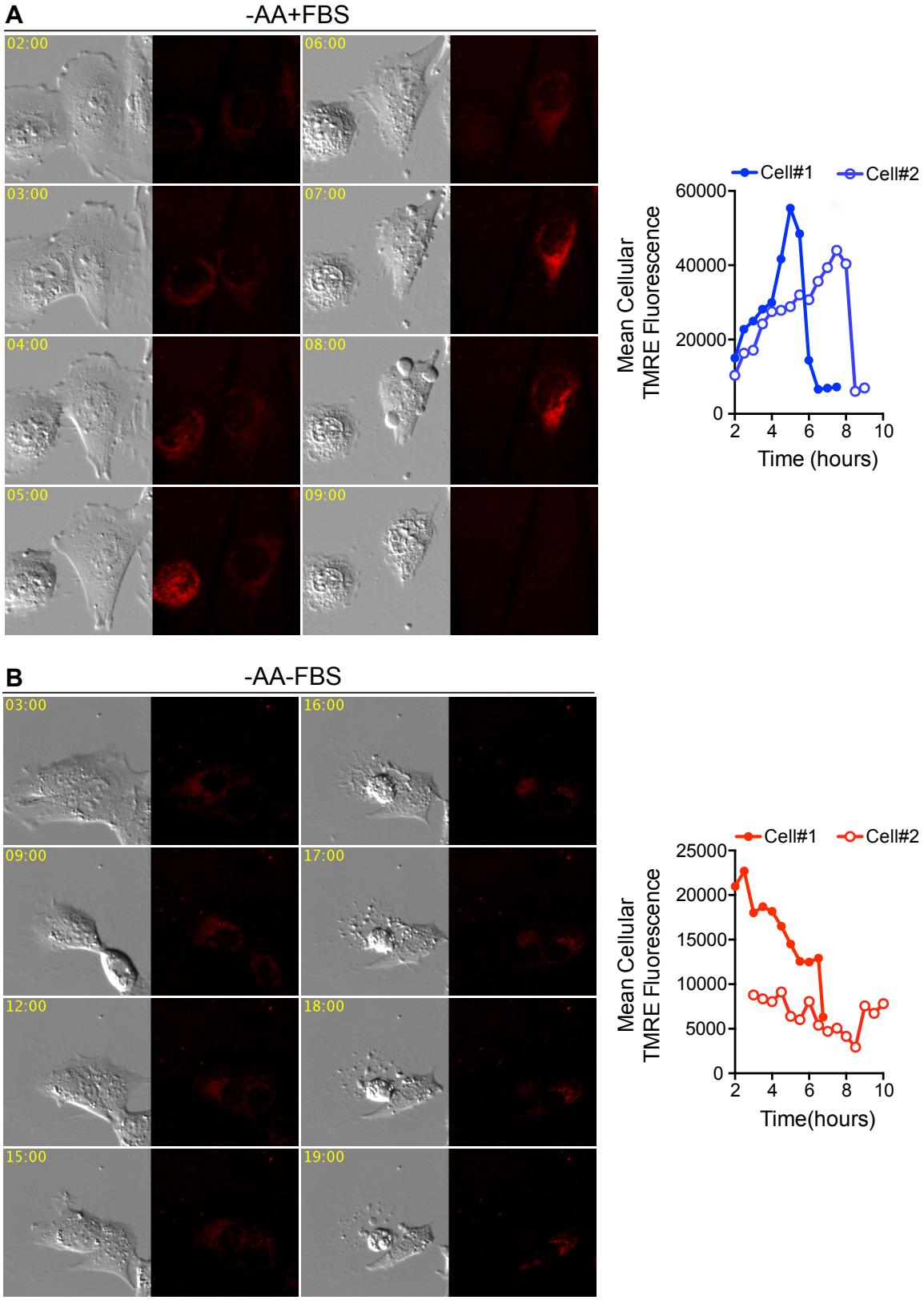
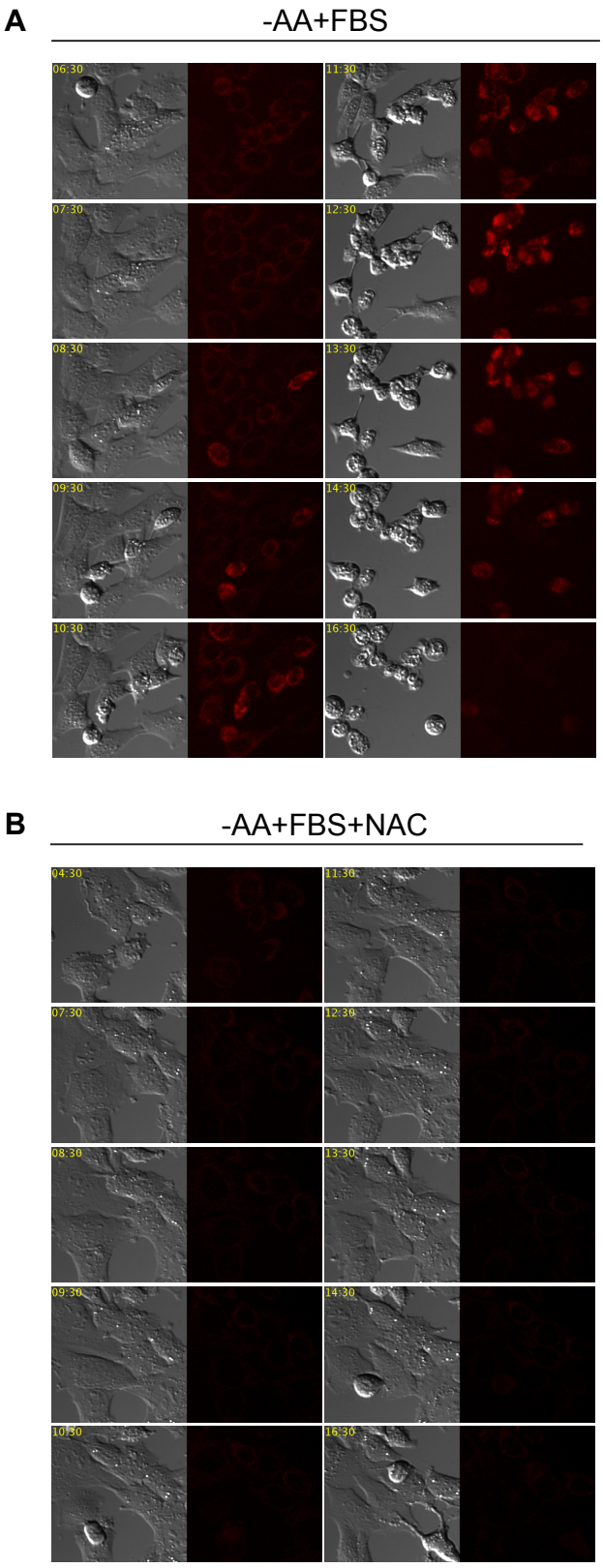


