Characterization of the Phenotypic Differences Between Wild type and Effector Caspase Deficient Mouse Embryonic Fibroblasts

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CHARACTERIZATION OF THE PHENOTYPIC DIFFERENCES BETWEEN WILD-TYPE AND EFFECTOR CASPASE-DEFICIENT MOUSE EMBRYONIC FIBROBLASTS

By

Matthew R. Brentnall

A DISSERTATION

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The caspase family of proteins consists of upstream initiator caspases that activate downstream effector caspases, which are responsible for the proteolytic cleavage of many hundreds of proteins during apoptosis. Caspases -7 and -3 cleave a variety of proteins that lead to the hallmarks of apoptosis including, nuclear condensation, DNA fragmentation and membrane blebbing. Classically, caspase-7 and -3 were thought of as redundant because they share a 49.2 % amino acid sequence identity, a 62.0 % amino acid sequence similarity and have the same preferred cleavage motif (DEVD). However, recent data have demonstrated that caspase-7 and -3 are distinct proteases that have differential activity toward a variety of substrates. Therefore, we examined the distinct roles of these caspases during serum withdrawal-induced cell death by using effector caspase-knockout MEFs. Here, we find that during serum withdrawal-induced cell death caspase-7-deficient MEFs display no ROS production and are not resistant to intrinsic apoptosis stimulation, but remain attached to the ECM. In contrast, caspase-3-deficient MEFs display increased ROS production and are less sensitive to intrinsic cell death stimulation. These data suggest that while caspase-3 is the dominant executioner caspase, caspase-7 plays a distinct role...
in the degradation phase of apoptosis. We also demonstrate that caspase-7 and -3 have distinct non-apoptotic functions that regulate processes such as morphology, adhesion and motility. Caspase-7-deficient MEFs display an elongated and spread out morphology with increased actin stress fibers, while caspase-3-deficient MEFs display only an elongated morphology with no increase in the size of the cell footprint in contact with the ECM. Also, caspase-7-deficient MEFs and caspase-3-deficient MEFs display an increase in adhesion. Interestingly, previous studies have shown that caspase-3-deficient MEFs display a delay in the upstream events of apoptosis such as bax activation, cytochrome c release and MOMP. Therefore, we hypothesized that the delay in these events was controlled by increased adhesion and that in the absence of caspase-3, cells have increased adhesion signaling and a higher apoptotic threshold. Consistent with this, we find that when caspase-3-deficient MEFs are subjected to anoikis conditions, they have no resistance to cell death. Since there is crosstalk between the pathways that regulate morphology and adhesion, and migration, we examined the role of caspase-7 and -3 during in vitro wound healing. Caspase-7-deficient MEFs close wounds as efficiently as WT MEFs, however they display an increase in average velocity and a decrease in directional migration. In contrast, caspase-3-deficient MEFs do not close wounds as efficiently as WT MEFs and this can be attributed to a decrease in average velocity and a decrease in directional migration. Interestingly, we demonstrate that changes in morphology and motility in caspase-deficient MEFs revert back to a WT phenotype by reconstituting the MEFs with the appropriate caspase or
catalytically inactive caspase, suggesting that caspase-7 and -3 regulate these processes through a mechanism that is independent from their catalytic activity. To further investigate the role of caspase-3 in adhesion and motility, we examined adhesion signaling and production and secretion of the ECM component, fibronectin. In the absence of exogenous factors from serum, caspase-3-deficient MEFs adhere more quickly than WT MEFs and display increased P-FAK expression and increased production and secretion of fibronectin. Taken together, these data suggest that caspase-7 and -3 are distinct proteases that have complementing roles during apoptosis and have novel non-apoptotic roles in regulating morphology, adhesion and motility through a mechanism that is independent of their catalytic activity.
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Chapter I: Introduction
A. The history of programmed cell death

The concept that cell death occurred naturally during development was first described in 1842, but was left largely forgotten until 1951 when Alfred Glucksmann wrote an influential review of cell death during vertebrate development (1,2). Around this time, the most common term used to describe these cell deaths was ‘programmed cell death,’ first used by Lockshin and Williams in 1965 to describe developmental cell death in invertebrate systems (3). Shortly after, parallels were made between invertebrates and vertebrates by comparing programmed cell death during limb development in the fly and duck (4). Initially, the analysis of cell death was conducted through morphological studies using light and electron microscopy to demonstrate features of cell death such as cell shrinkage and chromatin condensation (5). This led to the term ‘apoptosis,’ which was proposed by Kerr and colleagues in 1972, while describing the morphology of dying cells which resembled the “falling off” of leaves from a tree and included the formation of apoptotic bodies with nuclear and cytoplasmic condensation followed by phagocytosis and degradation (Figure 1) (6). This was the culmination of work Kerr started in 1965, which described the morphology of cells dying during ischemic liver injury and in untreated neoplasms, as well as cell death induced by toxins or hormones. This death was distinct from necrosis and resembled the morphology of the developmental cell death described by Glucksmann (7-10). Some of these morphological features became known to describe the 'hallmarks of apoptosis,' which was thought of as
Figure 1. The initial observations of apoptosis during development. Apoptosis was first coined in 1972 by Kerr and colleagues, while describing the morphology of dying cells, which resembled the “falling off” of leaves from a tree. The morphological features of apoptosis include cell shrinkage, fragmentation and the formation of apoptotic bodies with nuclear and cytoplasmic condensation followed by phagocytosis and degradation.
an active and controlled process. This led the field to study the intracellular factors that control apoptosis and the initiators and inhibitors involved in the process.

The first non-morphological marker of cell death to be characterized was the observation that cell death was concurrent with endonucleolytic degradation of DNA, which was subsequently associated specifically with apoptosis (11,12). Then as cell death began to be described in the nematode, Caenorhabditis elegans, a genetic approach to studying cell death developed (13). C elegans are 1 mm long roundworms that as an adult consist of only 959 somatic cells. They are well suited for research, specifically for the study of programmed cell death, because they are small and transparent, and have cellular simplicity with rapid generation time. These characteristics allowed researchers to map the fates and deaths of individual cells during development, which led to a model where 1,090 somatic nuclei are generated in an essentially invariant pattern of cell divisions and 131 of these undergo programmed cell death (13-17). To analyze the genetic control of this process, random mutagenesis was used to discover loss-of-function or gain-of-function mutations that affect the pattern and process of programmed cell death. The first genes discovered to be involved in the process of programmed cell death were ced-1 and ced-2, which were involved in the removal of dead cells by phagocytosis (18). Although these mutations did not directly affect programmed cell death, the mutant-nematode strains allowed for the persistent visualization of dead cells and were used to discover other cell death related mutations. In the late 1980’s and early 1990’s, a linear-genetic
model of programmed cell death was characterized in *C. elegans* with the discovery of the genes egl-1, ced-9, ced-4 and ced-3, which were involved in the initiation and execution of programmed cell death in the worm (Figure 2) (19-21). The first component of mammalian apoptosis to be characterized was Bcl-2, which was originally discovered through analysis of the t(14;18) chromosome translocation in human follicular lymphoma, where it was shown to provide a hematopoietic cell survival signal and prevent cell death (22-26). Then, in 1992, Vaux, Weissman and Kim showed that human Bcl-2 could inhibit cell death in *C. elegans*, unifying apoptosis in vertebrates and programmed cell death in invertebrates (27). As the components of the genetic model of cell death in *C. elegans* were cloned and sequenced, the mammalian orthologs BH3-only (egl-1), Bcl-2 (ced-9), Apaf-1 (ced-4) and caspase-3 (ced-3) were identified and observed to operate in a similar manner, revealing a evolutionarily-conserved process of cell death that was soon discovered to be regulated mainly by the caspase family and Bcl-2 family of proteins (28-33) (Figure 2).

**B. The caspase family of proteins**

Caspases are an evolutionarily conserved family of cysteine proteases that cleave substrates after specific aspartate (aspartic acid) residues. There are three main types of caspases that can be distinguished by their function, structure of the proforms and how they are activated. In mammals, there are two types of caspases involved in apoptosis, initiator caspases (such as caspase-8 and -9) and executioner caspases (such as caspase-3, -6 and -7) (34). The third
Figure 2. Apoptosis is an evolutionarily conserved process of cell death. The genetic model of programmed cell death in *C. elegans* and the corresponding mammalian orthologs.
type of caspase includes the inflammatory caspases (such as caspase-1, -4 and -5 in humans and caspase-1 and -11 in rodents), which are related to initiator caspases and are involved in the maturation of cytokines and cell death through pyroptosis, but their role in apoptosis is minimal (Figure 3) (35-37).

Although these caspases all cleave at aspartate residues, the presence of an aspartate in a substrate does not solely predict that a caspase will cut it. Caspases preferentially cleave substrates at different cleavage motifs based on at least a five amino acid sequence. The aspartate (P1) must be exposed so that the caspase can access it and the surrounding amino acids, labeled P4-P3-P2-P1/P1’, contribute to recognition by the caspase. Peptide libraries and cleavage profiles were used to predict the preferred sequences cleaved by each of the caspases (Figure 3) (38,39).