Figure 3.10. (Continued)



Disrupting mitochondrial membrane potential inhibits ferroptosis

The potential across the inner mitochondrial membrane is primarily established and maintained by the action of the enzymes in the electron transport chain (ETC), which couple the reductive transfer of electrons with pumping protons (H^+) into the inter-membrane space. This generates an electrochemical gradient that is used by the F_0F_1-ATP synthase complex to generate ATP. Inhibiting the activity of the ETC thus affects membrane potential (Smeitink et al., 2001).

To examine the significance of mitochondrial membrane potential in ferroptosis, I tested the effects of several inhibitors of the ETC. Use of rotenone and antimycin, inhibitors of Complex I and Complex III of the ETC respectively, reduced erastin-induced cell death in HT1080 cells (Figure 3.11A and B). While these inhibitors were both inherently toxic with prolonged exposure themselves, I found that carbonyl cyanide 3-chlorophenylhydrazone (CCCP) was especially potent in inhibiting both serum-dependent and erastin-induced cell death in multiple cell lines, as measured by PI exclusion and clonogenic growth assays (Figure 3.11A, B, C and D). CCCP is a protonophore that rapidly dissipates the H^+ gradient generated by the ETC, effectively depolarizing mitochondria and shutting down oxidative phosphorylation. These results suggest that a healthy mitochondrial membrane potential is needed to mediate ferroptosis.

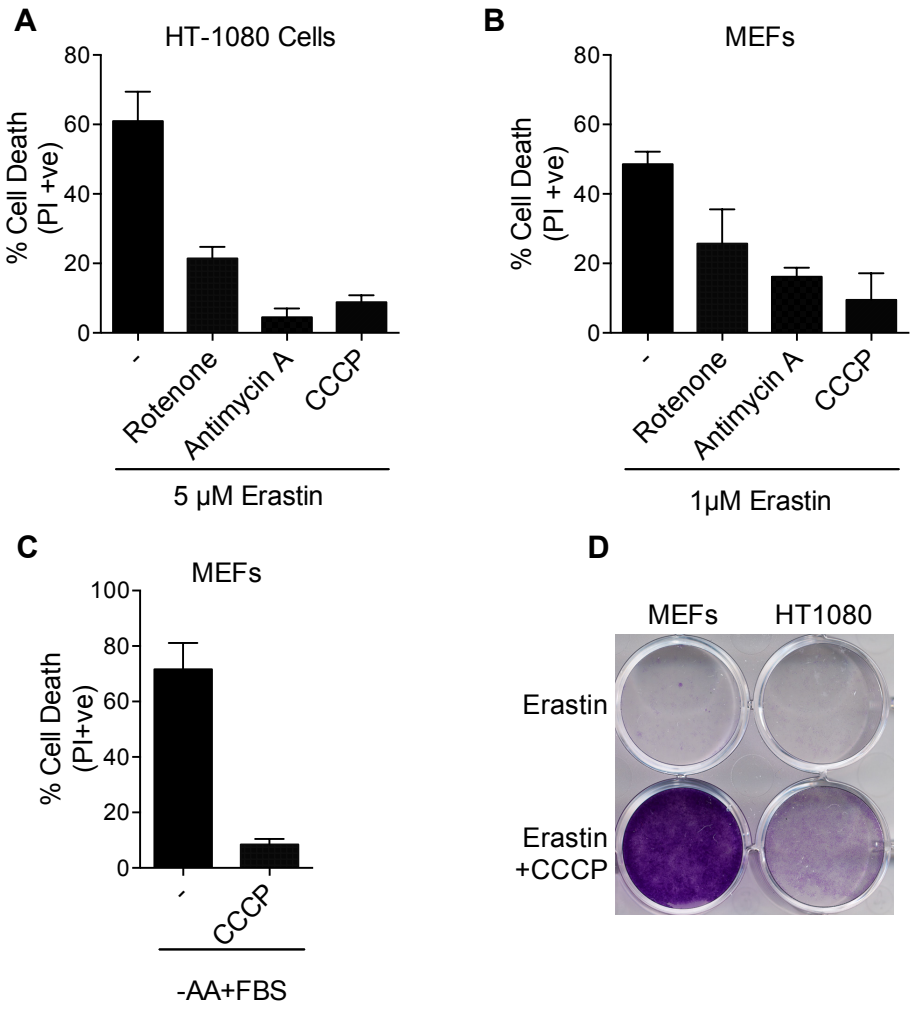


Figure 3.11. Disrupting mitochondrial membrane potential inhibits ferroptosis induced by erastin or amino acid deprivation.
(A) HT1080 cells were treated with 5μM erastin and indicated inhibitors for 16 hrs and assayed for cell death. Data are mean ± SD, n=3 independent experiments **(B)** MEFs were treated with 1μM erastin and indicated inhibitors for 16 hrs and assayed for cell death. Data are mean ± SD, n=2 independent experiments **(C)** MEFs were treated as indicated and assayed for cell death. Data are mean ± SD, n=3 independent experiments. In all cases, cell death was measured by PI staining coupled with flow cytometry. Rotenone (complex I inhibitor, 10μM in MEFs, 5μM in HT1080); Antimycin A (complex III inhibitor; 50μM); CCCP (membrane decoupler; 10μM). **(D)** MEFs and HT1080 cells were seeded at low density in 12 dishes and treated with erastin for 16 hrs. Cells were then allowed to recover for 3 days and subsequently fixed and stained with crystal violet.

The formation of lipid-based ROS is considered to be the driving force behind ferroptosis (Dixon et al., 2012). I thus examined the effect of CCCP on the accumulation of lipid ROS using the redox-sensitive dye BODIPY 581/591 C11. Intriguingly, although CCCP potently inhibited erastin-induced cell death, I found that it only had a minor effect on the accumulation of lipid ROS (Figure 3.12A and B). In contrast, compound 968, a glutaminase inhibitor that also inhibits erastin-induced ferroptosis, virtually abolished lipid peroxidation. These results demonstrate that CCCP and Compound 968 mediate their inhibition of ferroptosis through different means; while compound 968 blocks the formation of lipid ROS altogether, CCCP inhibits cell death downstream of lipid peroxidation. Furthermore, this result also suggests that the formation of lipid ROS is necessary, but not sufficient to induce ferroptosis.

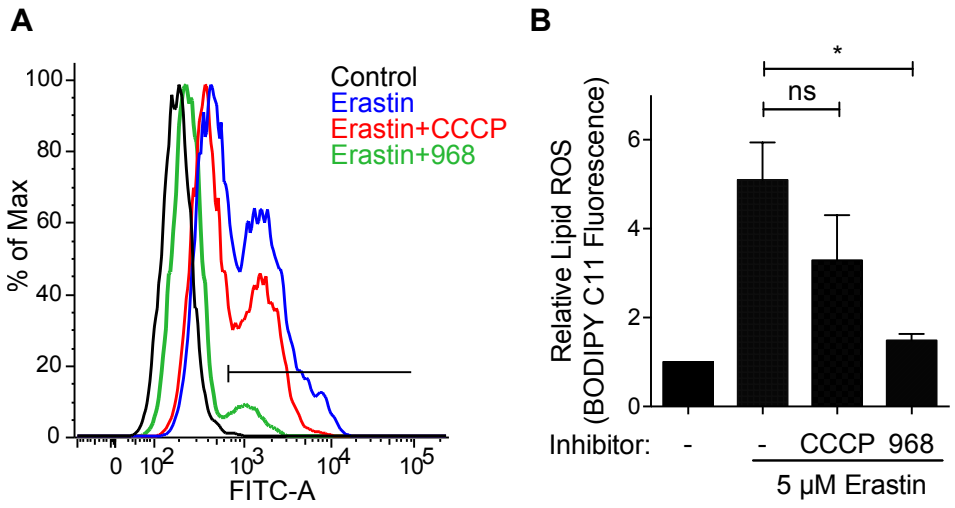


Figure 3.12. Disrupting mitochondrial membrane potential does not inhibit lipid ROS production.

HT1080 cells were treated as indicated for 10 hrs following which accumulation of lipid ROS was assessed by BODIPY C11 staining. **(A)** A representative histogram from flow cytometry analysis. **(B)** Quantification of median lipid ROS levels. Data are mean ± SD, n=3 independent experiments. (Student's t-test: ns; not significant, **P*<0.05).

Mitochondrial depletion by Parkin-mediated mitophagy inhibits erastin-induced ferroptosis

To directly test whether mitochondria are required to mediate ferroptosis, I adopted a previously characterized approach that allows for depletion of virtually all mitochondria from cells (Narendra et al., 2008; Tait et al., 2013). This system relies on overexpression of the Parkin protein, which selectively mediates the clearance of damaged mitochondria that lack membrane potential through the process of mitophagy. Treatment of Parkin-overexpressing HT1080 cells with CCCP (to induce depolarization) for 48 hours resulted in virtually no detectable mitochondria as assessed by immunostaining for TOM20 or western blotting for TIM23, both endogenous mitochondrial proteins (Figure 3.13A and B). These mitochondria-depleted cells persisted in such a state for a few days, as previously described (Narendra et al., 2008). In contrast, CCCP treatment in wild type cells had no major effect on mitochondrial volume in the same time frame.

Following two days of CCCP treatment to deplete mitochondria, I exposed WT and Parkin-overexpressing cells to erastin. Of note, since co-treatment with CCCP itself inhibits erastin-induced cell death (as described in the previous section), cells were extensively washed to remove traces of CCCP prior to addition of erastin. Additionally, a significantly higher concentration of erastin and extended treatment duration was used to induce robust cell death in this experiment. Remarkably, mitochondria-depleted cells displayed significant resistance to erastin-induced cell death relative to WT cells (Figure 3.13 C**)**.