All caspases are present in cells as catalytically inactive precursors, known as procaspases or caspase zymogens, which are generally localized to the cytoplasm. Procaspses have three regions, the N-terminal prodomain, and two protease subunits, the large subunit (p20) and the small subunit (p10). The catalytic cysteine resides in the p20 region and each of the regions are separated by one or more cleavage sites (Figure 3). These sites are either a target of the caspase itself, as it becomes active, or another protease, and the functions of these cleavage events are unique between initiator and effector caspases.

The proforms of initiator caspases are present in cells as inactive monomers and are activated by homodimerization not cleavage. Once dimerized, the initiator caspases become active and cleave themselves between the large
Figure 3. The caspase family of proteins. Members of the caspase family of proteins. All caspases consist of a prodomain, a large (~p20) subunit with a catalytic cysteine and a small (~p10) subunit. Inflammatory and initiator caspases have large prodomains that contain protein-protein interaction domains, including the caspase recruitment domain (CARD) and the death effector domain (DED). The preferred caspase cleavage motif is shown and the main caspase cleavage site is indicated with an arrow and corresponding amino acid.
and small protease subunits to stabilize the dimer (Figure 4). The activation of initiator caspases through dimerization is known as the induced proximity model, which activates initiator caspases by causing a conformational change that forms an active site between the substrate-specificity pocket and the cysteine-histidine catalytic diad (40-43). These dimerization events are facilitated by the binding of a protein-protein interaction motif, known as a death fold, in the prodomain of initiator caspases with specific adaptor proteins, which form oligomeric activation platforms. The protein-protein interaction region in the prodomain of the ‘extrinsic’ initiator caspases (caspase-8 and -10) is the death-effector domain (DED), which can bind the adaptor protein Fas associated death domain (FADD) (44,45). While the protein-protein interaction region in the prodomain of the ‘intrinsic’ initiator caspase (caspase-9) is the caspase-recruitment domain (CARD), which can bind the adaptor protein apoptotic protease activating factor-1 (Apaf-1) (46-48). After activation, initiator caspases cleave and activate executioner caspases, which continues the apoptotic-associated caspase cascade and the downstream events of apoptosis (Figure 4) (49).

The proforms of executioner caspases are present in cells as inactive homodimers and are activated through cleavage between the large and small subunits by initiator caspases or granzyme B. Upon cleavage, the executioner caspases remain heterotetramers and become active through a conformational change that forms the specificity pocket and enables the cysteine-histidine catalytic diad to access the substrate (Figure 4). Following activation, executioner caspases act in trans to remove the small prodomains and although this does not
Figure 4. The activation of initiator and effector caspases. All caspases are present in cells as catalytically inactive precursors, known as procaspases. Initiator caspases are present in cells as inactive monomers and are activated by homodimerization with adapter proteins through the induced proximity model. Effector caspases are present in cells as inactive homodimers and are activated through cleavage between the large and small subunits by initiator caspases.
currently seem to be necessary for activation, it may play a role in stabilization (50,51). After activation, executioner caspases cleave at least hundreds of specific substrates that coordinate all of the features that characterize apoptosis. In particular, executioner caspases cleave iCAD to coordinate DNA fragmentation, and gelsolin, PAK and ROCK-1 to coordinate membrane blebbing. The DNA fragmentation observed during apoptosis is completed by the enzyme caspase-activated DNase (CAD), which is held in an inactive complex by the inhibitor iCAD. When iCAD is cleaved by caspases, it releases active CAD, which then cuts the DNA into 180 base pair fragments (52-54). Membrane blebbing is initiated during apoptosis when executioner caspases cleave the regulatory domains of gelsolin, PAK and ROCK-1, which control the organization of actin and myosin (55-60). These proteolytic events that occur during the digestion phase of apoptosis assist in the efficient and silent removal of apoptotic cells.

Caspases are highly regulated at the activation stage, but there are also some viral, endogenous, and synthetic inhibitors that have been discovered. A conserved inhibitor of apoptosis (IAP) family of proteins was originally identified in the insect virus, baculovirus, along with another unrelated viral-caspase inhibitor, p35 (61-63). Based on sequence similarities containing a motif known as the baculoviral IAP repeat (BIR), it was shown that IAP proteins also exist in many animals including mammals (64-67). However, despite their name, only one of the eight mammalian IAP proteins has significant and direct inhibition of caspases. X-linked IAP (XIAP) can directly inhibit caspase-9 or caspase-3 and -7
through interaction with the BIR3 domain or the linker / BIR2 domain, respectively (68,69). Along with direct inhibition through BIR domains, XIAP has a really interesting new gene (RING) domain, which functions as an E3-ubiquitin ligase that adds a ubiquitin chain to the caspase and targets it for proteasomal degradation (Figure 5) (70). Some studies suggest that cIAP1, cIAP2, NAIP and survivin can also inhibit caspases, but the interaction is not direct and inhibition appears minor (71,72). Another viral caspase inhibitor CrmA, which was discovered in poxviruses and belongs to the serpin family of proteins, can inhibit caspase-8, but not caspase-9 or the executioner caspases (73-76). Caspases can also be inhibited by synthetic pan-caspase inhibitors, such as benzyloxycarbonyl-Val-Ala-Asp(OMe)-fluoromethylketone (zVAD-fmk), Boc-Asp(OMe)-fluoromethylketone (BocD-fmk) and Quinolyl-Val(OMe)-[2,6-difluorophenoxy]-methylketone (Q-VD-OPh) that were developed by attaching known caspase peptide recognition sequences to fmk or OPh, which can irreversibly bind caspases (77-79). Activation of caspases must be regulated at many levels in order to ensure there is no accidental activation of such damaging proteins.

C. The Bcl-2 family of proteins

While caspases control the final execution phase of apoptosis, the threshold for determining whether or not a stressed cell undergoes intrinsic apoptosis, and in some cases extrinsic apoptosis, is controlled by the Bcl-2 family of proteins. The first member, B-cell lymphoma-2 (Bcl-2), was cloned from the t(14:18) chromosome translocation in B-cell follicular lymphoma, where Bcl-2
Figure 5. The inhibition of caspases by XIAP. The caspase inhibitor XIAP contains three BIR domains and a RING domain. The BIR3 and linker/BIR2 domains inhibit caspase-9 and caspase-3 respectively. The RING domain of XIAP ubiquitinates bound caspases, which are targeted to the proteasome for degradation. Smac is released from the mitochondria during apoptosis stimulation and can inhibit the activity of XIAP.
becomes over expressed because its transcription is driven by the immunoglobulin heavy chain gene promoter and enhancer (22-25). The Bcl-2 family of proteins can be categorized by the presence of Bcl-2 homology domains (BH1-4) and their function during apoptosis. The three types of Bcl-2 proteins are the multi-domain pro-apoptotic Bcl-2 effectors, the multi-domain anti-apoptotic Bcl-2 proteins and the pro-apoptotic BH3-only proteins (Figure 6). The pro-apoptotic effectors, which include Bax and Bak, promote apoptosis through inducing mitochondrial outer membrane permeabilization (MOMP), while the anti-apoptotic proteins, which include Bcl-2, Bcl-xL and Mcl-1, inhibit apoptosis by preventing MOMP (80). The BH3-only proteins, which include Bid, Bim and Bad, promote apoptosis by regulating the previous two types of Bcl-2 family members (81).

The Bcl-2 family of proteins regulates the release of apoptogenic proteins from the mitochondria through MOMP, which is controlled by Bax and Bak (82,83). When activated, Bax and Bak oligomerize in the outer mitochondrial membrane (OMM) and permeabilize the mitochondria allowing for the release of proteins including cytochrome c (84). The exact mechanism of MOMP remains unclear, but it is thought that Bax and Bak disrupt the organization of lipids in the membrane to form holes (85,86). The regulation of Bax and Bak activation is also debatable and there are two models of activation, the neutralization/indirect model and the direct activator/derepressor model. Both of these models were theorized based on the different binding interactions and displacements of the Bcl-2 family of proteins (87-89). The anti-apoptotic proteins, including Bcl-2,
**Figure 6. The Bcl-2 family of proteins.** Select members of the Bcl-2 family of proteins. The Bcl-2 family of proteins can be categorized by the presence of Bcl-2 homology domains (BH1-4) and their function during apoptosis. Some members also have a transmembrane region (TM), which enables localization to the mitochondria.

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Bcl-x<sub>L</sub> and Mcl-1, differentially inhibit Bax and/or Bak by binding the BH3 domain and preventing their oligomerization, while the BH3-only proteins, including Bid, Bim and Bad, differentially bind the anti-apoptotic proteins and/or directly activate Bax and Bak (Figure 7) (90,91). In the neutralization model, the BH3-only proteins bind to Bcl-2/x<sub>L</sub> in order to release or prevent their binding to active Bax and Bak. In the direct activator/derepressor model, derepressor or sensitizer BH3-only proteins, such as Bad and Noxa, bind to Bcl-2/x<sub>L</sub> in order to release or prevent their binding to the direct activator BH3-only proteins, Bid and Bim, which can then activate Bax and Bak (Figure 8) (92). These two models are similar and recently a unified model has been proposed, where Bcl-2 proteins initially sequester direct activator BH3-only proteins, but as the concentration of activator BH3-only proteins increases, Bcl-2 proteins begin to sequester active Bax and Bak (Figure 9) (93). Therefore, both models may be correct in certain circumstances and it will be important to understand them when developing drugs to manipulate apoptosis for potential therapy. Also, it is important to understand the upstream signals that change the interaction of the Bcl-2 family of proteins and activate MOMP during intrinsic apoptosis activation.