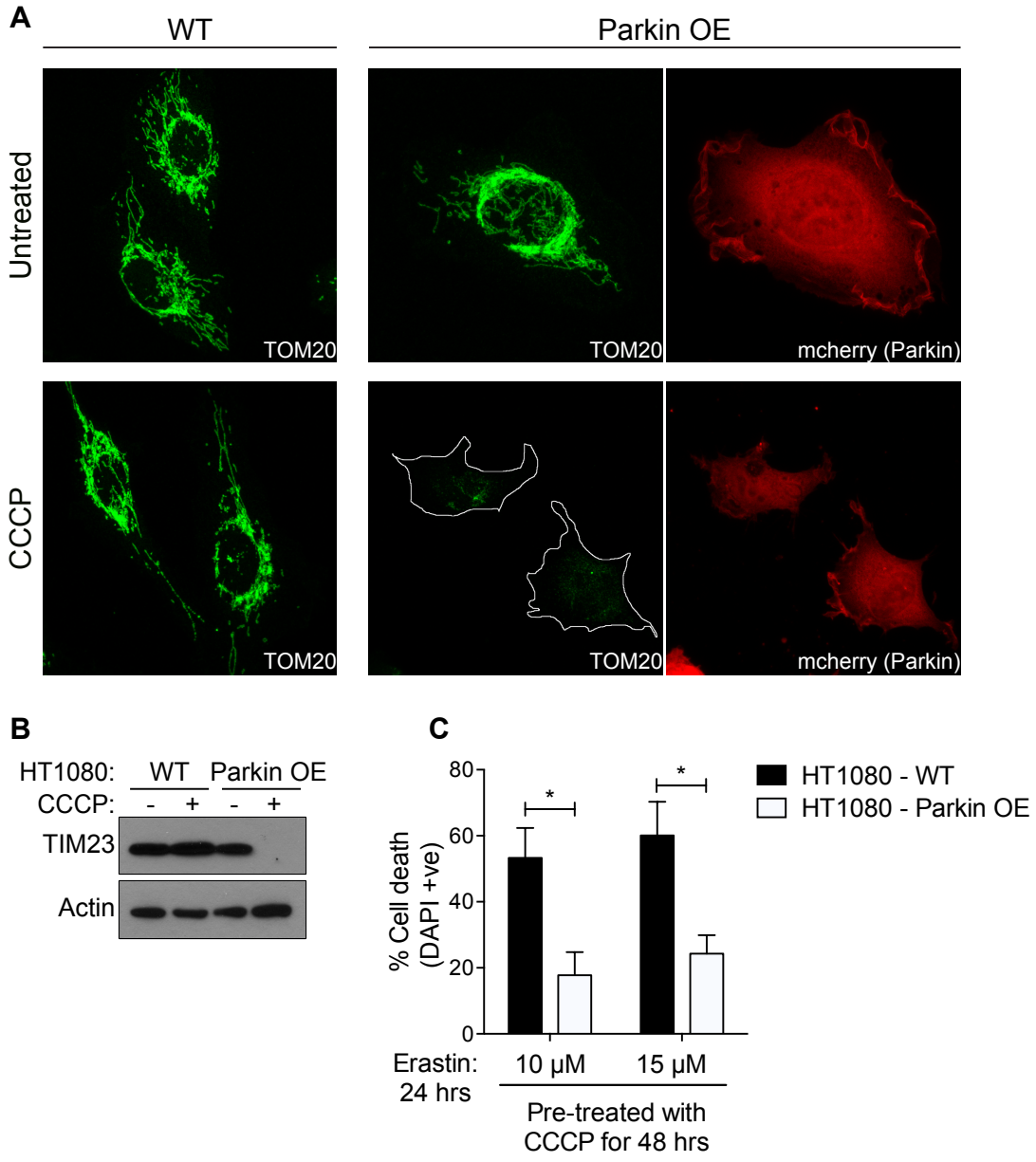


Figure 3.13. Mitochondrial depletion by parkin-mediated mitophagy inhibits ferroptosis.
Wildtype or mcherry-Parkin overexpressing HT1080 cells were treated with 30μM CCCP for 48 hrs to induce mitochondrial depletion. **(A)** The presence of mitochondria was assessed by immunostaining for endogenous TOM20 or by **(B)** Western blot for endogenous TIM23. **(C)** Cells were pre-treated with CCCP for 48 hrs and then thoroughly washed before being treated with erastin for 24 hrs. Cell death was then measured by DAPI staining coupled with flow cytometry. Data are mean ± SD, n=3 independent experiments. (Student's t-test: **P*<0.05).

Given that ferroptosis requires the formation of lipid ROS and mitochondria are generally considered to be among the most important sources for cellular ROS (Murphy, 2009), I examined the accumulation of lipid peroxides in mitochondria-depleted cells using the BODIPY-C11 redox sensor. Erastin-induced lipid peroxidation was slightly reduced but not completely inhibited in mitochondria-depleted cells (Figure 3.14A and B), implying that mitochondria are not the major source of lipid ROS during ferroptosis. Collectively, these results implicate a functional role for mitochondria in mediating ferroptosis downstream of lipid ROS production.

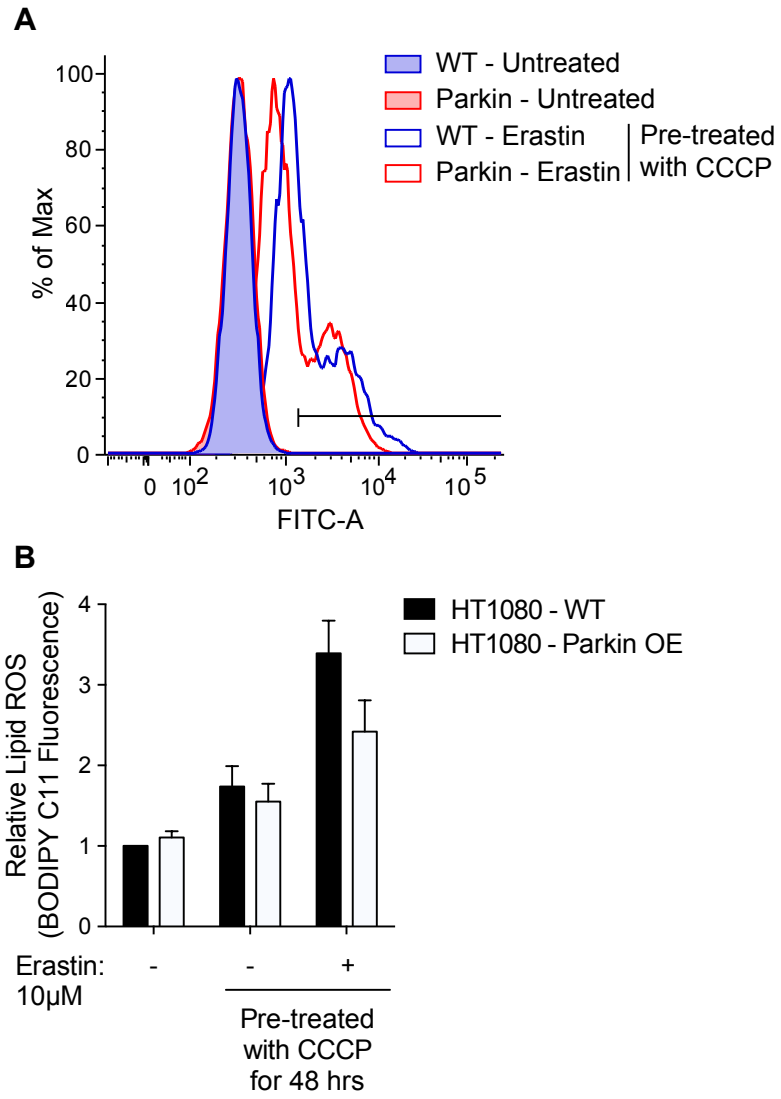


Figure 3.14. Mitochondrial depletion does not affect lipid ROS production during ferroptosis.

Wildtype or mcherry-Parkin overexpressing HT1080 cells were pre-treated with 30μM CCCP for 48 hrs to induce mitochondrial depletion and then thoroughly washed before being treated with 10μM erastin for 10 hrs. The accumulation of lipid ROS was then assessed by BODIPY C11 staining. **(A)** A representative histogram from flow cytometry analysis. **(B)** Quantification of median lipid ROS levels. Data represent mean ± SD, n=4 independent experiments.

3.3. Discussion

We began this project aiming to identify specific molecules present in serum that can induce cell death in amino-acid free conditions. These death-inducing factors were eventually revealed to be the iron-carrier protein transferrin, and the amino acid glutamine. Additionally, the metabolic process of glutaminolysis, which provides substrates for the TCA cycle and synthesis of various macromolecules, is also essential in mediating this cell death process. Several lines of evidence suggested that such serum-dependent cell death is in fact ferroptosis, a recently described form of iron-dependent oxidative cell death (Dixon et al., 2012). These include the requirement of cystine starvation, cellular glutathione depletion, and the involvement of iron-carrier transferrin in mediating cell death. Further testing confirmed that indeed, cystine starvation in the presence of glutamine was sufficient to induce ferroptosis. Thus our studies have identified several novel regulators of ferroptotic cell death.

Subsequently, I aimed to characterize the role of mitochondria in serum-dependent and erastin-induced ferroptosis. I found that mitochondria undergo significant fragmentation during ferroptosis. Inhibiting mitochondrial division genetically (by knockdown of Drp1) or pharmacologically (using Mdivi-1) significantly reduced ferroptosis induced by these stimuli. However, it should be noted that Drp1-/- MEFs were susceptible to ferroptotic stimuli, and Drp1 knockdown cells did eventually die as well, indicating that Drp1 is not essential for ferroptosis. Rather, it is likely that cells with hyper-fused mitochondria are intrinsically more resistant to ferroptotic cell death. The mechanism underlying

this resistance is yet to be defined, but it is tempting to speculate that it involves favorable alterations in the basal metabolic state of Drp1-knockdown cells, such as increased ATP production and decreased mitophagy (Hoppins, 2014).

Using live-cell imaging, I found that ferroptosis is accompanied by robust mitochondrial hyperpolarization prior to plasma-membrane rupture. The significance of this hyperpolarization event remains unclear. We originally suspected the hyperpolarization to be indicative of an oxidative burst occurring in the mitochondria. However, the lack of hyperpolarization in the presence of the ROS scavenger NAC, strongly suggests that ROS production precedes hyperpolarization. Thus, mitochondrial hyperpolarization is likely a consequence rather than the cause of ROS production. The effectiveness of the depolarizing agent CCCP in inhibiting erastin and serum-dependent ferroptosis implies that this hyperpolarization event is consequential in ultimately mediating cell death.

Mitochondria-mediated ROS formation has been implicated in several other forms of cell death (Fleury et al., 2002; Chen et al., 2007; Ott et al., 2007). Yet, I found that erastin induced significant lipid peroxidation in mitochondria depleted cells, indicating that ferroptosis involves the production of lipid ROS from non-mitochondrial sources. This is consistent with a previous study demonstrating that a mitochondria targeted lipid redox sensor undergoes oxidation with significantly slower kinetics than a non-targeted sensor, and that a mitochondria targeted antioxidant is significantly weaker at inhibiting ferroptosis. (Friedmann Angeli et al., 2014). Further experiments will be necessary to

precisely pinpoint the cellular source of ROS production during ferroptosis. It should be noted that erastin-induced lipid ROS production in mitochondria depleted cells was slightly lower in comparison to wild type cells. Mitochondria play a key role in fatty acid synthesis through the production of citrate from the TCA cycle (Vander Heiden et al., 2009). Thus, this slight decrease in lipid ROS production could be due to diminished mitochondrial fatty acid metabolism in these cells, which is believed to supply specific lipid substrates for oxidation during ferroptosis (Dixon et al., 2012).

Despite significant lipid ROS production, mitochondria depleted cells were highly resistant to erastin induced cell death. There are two important implications from this finding. Firstly, it suggests that lipid peroxidation is necessary, but not sufficient, to induce ferroptosis. This is additionally substantiated by the finding that CCCP, which potently inhibits cell death, also fails to inhibit lipid ROS production. Secondly, it further implicates a functional role for mitochondria in mediating ferroptosis, downstream of lipid ROS. The precise nature of such a function for mitochondria remains to be defined. One intriguing possibility is that lipid peroxides generated elsewhere could be transported into the mitochondria and cause damage inside the organelle, leading to cell death. In this regard, it is worth noting that several lipid trafficking proteins have been implicated in transporting oxidized lipids into the mitochondria (Vila et al., 2004; Korytowski et al., 2015). Import of proteins into the mitochondrial matrix via the TIM23 channel is known to require a healthy membrane potential (Martin et al., 1991; Pfanner and Geissler, 2001). It is thus

conceivable that CCCP treatment (which leads to depolarization) inhibits cell death by preventing the import of toxic lipids into the mitochondria. Comparing the lipidomic profiles of mitochondria isolated from cells treated with erastin± CCCP would be an ideal strategy to test this hypothesis.

The involvement of mitochondria in ferroptosis induced by other stimuli also remains to be tested. Just as mitochondria are dispensable for execution of extrinsic apoptosis in certain cell types (Scaffidi et al., 1998), the existence of mitochondria-independent ferroptotic pathways is certainly plausible. Identifying such pathways and their relevant stimuli would be mechanistically intriguing.

In summary, our studies identified several novel regulators of ferroptotic cell death (Figure 3.15). Mechanistically, the data presented here support a model where mitochondria play an essential role in mediating erastin-induced and serum-dependent ferroptosis, downstream of lipid ROS production. Future work directed at teasing apart how lipid ROS act in concert with mitochondria to induce cellular demise would further our understanding of this unique cell death process. Ultimately, this could aid in the development of novel therapeutics that can unleash or inhibit ferroptosis for clinical benefit.