**D. Mitochondrial mediated intrinsic apoptosis**

The mitochondrial mediated intrinsic pathway of cell death can be activated by intracellular stress, including growth factor withdrawal, DNA damage and chemotherapeutic agents. These stressors lead to the initiation of MOMP and the release of cytochrome c, which is controlled by the Bcl-2 family of
Figure 7. The differential binding and inhibition of the Bcl-2 family of proteins. The BH3-only proteins (activators or sensitizers) can differentially inhibit the anti-apoptotic Bcl-2 proteins. The anti-apoptotic proteins inhibit Bax and Bak (effectors) and the BH3-only activators can directly activate Bax and Bak.
Figure 8. The neutralization and direct activator / derepressor models of Bax and Bak activation. In the neutralization model, BH3-only proteins bind to anti-apoptotic Bcl-2 proteins to disrupt their binding to and inhibition of Bax and Bak. In the direct activator / derepressor model, sensitizer BH3-only proteins bind to anti-apoptotic Bcl-2 proteins to disrupt their binding to and inhibition of activator BH3-only proteins, which can then activate Bax and Bak.
Figure 9. The unified model of Bax and Bak activation. Llambi et al. proposed a unified model of Bax and Bak activation. Initially, Bcl-2 proteins sequester direct activator BH3-only proteins to inhibit MOMP (Mode 1), but as levels of BH3-only proteins increase, Bcl-2 proteins sequester active Bax and Bak to inhibit MOMP (Mode 2).
proteins. The mitochondria were first discovered to be at the center of this pathway when the anti-apoptotic protein Bcl-2 was shown to localize to the mitochondria (94). Subsequently, the role of cytochrome c release in the activation of the caspase cascade was determined and Bcl-2 was shown to inhibit this process (95-97). Upon induction of intrinsic apoptosis, the BH3-only proteins sense apoptotic signals and can translocate to the mitochondria where they inhibit the anti-apoptotic Bcl-2 proteins and/or directly activate Bax and Bak to initiate MOMP. MOMP results in the release of molecules from the intermembrane space, including cytochrome c (98). Once released, cytochrome c associates with Apaf-1 allowing for the recruitment and binding of caspase-9 forming the apoptosome. The apoptosome dimerizes and activates caspase-9, which can then cleave and activate the downstream effector caspases -3 and -7 (46,99,100). Other factors released from the mitochondria include Smac and Omi, which can neutralize the caspase-inhibitory effects of XIAP (Figure 10) (101,102). If caspase activation is inhibited or disrupted, cells can still die through a caspase-independent cell death (CICD). CICD occurs because MOMP causes mitochondrial catastrophe and normal mitochondrial functions, including ATP production, can no longer occur (103). Also, other proteins released from the intermembrane space, including endonuclease G and apoptosis-inducing factor (AIF) can lead to cell death without caspase activation (104).
Figure 10. The mitochondrial mediated intrinsic pathway of apoptosis. The BH3-only proteins sense apoptotic signals and inhibit the anti-apoptotic Bcl-2 proteins and/or directly activate Bax and Bak to initiate MOMP. MOMP results in the release of cytochrome c and formation of the apoptosome with Apaf-1 and caspase-9. The apoptosome activates caspase-9, which then cleaves and activates the effector caspases -3 and -7. Other factors released from the mitochondria include Smac and Omi, which neutralize the caspase inhibitor XIAP.
E. Death receptor mediated extrinsic apoptosis

In addition to intracellular stress signals, extracellular signals can initiate another form of apoptosis that is triggered by cell surface receptors known as death receptors. The extrinsic apoptosis pathway is controlled by death ligands that are part of the tumor necrosis factor (TNF) family and activate death receptors from the TNF receptor (TNFR) family. TNF ligands, including TNF, CD95-L (Fas-L) and TNF-related apoptosis inducing ligand (TRAIL), bind and activate their respective TNF receptors, including TNFR1, CD95 (Fas), and TRAIL receptors (DR4/5). Upon activation, the TNF receptors expose a death domain (DD) and recruit adaptor proteins and pro-caspase-8 and/or -10 into a complex termed the death-inducing signaling complex (DISC). The DISC complex dimerizes and activates the initiator caspases, which can cleave and activate executioner caspases, leading to cell death (Figure 11) (105,106). The DISCs of CD95 and TRAIL include a DD-DD interaction between the receptor and adapter protein Fas-associated death domain (FADD), which exposes a death effector domain (DED) that can bind and activate pro-caspase-8 or -10 by induced proximity (40,45,107). The TNFR1 DISC includes a DD-DD interaction between TNFR1, TNF-receptor associated factor-2 (TRAF2), TNFR1-associated death domain (TRADD) and FADD, which activates pro-caspase-8 or -10 in a similar manner (108). However, the presence of TRAF2 and TRADD allow the DISC to also recruit receptor-interacting protein kinase-1 (RIPK1) and activate NF-κB signaling, which includes the transcription of many cell survival and inflammation proteins (109,110).
**Figure 11. The death receptor mediated extrinsic apoptosis.** The extrinsic apoptosis pathway is controlled by death ligands and receptors that control the formation of the death-inducing signaling complex (DISC), which activates initiator caspases. Type I cell death occurs from direct activation of effector caspases, whereas type II cell death is dependent on the activation of the mitochondrial pathway of cell death.
Death receptor mediated apoptosis can also lead to activation of the mitochondrial pathway of apoptosis. After DISC formation, caspase-8 is active and cleaves the BH3-only protein, Bid, into an active form, tBid (111). tBid is a direct activator of Bax and Bak, which when activated cause MOMP, the release of cytochrome c and downstream activation of the mitochondrial pathway of apoptosis (112). In some cells, termed type I cells, the activation of intrinsic apoptosis is not needed for death receptor-mediated cell death and inhibition of MOMP has no effect. In contrast, type II cells are dependent on MOMP for cell death and this may be due to the expression levels of XIAP (Figure 11) (113). In type II cells caspase-8 activation of the executioner caspases -3 and -7 may be inhibited by XIAP and MOMP is needed in order to release the inhibitors of XIAP, Smac and Omi (114,115).

F. Alternate roles for cell death related proteins

The role of the Bcl-2 family and caspase family of proteins is well described in regards to cell death, but data have also suggested non-cell death roles or ‘day jobs’ for these proteins (Table 1). For example, the Bcl-2 family of proteins has been implicated in the regulation of autophagy and mitophagy (116). Autophagy is a mechanism of cell survival in which cellular contents, including organelles such as mitochondria (mitophagy), are engulfed by double-layered membrane structures known as autophagosomes. These autophagosomes fuse with lysosomes, which allow for the breakdown of their contents and the recycling of essential nutrients for preservation of cellular bioenergetics (Figure 12) (117).
Table 1. Alternate functions of cell death related proteins.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Non-apoptotic function</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mammals</strong></td>
<td></td>
</tr>
<tr>
<td>Caspase-1</td>
<td>Inflammation (IL-1) / Migration</td>
</tr>
<tr>
<td>Caspase-3</td>
<td>Cell differentiation / Cell proliferation</td>
</tr>
<tr>
<td>Caspase-8</td>
<td>Cell proliferation / Cell differentiation / Migration</td>
</tr>
<tr>
<td>Caspase-9</td>
<td>Cell differentiation</td>
</tr>
<tr>
<td>Caspase-11</td>
<td>Inflammation (IL-1) / Migration</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>Autophagy</td>
</tr>
<tr>
<td>BNIP3/NIX</td>
<td>Mitophagy</td>
</tr>
<tr>
<td>Bad</td>
<td>Gluconeogenesis</td>
</tr>
<tr>
<td>Cytochrome c</td>
<td>Electron transport</td>
</tr>
<tr>
<td>AIF</td>
<td>NADH oxidase</td>
</tr>
<tr>
<td><strong>Drosophila</strong></td>
<td></td>
</tr>
<tr>
<td>Dronc</td>
<td>Border cell migration / Shape antenna aristae</td>
</tr>
</tbody>
</table>
Figure 12. The autophagy pathway. Autophagy is a mechanism of cell survival in which cellular contents are engulfed and digested to recycle essential nutrients. Autophagy is controlled by the Atg family of proteins, including LC3, which initiate elongation and maturation of autophagosomes. Autophagosomes are fused to lysosomes and their contents are digested.
The Beclin-1/Vps34 interaction is a major regulator of autophagy and is required for autophagy induction (118). Beclin-1 was first identified as a Bcl-2 interacting protein suggesting crosstalk between apoptosis and autophagy, and future studies showed that the interaction between Beclin-1 and Bcl-2, Bcl-x\textsubscript{L}, Bcl-w or Mcl-1 lead to decreased Vps34 activity and autophagosome formation (119-122).

These interactions are regulated through the BH3 domain in Beclin-1 and the hydrophobic BC groove of the Bcl-2 proteins, which inhibit Beclin-1 and Vps34 binding (123,124). Mitophagy is also regulated through a similar mechanism, but also involves the BH3-only proteins BNIP3 and NIX. BNIP3 and NIX are upregulated in response to metabolic stress and can disrupt the interaction of Beclin-1 with the Bcl-2 proteins, promoting mitophagy (125,126).