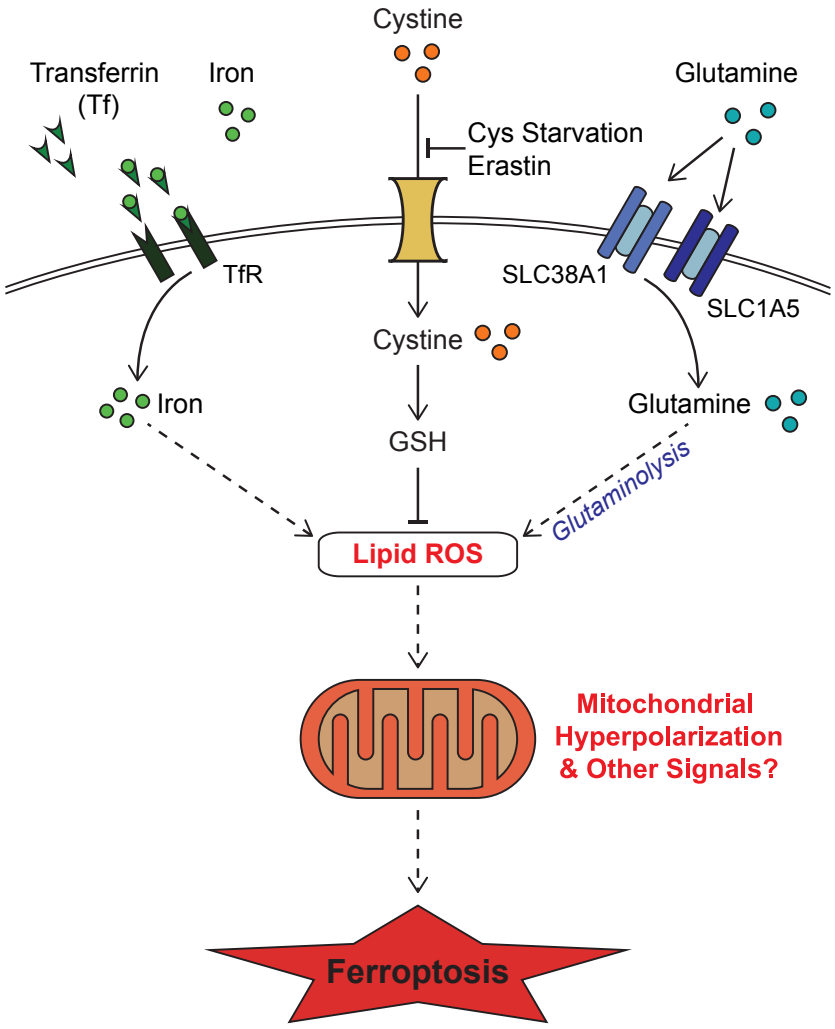


Figure 3.15. Proposed model for the role of glutamine, transferrin and mitochondria in regulating ferroptosis

Cystine starvation or erastin treatment leads to depletion of glutathione (GSH), a key cellular antioxidant. In this scenario, the import of glutamine and subsequent byproducts from glutaminolysis lead to an iron-dependent production of oxidized lipids from a non-mitochondrial source. The accumulation of lipid ROS triggers structural alterations and hyperpolarization in the mitochondria, ultimately leading to ferroptotic cell death.

3.4. Experimental procedures

Cell Culture

MEFs, HT1080 and 293T cells were cultured in DMEM-high glucose supplemented with 10% FBS, L-glutamine (2 mM) and penicillin-streptomycin. Lentiviral and retroviral constructs were co-transfected with the respective packaging vectors into 293T cells for virus production. Virus containing media was passed through a 0.45 μM PES filter and supplemented with polybrene before being used to transduce cells.

Reagents, Antibodies and plasmids

The following antibodies were used: Drp1 (BD Biosciences, #611112), RIP3 (Prosci, #2283), TIM23 (BD Biosciences, #611222), TOM20 (Santa Cruz, #11415). The following small molecule inhibitors and chemicals were used: Mdivi1 (Sigma, #M0199), Rotenone (Sigma, # R8875), antimycin (Sigma, #A8674), CCCP (Sigma, #C2759), Compound 968 (Millipore, #352010). Erastin (Millipore, #329600 or Sigma, #E7781), Ferrostatin-1 (XcessBio, # M60042), NAC (Sigma, # A7250), AOA (Sigma, # C13408) and GPNA (Sigma, G6133). The Drp1 shRNA sequences were sh#1: GCTTCAGATCAGAGAACTTAT and sh#2: CGGTGGTGCTAGGATTTGTTA. Lentiviruses harboring the knockdown sequences were used to infect MEF cells. Cells when then selected and maintained in puromycin. Su9 cDNA (containing amino acids 1-69 of subunit 9 of F_0-ATPase) was purchased from Addgene (#23214) and subcloned into the pQCXIP vector with a C-terminal GFP tag. The mCherry-parkin construct was generously provided by Dr. Cole Haynes (MSKCC).

Cell death induction and measurements

To induce cell death, 80%-confluent cells were washed with PBS twice and then cultured in amino acid-free medium, with specific factors added as indicated in individual experiments. Alternatively, cells were treated in full media with the indicated concentration of erastin. Cell death was analyzed by propidium iodide (PI) or DAPI staining. Briefly, following treatment, adherent and floating cells were collected by trypsinization and resuspended in FACS buffer (1% FBS in PBS) containing PI (0.1μg/uL) or DAPI (1 μg/ml) and analyzed immediately by flow cytometry. A minimum of 10,000 cells were recorded per condition. Alternatively, cell viability was determined using the CellTiter-Glo luminescent Cell Viability Assay (Promega). In assays using WT MEFs, viability was calculated by normalizing ATP levels to cells treated with amino acid starvation in the presence of 10% (v/v) diFBS, while in assays using *bax/bak*-DKO MEFs, ATP levels were normalized to cells treated with amino acid and FBS double starvation. Clonogenic assays were performed as described in chapter two.

Lipid ROS measurements

Cells were plated in 6-well dishes and treated as indicated. 1.5 μM BODIPY 581/591 C11 (Invitrogen) was added for the final 1 hour of treatment. Cells were then washed, collected by trypsinization, resuspended in FACS buffer, and analyzed by flow cytometry. A 488nm laser was used for excitation, and data was collected from the FITC (530/50 BP) channel. A minimum of 20,000 cells was recorded per condition. All FACS data Analysis were analyzed using FlowJo

software (Tree Star). Relative lipid ROS was calculated in relation to control cells using median fluorescence intensity in the FITC channel.

qRT-PCR

qRT-PCR was performed as described in chapter 1, with the following primers:

MLKL_FW: GGAAGATCGACAGGATGCAG

MLKL_Rev: GTCCACGGAGGTCCAAGATG

Actin_FW: GGCACCACACCTTCTACAATG

Actin_Rev: GGGGTGTTGAAGGTCTCAAAC

Immunofluorescence and fluorescence microscopy

For imaging mitochondria morphology, MEFs expressing Su9-GFP were plated on 35-mm glass-bottom plates (MatTek) and imaged live on a NikonEclipse Ti-U confocal microscope. For immunofluorescence, cells were plated onto glass coverslips 24 hours prior to the experiment. Following treatment, cells were fixed in 3.7% formaldehyde for 20 min, permeabilized with 0.1% Triton X-100 in PBS for 5 minutes and incubated in blocking buffer (1% BSA in PBS) for 15 min. After washing, the coverslips were incubated with TOM20 antibody (1:500) in blocking buffer for 90 min at 37°C. After washing three times, cover slips were incubated with Alexa Fluor secondary antibody (ThermoFisher, # A11012 or A11029, 1:1000) for 30 min at 37°C. After washing, coverslips were mounted on microscope slides using ProLong Gold antifade reagent (Life Technologies, # P36935). Slides were visualized on a Nikon Eclipse TE2000-U microscope or a NikonEclipse Ti-U confocal microscope. Acquired images were processed and

analyzed with ImageJ software (NIH).

Time-lapse imaging

Live-cell imaging of H2b-mCherry-expressing and WT MEFs was performed on glass-bottom six-well plates (MatTek) using a Nikon Ti-E inverted microscope attached to a CoolSNAP CCD camera (Photometrics). For imaging of mitochondrial membrane potential, cells were pre-loaded with 200nm TMRE (Enzo Life Sciences) for 1 hour. Subsequently, cells were washed to remove traces of TMRE from the media and treated as indicated. Fluorescence and differential interference contrast (DIC) images were acquired every 5-7 min, and images were analyzed using NIS elements software (Nikon) and ImageJ software (NIH). For confocal imaging, MEFs were grown on 35-mm glass-bottom plates (MatTek), and DIC images were acquired every 5 min with the Ultraview Vox spinning disc confocal system (PerkinElmer) equipped with a Yokogawa CSU-X1 spinning disc head and EMCCD camera (Hamamatsu C9100-13) and coupled with a Nikon Ti-E microscope. Image analysis was performed with Volocity software (PerkinElmer). All imaging was carried out in incubation chambers at 5% $CO_2$and 37°C.

Statistical analysis

Results were analyzed by a Student's t-test, performed using the GraphPad Prism 6 software. Significance was defined as $P<0$.

CHAPTER 4

PERSPECTIVES AND FUTURE DIRECTIONS

4.1. Regulation of apoptosis post-MOMP

There has been mounting evidence against MOMP being a universal point-of-no-return for cell death. As one might expect intuitively, caspase-3 activity has been identified as a key determinant of whether cells can recover from MOMP (Colell et al., 2007; Tait et al., 2010). This raises the question, how can caspase-3 activation be controlled once cytochrome C is liberated from the mitochondria? To address this question, I examined the regulation of CAS, a stimulant of apoptosome formation. In the context of the extrinsic apoptosis pathway induced by TRAIL, I found that caspase-8 induces a signaling cascade that leads to upregulation of CAS, and thus contributes to increased apoptosome formation and subsequent caspase-3 activation. Caspase-8 can induce caspase-3 activation through at least two other related mechanisms: by direct cleavage of caspase-3, or by cleavage of Bid, which induces MOMP. The upregulation of CAS could thus represent a third tool in caspase-8's repertoire of death.

cIAP1 mediates CAS degradation

I found that caspase-8 mediates its effect on CAS levels through degradation of cIAP1, an E3-ubiquitin ligase that targets CAS for proteasomal degradation. The precise mechanism underlying caspase-8 mediated cIAP1 degradation remains to be elucidated. Further studies into this mechanism could lead to the generation of a cIAP1 mutant that is resistant to such degradation.

Overexpression of degradation-resistant cIAP1 would presumably inhibit CAS upregulation, allowing us to directly determine if inhibiting CAS upregulation (as opposed to knocking down CAS) dampens apoptosis.

cIAP1 has been characterized to promote cell survival primarily by activating the NF-κB pathway through ubiquitination of several substrates (Li et al., 2002; Bertrand et al., 2008; Mahoney et al., 2008; Gyrd-Hansen and Meier, 2010). CAS could be a functionally relevant substrate of cIAP1 in the specific context of surviving apoptotic insults. The biological significance of this regulatory pathway could be assessed by testing the pro-survival activity of cIAP1 in the presence of a CAS mutant that is refractory to ubiquitination. This would first require identification and mutation of the relevant ubiquitinated lysine residues in CAS.

In this study, I have focused exclusively on understanding the mechanism of TRAIL-induced CAS upregulation. We have previously observed that UV-radiation can also induce upregulation of CAS (Kim et al., 2008). UV-induced DNA damage leads to activation of the cell intrinsic apoptotic pathway, and this is thought to occur independent of caspase-8 activity (Figure 1.1). How UV-radiation upregulates CAS thus remains an outstanding question, with several possible explanations. Firstly, while I demonstrate that caspase-8 is required for TRAIL-induced CAS upregulation, this does not rule out a redundant function for caspase-3 if it is activated directly (as in the cell intrinsic pathway). In this regard, it is worth noting that cIAP1 has been reported to be a direct substrate of

caspase-3 *in vitro* (Clem et al., 2001). Secondly, a few studies have reported that UV-radiation can activate death receptor signaling through ligand-independent clustering of receptors at the cell surface (Rosette and Karin, 1996; Rehemtulla et al., 1997; Aragane et al., 1998). While this remains to be proven more conclusively, low levels of caspase-8 activation thus achieved could be sufficient to induce CAS upregulation. Finally, the existence of a caspase-8-independent pathway that mediates CAS upregulation in response to intrinsic apoptotic stimuli cannot be ruled out. Further studies should help tease apart these possibilities.

CAS in cancer

CAS is a pro-apoptotic protein, but also appears to play a critical role in cell growth. Can this intriguing dichotomy be exploited for therapeutic purposes? I found that oncogene-induced upregulation of basal CAS levels plays a significant role in sensitizing cells to apoptotic insults. While this correlation needs to be tested more rigorously in transformed cells with additional stimuli, it suggests that tumors that have increased basal expression of CAS may be especially sensitive to select apoptotic triggers. However, the relationship between apoptosis and cancer is complicated, and it is certainly possible that these tumors develop additional mutations to evade apoptosis *in vivo.*

Conversely, could artificially raising CAS expression in tumors sensitize them to apoptosis? In this regard, it is worth noting the development of a novel class of therapeutics known as SMAC-mimetics. These IAP-antagonist compounds bind to, and induce auto-ubiquitination mediated degradation of

cIAP1 and cIAP2 (Petersen et al., 2007; Varfolomeev et al., 2007; Vince et al., 2007). SMAC-mimetics have been shown to sensitize cells to apoptosis in several contexts. It would be intriguing to test if these compounds can also induce expression of CAS, especially in cells where basal expression is low, and if so, whether this can sensitize cells to apoptosis. Manipulating CAS expression levels to therapeutically target cancer presents an interesting avenue for future research.