Besides their role in inflammation and cytokine production, the caspase family of proteins has also been implicated in cell adhesion, motility, and proliferation, among other processes (127-131). Procaspase-8 has been shown to increase cell adhesion and promote motility in a Src and phosphatidylinositol 3-kinase (PI3K) dependent manner (132). Procaspase-8 can be phosphorylated by Src, which blocks auto-cleavage and suppresses the pro-apoptotic function of caspase-8 (133,134). Phosphorylated procaspase-8 then interacts with the p85\textsubscript{α} regulatory subunit of PI3K, which promotes calpain, Rac and Rab5 activity, inducing adhesion and motility (135-137). Caspase-8 and -3 have also been implicated in cell proliferation of T- and B-cells. Caspase-8 increases IL-2 production in T-cells, while caspase-3 controls B-cell proliferation by cleaving the cyclin-dependent kinase (cdk) inhibitor p21 (138,139). Also, in an alternate
method, activated caspase-3 and -7 in apoptotic cells were shown to increase cell proliferation and promote wound healing of surrounding tissue in a process termed ‘Phoenix Rising’ (140). Caspase-3 was also shown to function in the differentiation of multiple cell types, including neuronal cells and skeletal-muscle cells (141,142). Similarly, in Drosophila, the initiator caspase Dronc was shown to have a non-apoptotic function in regulating border cell migration (143). Ongoing research has shown that many of the members of the Bcl-2 family and caspase family of proteins have ‘day jobs,’ and there are probably more, which are yet to be discovered.

G. Cell adhesion and migration

Cell adhesion is regulated by the integrin family of transmembrane receptors, which can be divided into four subfamilies, the RGD receptors, the collagen receptors, the laminin receptors and the leukocyte-specific receptors (Figure 13) (144). 8 β subunits assort with 18 α subunits to form 24 distinct integrins and these receptors bind to specific sequence motifs on extra-cellular matrix (ECM) proteins, including fibronectin and collagen, causing a conformational change to expose the cytoplasmic tail of the receptor. The integrin receptor is then linked to the actin cytoskeleton through interaction with adaptor proteins, including talin, α-actinin, and vincullin, which can recruit scaffold proteins, such as FAK and paxillin, to form a structure known as the adhesome (145-148). The adhesome regulates the activity of the Rho GTPase family of proteins that control adhesion assembly and organization, and leads to
Figure 13. The Integrin receptor family. 8 β subunits assort with 18 α subunits to form 24 distinct integrins that preferentially bind components of the ECM or are leukocyte specific. (adapted from Hynes, R.O., Cell 2002)(144)
Figure 14. The formation of focal adhesion complexes. α and β integrins bind ECM, oligomerize and allow for the recruitment of a variety of proteins to form the adhesome. Signaling from the adhesome leads to proliferation, survival, and migration.
activation of proliferation, survival and migration (Figure 14) (149). Specifically, integrin signaling recruits guanine nucleotide exchange factors (GEFs) and GTPase activating proteins (GAPs) that regulate the activation state of Rac, Rho and CDC42, which control actin polymerization and disassembly (150,151). The dynamic process of formation and disassembly of adhesions that is controlled by the integrin signaling network is essential for cell migration.

Cell migration is a cyclic process of polarizing, extending protrusions, forming stabilizing-adhesions and disassembling adhesions at the rear of the cell to allow for forward movement (Figure 15) (152). The polarity of the cell during new protrusion formation and cell migration is established by a gradient of asymmetrical G-protein coupled receptor (GPCR) activation, which is amplified by activation of signaling adaptors, such as PI3K and PKC (153-155). CDC42 is thought to be the major GTPase involved in polarization and it contributes to recruitment of proteins to the leading edge (156). Protrusions are extended outward from the cell lamellum through actin polymerization to form either lamellipodia from a branching actin network or filopodia from parallel actin bundles (157). Lamellipodia are fast growing broad protrusions that provide the basis for directional movement and are formed downstream of Rac through activation of WASP/WAVE proteins and the Arp2/3 protein complex (Figure 16) (158,159). Filopodia are slow growing pointed protrusions that act as sensors used to explore the local environment and are formed downstream of CDC42 through ENA/VASP proteins and the actin filament-bundling protein fascin (Figure 16) (160,161). Protrusions on the leading edge of the cell are stabilized
Figure 15. The cyclic process of cell migration. Migration is initiated by environment sensing structures known as filopodia. As migration progresses nascent adhesions and lamellipodia are formed at the leading edge of the cell. As migration continues, retraction of the lagging end of the cell occurs through disassembly of focal adhesions.
Figure 16. The formation of lamellipodia and filopodia at the leading edge of cell migration. Lamellipodia are formed from a branching actin network and are fast growing broad protrusions that provide the basis for directional movement. Filopodia form from parallel actin bundles and are slow growing pointed protrusions that act as sensors used to explore the local environment.
by short-lived adhesions, termed nascent adhesions, which can turn over rapidly or mature into larger focal complexes (162). As migration continues, some of the focal complexes will mature into focal adhesions, which is dependent on actomyosin complexes and Myosin II-generated tension (163). Focal adhesions that reach the rear of the cell must be disassembled in order for cell migration to continue and this occurs through the cleavage activity of calpains, which includes the cleavage of talin and FAK (164-166).

H. Statement of the problem

The Bcl-2 family and caspase family of proteins are well known for their roles in cell death. The Bcl-2 proteins sense cellular stress and regulate the integrity of the mitochondria, while caspases can cause cell death through a variety of mechanisms involving the cleavage of many proteins. However, more and more data are surfacing about alternate ‘day jobs’ for these proteins that are classically thought to act in the process of cell death. Bcl-2, Bcl-xL, Bcl-w and Mcl-1 have been shown to function in autophagy, BNIP3 and NIX have been implicated in mitophagy, and Bad was discovered to regulate glucose metabolism (116,125,126,167). In regards to caspases, caspase-1, -4 and -5 have been known to regulate inflammation for some time, but more recent data have shown that caspase-8 is involved in adhesion and motility, and other caspases contribute to cell differentiation and proliferation (127,128,138-140). Genetically engineered knockout mice have helped in discovering these novel ‘day jobs’ through studying the changes in the development and phenotypes of
the mice, but some of the mice are embryonic or perinatally lethal and most of
the developmental defects occur because cell death is delayed or absent
(168,169). However, isolation of mouse embryonic fibroblasts (MEFs) from the
genoex knockoout mice has allowed for a more extensive analysis of the direct effects of
caspase knockouts on both apoptosis and non-apoptotic mechanisms.

In particular, effector caspase-3 and -7 knockout mice and MEFs were
developed to study the roles of these downstream caspases (170). Caspase-3
knockout mice are born in a slightly lower frequency than expected from
Mendelian genetics and are perinatally lethal after about 1-3 weeks due to
defects in brain development (171). Caspase-7 knockout mice are born in normal
ratios expected from Mendelian genetics and have normal appearance, organ
morphology and development (172). Double caspase-3 and -7 knockout mice are
born in normal expected ratios, but die rapidly after birth due to defects in cardiac
development. MEFs from caspase-3 and -7 knockout mice display resistance to
a variety of apoptotic stimuli, which is paralleled with a delay in Bax translocation
to the mitochondria, loss of mitochondrial membrane potential and cytochrome c
release (172). Classically, these events are thought to be upstream of effector
caspase activation during apoptosis stimulation, which suggests that effector
caspases either feedback on the mitochondria or have novel roles that are
upstream of the mitochondria that can regulate the apoptotic threshold of cells.

We hypothesize that effector caspases have upstream roles, or ‘day jobs,’
that regulate signaling that feeds into the apoptosis machinery and regulate the
apoptotic threshold of cells. When caspases are present, apoptosis can be
triggered and activated quickly and efficiently allowing for Bax translocation, loss of mitochondrial membrane potential and release of cytochrome c to occur rapidly. However, in the absence of effector caspases, there are additional pro-survival signals present, which raise the apoptotic threshold and there is resistance to cell death upstream of the mitochondrial events of apoptosis. We have tested this hypothesis by studying the phenotypic differences between WT and effector caspase knockout MEFs and determining how these phenotypes relate to the delay in apoptotic events found in caspase knockout MEFs. Our data suggest that effector caspases have novel roles upstream of the mitochondrial events of apoptosis, some of which confer a survival advantage and resistance to apoptosis.
Chapter II: Characterization of the phenotypic differences between wild-type and effector caspase-deficient MEFs during serum withdrawal-induced cell death

A. Caspase-7 and caspase-3 have distinct effects on the mitochondria during serum withdrawal-induced cell death

Effector caspases are known to function downstream of mitochondrial outer membrane permeabilization (MOMP) during intrinsic cell death stimulation, however the exact role of each of these caspases remains unclear. It has been previously reported that caspase-3 and caspase-7 are distinct proteases that have differential activity toward multiple protein substrates, therefore we wanted to examine the effects of effector caspase knockout during serum withdrawal-induced cell death (173). We have previously demonstrated that blocking caspase activation downstream of MOMP during IL-3 withdrawal of FL5.12 cells resulted in a partial block in the loss of mitochondrial membrane potential ($\Delta\Psi_m$) and that there was an increase in ROS production in the absence of effector caspase activity (174). These studies demonstrated that caspases have sequential and distinct effects on the mitochondria during apoptosis stimulation. However, in these studies the role of effector caspases was determined using the caspase inhibitor BocD-fmk, which inhibited all DEVDase activity and we could not discriminate between inhibition of caspase-3 and caspase-7. Therefore, we wanted to use a genetic approach to study the effects of effector caspase knockout during apoptosis stimulation.

Wild-type (WT) and effector caspase-deficient MEFs (Casp7$^{-/-}$, Casp3$^{-/-}$ and Casp3$^{-/-}$7$^{-/-}$ MEFs) were developed by and obtained from Flavell and
colleagues (171,172). We performed western blot analysis for expression of caspase-3 and caspase-7 in order to check the genetic accuracy of the cell lines and determine if the other related effector caspase was upregulated to compensate for the loss of the caspase. Caspase-3 and caspase-7 have a 49.2% amino acid sequence homology, a 62.0% amino acid sequence similarity and share a similar preferred cleavage site recognition sequence (DEVD) (Figure 17A) (175). Therefore, it is important to know the expression of each of these caspases in the knockout MEFs compared to the endogenous level in WT MEFs. As expected, Casp7\(^{-/-}\) MEFs do not express caspase-7 and express caspase-3 protein levels comparable to WT MEFs. Similarly, Casp3\(^{-/-}\) MEFs do not express caspase-3 and do not over express caspase-7 to compensate for the loss of caspase-3 expression. Consistent with the genetic knockout, caspase-3 and caspase-7 double knockout MEFs display no effector caspase-3 or caspase-7 expression (Figure 17B). These data indicate that the development of effector caspase-7-deficient MEFs, caspase-3-deficient MEFs and the double knockout MEFs was successful and that there is no increase in the protein level of the other related effector caspase in order to compensate for the loss of the caspase.

To determine if caspase-3 and caspase-7 play specific roles in the regulation of ROS production during intrinsic cell death, WT, Casp7\(^{-/-}\), Casp3\(^{-/-}\) and Casp3\(^{-/-}\)Casp7\(^{-/-}\) MEFs were subjected to serum withdrawal for 12 hours and ROS production was determined by H\(_2\)DCFDA staining. WT MEFs display
Figure 17. Amino acid sequence homology of caspase-3 and -7 and expression in knockout MEFs. (A) Amino acid sequences were obtained from PubMed and homology was analyzed using EMBOSS Stretcher pair wise alignment tool (175). (B) Cells were grown in culture dishes for 24 hours, collected, lysed with RIPA buffer and effector caspase expression was analyzed by western blot. Expression of the other related effector caspase was not altered in caspase-deficient MEFs.
an increase in ROS production after serum withdrawal and consistent with our previous findings in IL-3 withdrawal of FL5.12 cells, this is augmented by the addition of BocD-fmk (174). Interestingly, Casp7<sup>−/−</sup> MEFs display no increase in ROS production following serum withdrawal and surprisingly BocD-fmk had no effect on ROS production. In contrast, Casp3<sup>−/−</sup> MEFs have an increase in ROS production during serum withdrawal and this is not altered by the addition of BocD-fmk (Figure 18). Together these data suggest that caspase-3 is responsible for limiting ROS production. However, they also suggest that caspase-7 may contribute to ROS production because loss of caspase-7 resulted in loss of ROS production by serum withdrawal as well as ROS induced by BocD-fmk addition. To directly test this possibility, ROS production was determined following serum withdrawal of caspase-3 and caspase-7 double knockout MEFs. Consistent with the possibility that caspase-7 plays a role in the production of ROS, no increase in ROS production was observed in Casp3<sup>−/−</sup>/Casp7<sup>−/−</sup> MEFs (Figure 18).

B. Caspase-7 and caspase-3 have distinct roles during serum withdrawal-induced cell death

During the course of these studies, we noted differences between the cell lines in the amount of cell death and the number of adherent cells remaining following serum withdrawal. Therefore, we wanted to further identify if specific effector caspases were responsible for these phenotypes. A serum withdrawal time course was completed and cell death and percent detachment were
Figure 18. Caspase-7 and caspase-3 have distinct roles in ROS production during cell death. Cells were grown in 6-well plates for 24 hours and then subjected to serum withdrawal for 12 hours. After, cells were collected, stained for ROS (H$_2$DCFDA) and analyzed by flow cytometry. 30 minutes antimycin A treatment was used as a positive control. Casp7 is responsible for ROS production, while Casp3 inhibits it. Data representative of at least 3 independent experiments.
determined by annexin V staining and cell counting, respectively. Casp7−/− MEFs display no protection from serum withdrawal-induced cell death when compared to WT MEFs at all time points measured. In contrast, Casp3−/− MEFs display significant protection from serum withdrawal-induced cell death at all time points measured. MEFs deficient in both caspase-7 and caspase-3 display significant protection from serum withdrawal-induced cell death compared to WT MEFs at all time points, however incur no additional survival advantage over MEFs that are caspase-3-deficient alone (Figure 19A). These data suggest that caspase-3 is the dominant executioner caspase and that caspase-7 activation is neither necessary nor sufficient for serum withdrawal-induced cell death. Following serum withdrawal, cell death of WT MEFs correlates with percent of cell detachment suggesting that all dead cells detach from the ECM. Similarly, Casp3−/− MEFs display a correlation between cell death and cell detachment following serum withdrawal, although less cells die, the ones that do detach from the ECM. In contrast, Casp7−/− MEFs display significantly lower levels of cell detachment when compared to cell death suggesting that dead cells remain attached to the ECM. Consistent with these results, Casp3−/−-Casp7−/− MEFs display both lower levels of cell death and lower levels of cell detachment when compared to cell death (Figure 19B). Taken together, these data indicate that caspase-3 is the dominant executioner caspase, while caspase-7 is responsible for cell detachment during serum withdrawal-induced cell death.
Figure 19. Caspase-7 and caspase-3 have distinct roles during apoptosis.
(A, B) Cells were grown in 6-well plates for 24 hours and then subjected to serum withdrawal for indicated time points. (A) Cell death was analyzed by annexin V staining on flow cytometry and (B) cell detachment was determined by cell counting. Casp3 is the dominant apoptotic effector caspase, while Casp7 is responsible for loss of adherence during cell death. Data presented as mean ± SEM of at least 3 independent experiments.
Chapter III: Characterization of novel non-apoptotic roles for caspase-7 and caspase-3

A. Caspase-7 and caspase-3 regulate cell morphology

We first noted differences between the cell lines when we made the empiric observation that the Casp3\(^{-/-}\) Casp7\(^{-/-}\) MEFs were more difficult to trypsinize from cell culture dishes when splitting. Also, we observed that under standard light microscope conditions, caspases-deficient MEFs displayed distinct changes in morphology, area of the cell footprint in contact with the ECM (size) and presence of cell protrusions, including lamellipodia and filopodia (data not shown). Therefore, we wanted to examine these changes using confocal-fluorescent microscopy, which is a more accurate and measurable method. WT, Casp7\(^{-/-}\), Casp3\(^{-/-}\) and Casp7\(^{-/-}\) MEFs were plated on fibronectin coated-glass coverslips and allowed to adhere for 24 hours. Then cells were fixed and stained with phalloidin (actin) and DAPI (nucleus) to observe cell morphology using fluorescent microscopy. WT MEFs display a small-rounded morphology with some cells having a distinct leading edge-lamellipodia structure. While Casp3\(^{-/-}\) MEFs share a similar overall size to WT MEFs, they have more of an elongated morphology with no distinct leading edge. Casp7\(^{-/-}\) MEFs display a larger and more spread out cell footprint compared to WT MEFs and have enhanced stress fiber staining with many distinct cell protrusions. MEFs deficient in both caspase-3 and caspase-7 have a combined morphology that consists of very spread out and elongated cells with well-defined stress fibers and many cell protrusions (Figure 20A). Cell elongation and area of the cell footprint were
Figure 20. Caspase-7 and caspase-3 control cell morphology by regulating cell elongation and spreading. (A) Cells were grown on fibronectin coated glass coverslips for 24 hours, stained with phalloidin (actin) and DAPI (nucleus) and then analyzed by fluorescent microscopy. (B, C) Cell elongation and cell footprint were measured on LSM 510 META software. Casp7 inhibits cell elongation and spreading, and Casp3 inhibits cell elongation. Data presented as mean ± SEM of at least 3 independent experiments. (***p<0.0001, **p=0.0004)
measured quantitatively using LSM 510 META software. WT MEFs have an average cell length of 42.2 µm ± 1.7 µm, while MEFs deficient in caspase-7 and/or caspase-3 display an almost 2-fold increase in cell length. Casp7−/− MEFs have an average cell length of 77.1 µm ± 2.5 µm, Casp3−/− MEFs have an average cell length of 67.8 µm ± 3.6 µm and Casp3−/−7−/− MEFs have an average cell length of 79.1 µm ± 2.3 µm (Figure 20B). However, MEFs deficient in caspase-7 display a consistently elongated morphology along all axes of the cell causing a spread out morphology, while caspase-3-deficient MEFs only have one elongated axis. These distinctions cause differences in the size of the cell footprint in contact with the ECM of MEFs deficient in caspase-7. WT MEFs display a cell footprint of 761.2 µm² ± 49.2 µm², while Casp3−/− MEFs display a similar cell footprint of 791.7 µm² ± 63.9 µm². In contrast, Casp7−/− MEFs display a cell footprint of 1449.0 µm² ± 67.9 µm², which is a 1.9-fold increase over the cell footprint of WT MEFs. Interestingly, Casp3−/−7−/− MEFs display a cell footprint of 1820.0 µm² ± 71.1 µm², which is significantly greater than the cell footprint of MEFs deficient in caspase-7 alone suggesting that the elongation phenotype of caspase-3-deficient MEFs contributes to further increase the cell footprint of caspase-3 and caspase-7 double knockout MEFs (Figure 20C).