4.2. Ferroptosis

In the third chapter of this dissertation, we identified ferroptosis as the specific cell death process triggered by amino acid starvation in the presence of serum. Subsequent work revealed that cystine starvation in the presence of glutamine and transferrin was sufficient to induce ferroptosis. This stimulus is reminiscent of erastin, which inhibits cystine import into the cell to induce ferroptosis (Figure 1.2). We also found glutamine metabolism to be a key regulator of ferroptosis. Inhibiting glutaminases with compound 968 dramatically inhibited the formation of lipid ROS, indicating that glutaminolysis functions upstream of ROS production. The precise contribution of glutaminolysis to lipid ROS production during ferroptosis remains to be defined, but it likely involves the synthesis of lipids via feeding substrates into the TCA cycle (Vander Heiden et al., 2009).

A role for mitochondria in ferroptosis

In the latter part of chapter 3, I describe a significant role for mitochondria in erastin-induced ferroptosis. While modulating mitochondrial dynamics or inhibiting the ETC led to a partial block of cell death, disruption of membrane potential or complete depletion of mitochondria dramatically inhibited ferroptosis. A functional ETC has previously been deemed dispensable for erastin-induced ferroptosis (Dixon et al., 2012). This conclusion was reached using mitochondrial DNA-deficient 143B (ρ^0) osteosarcoma cells, which lack mitochondria-encoded components of the ETC, and are thus defective for oxidative phosphorylation. However, it should be noted that mitochondria in such cells still possess significant membrane potential generated by the activity of an adenine nucleotide carrier and an incomplete F_0F_1-ATP synthase (Skowronek et al., 1992; Buchet and Godinot, 1998; Appleby et al., 1999). Thus, while this experiment indicates that ETC-derived ROS are dispensable for ferroptosis, it does not directly assess the importance of mitochondrial membrane potential in this process. Nonetheless, my finding that depletion of mitochondria does not dramatically effect erastin-induced lipid-ROS accumulation is consistent with the idea that ferroptosis involves the generation of ROS from non-mitochondrial sources.

In addition to pinpointing the cellular site of ROS generation, identifying which specific lipid species are oxidized during ferroptosis remains an open area of investigation. While non-specific lipid oxidation hasn't been conclusively ruled out, current evidence suggests the existence of a complex "lipid oxidation signature" associated with ferroptosis (Friedmann Angeli et al., 2014).

Identification of such lethal lipids could lead to the development of new ferroptosis inhibitors that target specific lipid peroxides.

The finding that cells can survive lipid ROS production (in the presence of CCCP or in the absence of mitochondria), indicates that lipid peroxidation is not a point of no return for ferroptosis. Rather, it appears that functional mitochondria are essential to “execute” this lethal lipid signal. Such a critical function for mitochondria in ferroptosis is in line with its pivotal role in making other cell fate decisions. Defining how mitochondria sense and respond to such a lipid signal promises to be an exciting area for future research. While it is possible that lipid peroxides directly damage mitochondria, the presence of intermediate molecules that sense and relay the ROS signal to mitochondria (similar to the function of the Bcl-2 family of proteins in apoptosis) would be intriguing and could provide another opportunity for therapeutic intervention.

Ferroptosis in disease

As discussed in chapter 1, the physiological relevance of ferroptosis continues to be actively explored. Several studies have demonstrated a functional role for ferroptosis in a myriad of diseases, ranging from ischemia-reperfusion induced organ injury to neurodegenerative conditions. However, in a majority of these scenarios, a role for ferroptosis has been inferred using small molecule inhibitors like ferrostatin-1 and liproxstatin-1 to decrease tissue damage cause by acute insults. While these inhibitors have been characterized to inhibit ferroptosis, the possibility that they inhibit other forms of cell death cannot be

ruled out conclusively. The discovery of molecular markers of ferroptosis, akin to caspase activation for apoptosis or MLKL phosphorylation for necroptosis, should help confirm and identify new roles for ferroptosis in disease.

To my knowledge, ferroptosis has only been observed *in vivo* as a detrimental cell death process that contributes to various pathologies. Could ferroptosis also play a role in developmental or homeostatic processes? The most obvious way to establish such a function would involve studying mouse models where genes essential for ferroptosis are knocked out, analogous to how RIP3-knockout mice were used to demonstrate a role for necroptosis in host antiviral response (Cho et al., 2009; Upton et al., 2010). However, to date, most proteins identified to play a critical role in ferroptosis (eg. system x_c^-, GLS2, transferrin, etc.) are also required for essential cellular processes, thus precluding this approach.

Hypothetically, targeting ferroptosis for clinical benefit could be achieved through two means. Firstly, inhibiting ferroptosis is a promising therapeutic approach in scenarios where ferroptotic cell death contributes to pathology. This approach is dependent on the development of ferroptosis-specific inhibitors with favorable pharmacodynamics and metabolic profiles. Conversely, inducing ferroptosis could be a novel approach to treat certain cancers and autoimmune conditions. While a few clinically used drugs like sulfasalazine and sorafenib have been shown to inhibit system x_c^-, it remains to be proven whether ferroptosis contributes to their respective therapeutic efficacies. Although

inhibiting system x_c^- can induce ferroptosis, it could also lead to other pleiotropic effects associated with cystine starvation. Thus, inhibiting GPX4 might present a more targeted approach to induce ferroptosis without leading to cystine starvation or GSH depletion.

The development of next-generation ferroptosis inducers needs to be accompanied by the identification of biomarkers that can accurately predict sensitivity to ferroptosis. Further understanding of the basic mechanism underlying ferroptosis should help in this regard. Notably, a recent study using an unbiased approach identified NADPH abundance to strongly correlate with sensitivity to ferroptosis inducers *in vitro* (Shimada et al., 2016). This adds to the intriguing possibility that the altered metabolic state associated with tumors, including increased glutaminolysis, higher NADPH production, and greater levels of endogenous ROS, could provide a therapeutic window for selective ferroptosis-dependent elimination of cancer cells (Toyokuni et al., 1995; Schumacker, 2006; DeBerardinis et al., 2007; Trachootham et al., 2009; Vander Heiden et al., 2009 180; Wise and Thompson, 2010).

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