Caspase-7-deficient MEFs have an increase in the area of the cell in contact with the ECM, which could be caused by an increase in total cell volume or increased spreading of the cell along the ECM. To test this, we determined cell volume by analyzing forward scatter (FSC) on a flow cytometer when cells were no longer adhered to an ECM. Casp7−/−, Casp3−/− and Casp3−/−7−/− MEFs display similar cell
volumes to WT MEFs (Figure 21). Taken together, these data indicate that
caspase-7 is regulating cell elongation and cell spreading across the ECM and
caspase-3 is regulating cell elongation across the ECM.

Previous work from the Flavell laboratory has shown that
caspase-deficient MEFs have a delay in Bax translocation to the mitochondria, a
delay in MOMP and a delay in the release of cytochrome c from the mitochondria
after apoptosis stimulation (172). They suggested that this delay is present
because caspases feedback on the mitochondria after activation and cause
complete activation of Bax, MOMP and release of cytochrome c. However, our
data indicate that effector caspases have roles upstream of the mitochondria,
which cause changes in morphology. Therefore, we hypothesize that effector
caspases have upstream functions, or ‘day jobs,’ that may regulate adhesion and
survival signaling that is implicated in downstream Bax activation, MOMP, and
cytochrome c release, which might explain the delay in the mitochondrial events
of apoptosis during cell death stimulation of caspase-deficient MEFs (Figure 22).

B. Caspase-7 and caspase-3 regulate cell adhesion

We have shown that effector caspases regulate cell spreading and
elongation, which are regulated in part through the binding of integrins to the
ECM to form focal adhesions. These focal adhesions are essential for cell-ECM
interactions and anchoring of the cell (176,177). Therefore, we hypothesized that
caspase-deficient MEFs also have changes in cell adhesion. To test this, cells
were seeded in 96-well plates, allowed to adhere, and then stained with crystal
Figure 21. Caspase-deficient MEFs have a similar cell volume to WT MEFs. Cells were grown in 6-well plates for 24 hours, collected and then cell volume was analyzed by flow cytometry on the FSC channel. Casp3−/−, Casp7−/− and Casp3−/−/Casp7−/− MEFs all have similar volumes to WT MEFs. Data representative of at least 3 independent experiments.
Figure 22. Caspase-7 and caspase-3 have novel functions upstream of the mitochondria that regulate downstream signaling. We hypothesize that caspase-3 and caspase-7 have functions upstream of the mitochondria that affect the integrin signaling pathway and regulate survival signaling.
violet to determine the amount of adhesion over time. Casp7−/− MEFs display a 2-fold increase in adhesion compared to WT MEFs after 30 minutes and a 1.5-fold increase in adhesion after 1 and 2 hours, however by 4 hours Casp7−/− MEFs have the same amount of adhesion as WT MEFs. Casp3−/− MEFs display a greater increase in adhesion over Casp7−/− MEFs and after 30 minutes have a 3-fold increase in adhesion compared to WT MEFs and at least a 2-fold increase in adhesion after 1, 2 and 4 hours. Similarly, Casp3−/−7−/− MEFs display a 3-fold increase in adhesion after 30 minutes and at least a 2-fold increase in adhesion after 1, 2 and 4 hours when compared to WT MEFs (Figure 23). Taken together these data suggest that caspase-7 and caspase-3 are inhibiting cell adhesion, but to a different extent, since MEFs deficient in caspase-3 have a greater increase in adhesion compared to Casp7−/− MEFs.

C. Caspase-3 regulates the apoptotic threshold of MEFs upstream of the mitochondria

We have shown that caspase-3-deficient MEFs are resistant to serum withdrawal-induced cell death and that caspase-3 regulates cell adhesion. Also, previous work has demonstrated that during cell death stimulation caspase-3-deficient MEFs have delays in Bax activation, MOMP, and cytochrome c release (172). Therefore, we hypothesize that caspase-3-deficient MEFs have an additional survival advantage that is gained through increased adhesion, which causes an increased apoptotic threshold and the previously demonstrated delay in apoptotic events. To test this hypothesis, we examined
Figure 23. Caspase-deficient MEFs display an increase in adhesion. Cells were collected and counted, then seeded into 96-well plates and allowed to adhere for 0.5, 1, 2 or 4 hours. Adherent cells were then fixed, stained with crystal violet and absorbance was read at 590 nm after reconstituting with SDS. Both Casp7 and Casp3 inhibit cell adhesion over time. Data presented as mean ± SEM of at least 3 independent experiments. (*p=0.02, **p<0.01, ***p≤0.0001)
the role of adhesion in the cell death of WT, Casp7−/−, Casp3−/− and Casp3−/−7−/− MEFs. Cells were seeded in 6-well plates with or without serum and 6-well plates coated with or without polyHEMA, which blocks adhesion. After indicated time points, cell death was analyzed by annexin V-FITC staining and flow cytometry. Consistent with our previous findings, Casp3−/− MEFs and Casp3−/−7−/− MEFs display significant protection from serum withdrawal-induced cell death when compared to WT MEFs at all time points analyzed. After 24 hours of serum withdrawal, WT MEFs are 38.3 % ± 6.6 % annexin positive, while Casp3−/− MEFs and Casp3−/−7−/− MEFs are only 7.7 % ± 1.2 % and 5.7 % ± 1.7 % annexin positive, respectively. After 96 hours of serum withdrawal, WT MEFs are 88.0 % ± 2.5 % annexin positive, while Casp3−/− and Casp3−/−7−/− MEFs are still significantly protected from serum withdrawal-induced death displaying 55.7 % ± 4.5 % and 51.7 % ± 3.3 % annexin positive cells, respectively (Figure 24).

However, when plated on polyHEMA coated plates in full serum to block adhesion alone, Casp3−/− and Casp3−/−7−/− MEFs are significantly less resistant to cell death induced by anoikis. After 24 hours of anoikis, Casp3−/− and Casp3−/−7−/− MEFs display 29.3 % ± 1.8 % and 34.0 % ± 4.7 % annexin positive cells, respectively and 71.0 % ± 1.5 % and 71.3 % ± 2.2 % annexin positive cells after 96 hours of anoikis-inducing conditions (Figure 24). Interestingly, at 24 and 48 hours, Casp3−/− MEFs and Casp3−/−7−/− MEFs are protected from anoikis-induced cell death when there is also no serum present, however this protection is lost at 72 and 96 hours, suggesting an initial protective effect of serum withdrawal. Taken together, these data indicate that caspase-3 is
Figure 24. The survival advantage seen in caspase-3-deficient MEFs during serum withdrawal is lost during anoikis-induced cell death. Cells were collected and then seeded into 6-well plates in full medium or serum-free medium and into polyHEMA coated 6-well plates in full medium or serum-free medium. After indicated time points, cell death was analyzed by annexin V staining and flow cytometry. Casp3 regulates cell adhesion and lowers the apoptotic threshold, allowing for more efficient activation of apoptosis. Data presented as mean ± SEM of at least 3 independent experiments. (Statistics Table 1)
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regulating adhesion dependent survival signaling upstream of the mitochondria, which alters the apoptotic threshold of the cell and allows the mitochondrial events of apoptosis to happen quickly and efficiently upon cell death stimulation.

D. Caspase-7 and caspase-3 regulate cell morphology through a mechanism that is independent from their catalytic activity

Our data indicate that caspase-7 and caspase-3 have non-apoptotic roles in regulating cell morphology and adhesion. Since these MEFs were developed in the absence of the knocked out caspase, we wanted to determine that these effects were a direct consequence of the absence of effector caspases and not changes in development of the MEFs. Also, these changes in morphology are displayed when there is no exogenous apoptosis stimulation suggesting that there is either localized and controlled activation of caspases or these roles are independent of the catalytic activity of effector caspases. In order to test these possibilities, we introduced the appropriate effector caspase and a catalytically inactive caspase into the caspase-deficient MEFs and used these cell lines to determine if the cells revert to a WT phenotype. New caspase-deficient cell lines that stably express the appropriate effector caspase were made by retroviral transduction and selected with puromycin. Casp7 pBabe, Casp7\textsuperscript{C186S} pBabe, Casp3 pBabe and Casp3\textsuperscript{C163S} pBabe were introduced into the appropriate caspase-deficient MEFs and caspase expression was determined by western blot, pBabe-puro was used as a vector control. Casp7 and Casp7\textsuperscript{C186S} are successfully expressed in the MEFs, although it is at a much higher level
compared to endogenous expression in WT MEFs (Figure 25A). Casp3 and Casp3\textsuperscript{C163S} are successfully expressed in the MEFs at similar levels compared to endogenous expression in WT MEFs (Figure 25B).

Cell death assays were repeated by conducting a 48-hour serum withdrawal on the reconstituted cell lines to check for effector caspase-catalytic activity. Consistent with our previous data, Casp3\textsuperscript{-/-} pBabe and Casp3\textsuperscript{-/-}7\textsuperscript{-/-} pBabe MEFs are not sensitive to serum withdrawal-induced cell death. However, Casp3\textsuperscript{-/-} C3 MEFs and Casp3\textsuperscript{-/-}7\textsuperscript{-/-} C3 MEFs display an increased sensitivity to serum withdrawal, suggesting that the reconstituted caspase-3 is active. Interestingly, Casp3\textsuperscript{-/-} C3\textsuperscript{C163S} MEFs are also sensitized to serum withdrawal, however Casp3\textsuperscript{-/-}7\textsuperscript{-/-} C3\textsuperscript{C163S} MEFs are not sensitized to serum withdrawal (Figure 26A). These data suggest that catalytically inactive caspase-3 is sufficient to increase sensitivity to cell death only when caspase-7 is present. Also, we find that Casp3\textsuperscript{-/-}7\textsuperscript{-/-} MEFs reconstituted with caspase-7 display an increased sensitivity to serum withdrawal, even though we previously found that caspase-7 alone is insufficient for apoptosis (Figure 26B). However, this may be explained by the increased levels of caspase-7 reconstituted into the cells. Consistent with this, Casp3\textsuperscript{-/-}7\textsuperscript{-/-} C7\textsuperscript{C186S} are not sensitized to serum withdrawal, suggesting there is no caspase-7 activity in these cells (Figure 26B).

We repeated the morphology assay with the reconstituted cell lines in which we introduced the specific WT caspase or catalytically inactive caspase and determined if this resulted in reversion to a WT morphology. Both introduction of Casp7 or Casp7\textsuperscript{C186S} reverts Casp7\textsuperscript{-/-} MEFs to a WT morphology.
Figure 25. Caspase expression in new cell lines created by retroviral transduction. (A, B) Protein expression was determined by western blot and compared to WT MEFs in all cell lines created. Casp7 and Casp7\textsuperscript{C186S} expression levels were much higher in the new cell lines compared to endogenous levels in WT MEFs, but Casp3 and Casp3\textsuperscript{C163S} expression was comparable to WT expression levels.
Figure 26. Cell death in reconstituted caspase-deficient MEFs. (A, B) Cells were subjected to serum withdrawal for 48 hours and cell death was analyzed by annexin V staining and flow cytometry. Data presented as mean ± SEM of at least 2 independent experiments. (*p<0.01 , **p<0.001)
by decreasing elongation and area of the cell footprint (Figure 27A, B, and C).

Similarly, both Casp3 and Casp3\(^{C163S}\) reverts Casp3\(^{-/-}\) MEFs to a WT morphology by decreasing elongation (Figure 27A and B). Interestingly, introduction of Casp7 or Casp7\(^{C186S}\) reverts Casp3\(^{-/-}\)-7\(^{-/-}\) MEFs to a WT morphology, even though no caspase-3 was present (Figure 27A, B and C). These data suggest that the high expression levels of caspase-7 introduced into the MEFs can compensate for the lack of caspase-3 or that there are similar mechanisms between the two caspases in regards to cell elongation. Taken together, these data indicate that caspase-7 and caspase-3 are regulating cell morphology through a mechanism that is independent from their catalytic activity.

**E. Caspase-7 and caspase-3 affect wound closure and motility through a mechanism that is independent from their catalytic activity**

The formation of cell protrusions, including filopodia and lamellipodia, the regulation of actin-cytoskeleton reorganization and focal adhesion assembly and disassembly are essential for cell migration (152,178,179). Since caspase-3 and/or caspase-7 are regulating cell morphology, cell adhesion and the presence of cell protrusions and actin stress fibers, we wanted to determine the role of these effector caspases in cell motility. *In vitro* wound healing assays were performed with WT, Casp7\(^{-/-}\), Casp3\(^{-/-}\) and Casp3\(^{-/-}\)-7\(^{-/-}\) MEFs and percent wound closure was analyzed by time-lapse microscopy. Both Casp3\(^{-/-}\) MEFs and Casp3\(^{-/-}\)-7\(^{-/-}\) MEFs display a deficiency in their ability to close wounds over time, while Casp7\(^{-/-}\) MEFs show no significant difference (Figure 28). Casp3\(^{-/-}\) MEFs
Figure 27. Caspase-7 and caspase-3 regulate cell morphology through a mechanism that is independent from their catalytic activity. (A) Cells were grown on fibronectin coated glass coverslips for 24 hours, fixed, stained with phalloidin (actin) and DAPI (nucleus), and then were analyzed by fluorescent microscopy. (B, C) Cell elongation and cell footprint were analyzed on LSM 510 META software. Cell elongation and cell spreading of caspase-deficient MEFs is reverted with the introduction of WT or catalytically inactive caspases. Data presented as mean ± SEM of at least 3 independent experiments. (**p<0.0001)
Figure 28. Caspase-3-deficient MEFs have defects in *in vitro* wound closure. Cells were grown to confluency and then the plates were scratched with a p10 pipette tip. Percent wound closure was analyzed by time-lapse microscopy for at least 15 hours. Caspase-3-deficient MEFs close wounds less efficiently than WT MEFs. Data presented as mean ± SEM of at least 3 independent experiments. (*p<0.05, **p<0.005)
are unable to close wounds as efficiently as WT MEFs and have 37.8 % ± 8.2 % and 50.5 % ± 9.4 % wound closure at 9 and 12 hours, respectively, while WT MEFs display 63.8 % ± 4.9 % and 84.0 % ± 7.2 % wound closure after the same time. Interestingly, Casp3<sup>-/-</sup>-7<sup>-/-</sup> MEFs have significantly less wound closure compared to WT MEFs at all time points analyzed and after 15 hours display only 52.6 % ± 10.3 % wound closure compared to 91.7 % ± 3.0 % wound closure for WT MEFs, suggesting an unseen role for caspase-7 in wound closure as well (Figure 28). Although Casp7<sup>-/-</sup> MEFs display no significant difference in their ability to close wounds, they have a consistently lower average wound closure and a non-uniform leading edge of the wound, suggesting a trend toward deficient wound healing, which may be revealed by other methods. Taken together these data suggest that caspase-3 and caspase-7 are regulating cell motility and that they may have complementing roles that create a scenario for the most efficient cell migration.

Wound closure can be accomplished through activation of cell migration and/or cell proliferation (140,180). Therefore, we determined the cell proliferation rate in WT, Casp7<sup>-/-</sup>, Casp3<sup>-/-</sup> and Casp3<sup>-/-</sup>-7<sup>-/-</sup> MEFs through cell cycle analysis using propidium iodide staining and by cell number counting. In a standard cell cycle assay, the percent of cells in G1, S, or G2 phase of the cell cycle is not significantly different between any of the MEFs tested (Figure 29A). However, this did not represent a wound healing situation where cells are at confluency and then released from contact inhibition with stimulation of migration and/or proliferation. Therefore, we examined the change in cell cycle when simulating
Figure 29. Caspase-deficient MEFs display a similar cell cycle to WT MEFs even during release from contact inhibition. (A) Cells were grown in 6-well plates for 24 hours, collected and then cell cycle was analyzed by PI staining and flow cytometry. (B, C) Cells were grown to confluency in 6-well plates and then the plates were scratched with a p10 pipette tip 0, 8, or 16 times and cells were allowed to migrate for 12 hours. Cell cycle was then analyzed by PI staining and flow cytometry. Casp3 and Casp7 do not regulate the cell cycle. Data presented as mean ± SEM of at least 3 independent experiments. (*p<0.05, **p<0.001)
wound healing by growing cells to confluency and then scratching the plates with 8 parallel scratches or a grid of 16 scratches. After 12 hours of migration, cell cycle was analyzed by propidium iodide staining and flow cytometry. WT, Casp7\(^{-/-}\), Casp3\(^{-/-}\) and Casp3\(^{-/-}\)/Casp7\(^{-/-}\) MEFs display no difference in percent of dividing cells after stimulation of cell migration for 12 hours under conditions of 8 scratches or 16 scratches (Figure 29B). Interestingly, MEFs deficient in caspase-7 display a slight increase in the percent of cells in G1 before stimulation of migration, which may be explained by an increase in contact inhibition due to the larger cell footprint of these cells. Also, when comparing the percent of cells in G1 before and after stimulation of migration, there is an increase in cell proliferation in Casp7\(^{-/-}\) and Casp3\(^{-/-}\)/Casp7\(^{-/-}\) MEFs under conditions of 8 and 16 scratches and in Casp3\(^{-/-}\) MEFs under conditions of 16 scratches, however not in WT MEFs (Figure 29C). Even though cell proliferation is activated in these cells because of release of contact inhibition, it is activated to similar levels as WT MEFs under conditions of 8 and 16 scratches and these results do not explain the changes seen in wound healing. Therefore, WT, Casp7\(^{-/-}\), Casp3\(^{-/-}\) and Casp3\(^{-/-}\)/Casp7\(^{-/-}\) MEFs were also seeded and counted over time to analyze cell proliferation and doubling time. There is no significant difference in the fold change over time of cell number in any of the cell lines tested (Figure 30A). The linear regression of the proliferation data was determined and used to calculate doubling time and slope of the line (Figure 30B). There is also no significant change in the doubling time of any of the cell lines or slope of the line (Figure 30C and D). Taken together, these data indicate that caspase-3 and
Figure 30. Caspase-deficient MEFs have a similar doubling time compared to WT MEFs. (A) 50,000 cells were seeded in a 6-well plate and after 24, 48 and 72 hours, they were collected and counted. (B, C and D) Linear regression of cell number fold change was determined and from that cell doubling time and the slope of the line were calculated. Casp7\(^{-/-}\), Casp3\(^{-/-}\) and Casp3\(^{-/-}\)Casp7\(^{-/-}\) MEFs all have similar doubling times to WT MEFs. Data presented as mean ± SEM of at least 3 independent experiments.
caspase-7 do not regulate the cell cycle and that the changes displayed in wound healing of Casp7−/−, Casp3−/− and Casp3−/−/Casp7−/− MEFs cannot be attributed to changes in cell proliferation.

Since cell proliferation was determined to not play a role in the motility deficiency displayed in caspase-deficient MEFs, we performed single cell tracking to identify changes in motility, including velocity and meandering. Cell tracks show that WT MEFs move relatively straight and far into the wound, while Casp7−/− MEFs do not take a direct path into the wound and Casp3−/− MEFs only move a short distance into the wound. MEFs deficient in both caspase-7 and caspase-3 take an indirect path into the wound and do not migrate as far into the wound as WT MEFs (Figure 31A). The cell tracks were analyzed for average cell velocity (distance / time) and meandering (displacement / distance). Meandering is a direct measure of how straight a cell moves on a scale from 0 to 1, where a meandering index of 1 is a straight line (displacement = distance) (Figure 32). WT MEFs display an average velocity of 37.9 µm/h ± 1.7 µm/h, while Casp7−/− MEFs display a 1.2-fold increase in average velocity and migrate at 43.9 µm/h ± 1.6 µm/h. In contrast, Casp3−/− MEFs display a decrease in average velocity and migrate at 21.7 µm/h ± 1.2 µm/h. Interestingly, Casp3−/−/Casp7−/− MEFs display a slower average velocity (28.6 µm/h ± 1.1 µm/h) compared to WT MEFs, but a higher average velocity compared to MEFs deficient in caspase-3 alone, suggesting competing roles of caspase-7 and caspase-3 in regulating average velocity (Figure 31B). WT MEFs display a meandering index of 0.79 ± 0.02, while Casp7−/− MEFs display a decrease in their meandering index (0.47 ± 0.02),
Figure 31. Caspase-deficient MEFs display defects in wound closure due to changes in velocity and directional migration. (A, B and C) Individual cells were tracked using Volocity software and average velocity and meandering were measured. Casp3 and Casp7 regulate cell velocity and meandering. Data presented as mean ± SEM of at least 36 cells from at least 3 independent experiments. (*p<0.05, ***p≤0.0001)
Figure 32. **Calculating the meandering Index during cell migration.** The meandering index is a direct measure of how straight a cell moves and is calculated by displacement / distance.
suggesting unsustained directional migration. Casp3\(^{-/-}\) MEFs display a slight, but significant, decrease in their meandering index (0.74 ± 0.02). Interestingly, MEFs deficient in both caspase-7 and caspase-3 display a meandering index of 0.43 ± 0.01, which is less directional migration compared to MEFs deficient in caspase-7 alone, suggesting complementing roles of caspase-7 and caspase-3 in regulating directional migration (Figure 31C). Taken together, these data indicate that caspase-7 and caspase-3 are regulating average velocity and directional migration during *in vitro* wound healing. Interestingly, caspase-7 and caspase-3 have competing roles in regulating average velocity, while they have complementing roles in regulating directional migration. This suggests that caspase-7 and caspase-3 have novel functions in regulating cell motility that are required for efficient *in vitro* wound closure.

Similarly to the adhesion assays, there was no exogenous apoptotic stimulation during the *in vitro* wound healing assays. Therefore, we wanted to determine if these novel roles were independent of caspase-7 and caspase-3 catalytic activity or dependent on localized and controlled caspase activation. We repeated the *in vitro* wound healing assays with our reconstituted cell lines that have introduction of the specific WT caspase or catalytically inactive caspase and determined reversion to a WT migration phenotype. Introduction of Casp7 or Casp7\(^{C186S}\) reverts Casp7\(^{-/-}\) MEFs to a WT motility by decreasing average velocity and increasing directional migration (Figure 33, 34A and B). Similarly, introduction of Casp3 or Casp3\(^{C163S}\) reverts Casp3\(^{-/-}\) MEFs to a WT motility by increasing average velocity and increasing directional migration, which allows
Figure 33. Caspase-7 and caspase-3 regulate cell motility through a mechanism that is independent from their catalytic activity. Cells were grown to confluency and then the plates were scratched with a p10 pipette tip. Percent wound closure was analyzed by time-lapse microscopy. Ability to close wounds is reverted in caspase-deficient MEFs with the introduction of WT or catalytically inactive caspases. Data presented as mean ± SEM of at least 3 independent experiments. (*p<0.05, **p<0.01, ***p≤0.0001)
Figure 34. Effector caspases regulate velocity and directional migration through a mechanism that is independent from their catalytic activity. (A, B) Individual cells were tracked using Volocity software and average velocity and directional migration were measured. Average velocity and directional migration are reverted in caspase-deficient MEFs with the introduction of WT or catalytically inactive caspases. Data presented as mean ± SEM of at least 36 cells from at least 3 independent experiments. (*p<0.05, **p<0.01, ***p≤0.0001)
Casp3\(^{-/-}\) MEFs to close wounds as efficiently as WT MEFs (Figure 33, and 34A and B). Interestingly, introduction of either caspase-7 or caspase-3 with or without catalytic activity can revert Casp3\(^{-/-}\) MEFS to meandering levels equal to WT MEFs (Figure 34B). However, only Casp3 or Casp3\(^{C163S}\) can allow Casp3\(^{-/-}\) MEFs to close wounds as efficiently as WT MEFs, suggesting that average velocity plays a more significant role in wound closure than meandering, which is consistent with our previous data that shows MEFs deficient in caspase-7 alone display no significant deficiency in percent wound closure. Taken together, these data indicate that caspase-7 and caspase-3 are regulating cell motility through a mechanism that is independent from their catalytic activity.

F. Caspase-3 controls adhesion through regulating fibronectin production and secretion

Caspase-3-deficient MEFs display increased cell adhesion and spreading along the ECM, and a slower average velocity compared to WT MEFs. These phenotypes could be explained by increased focal adhesion complexes and/or increased production and secretion of ECM. The previous adhesion experiments were performed in complete medium and we wanted to remove exogenous factors, including components of the ECM, from our analysis. Therefore, to test this hypothesis, exogenous factors were removed through a 4-hour serum withdrawal and then an adhesion assay was performed on glass coverslips with or without fibronectin coating. After 1 hour of adhesion, cells were fixed, stained with phalloidin (actin) and DAPI (nucleus) as well as a phospho-focal adhesion...
kinase (P-FAK) antibody to analyze cell adhesion, spreading and FAK signaling. All cell lines tested adhere to and spread on fibronectin with some localized P-FAK staining on the cellular edge, however only Casp3$^{+/−}$ MEFs and Casp3$^{−/−}$/Casp7$^{−/−}$ MEFs adhere and spread during this time in the absence of a supplied ECM (Figure 35). Increased P-FAK staining was seen in the Casp3$^{−/−}$ and Casp3$^{−/−}$/Casp7$^{−/−}$ MEFs, however this could not be validated by western blot due to poor reagents. Introduction of Casp3 or Casp3$^{C163S}$ reverts Casp3$^{−/−}$ MEFs to a WT adhesion and spreading phenotype (Figure 35). These data suggest that caspase-3-deficient MEFs are able to produce and/or secrete their own ECM more efficiently then WT MEFs, suggesting that caspase-3 regulates secretion of ECM through a mechanism that is independent of its catalytic activity.

To analyze production and secretion of ECM, we repeated the adhesion assay, but cells were fixed, stained with phalloidin (actin) and DAPI (nucleus) as well as with a fibronectin specific antibody to analyze fibronectin levels. Fluorescent microscopy shows that Casp3$^{−/−}$ MEFs and Casp3$^{−/−}$/Casp7$^{−/−}$ MEFs adhere and spread faster then WT MEFs and display increased fibronectin staining after 1 hour of adhesion (Figure 36A). We also analyzed fibronectin secretion by ELISA and found that when compared to WT MEFs, Casp3$^{−/−}$ MEFs display higher levels of fibronectin secretion into the medium and secretion is blocked with addition of the ER/Golgi transport inhibitor brefeldin A (Figure 36B). Production of fibronectin was determined by western blot and ELISA of protein lysates in the presence of brefeldin A to block fibronectin secretion. Western blot
Figure 35. Caspase-3-deficient MEFs do not require exogenous ECM for cell adhesion and spreading. Cells were collected and counted, then seeded in serum free media into polypropylene tubes and incubated at 37°C for 4 hours. After serum starvation, cells were plated onto fibronectin coated or uncoated glass coverslips in HBSS and allowed to adhere and spread for 1 hour. Adherent cells were then fixed, stained with Anti-P-FAK, Phalloidin and DAPI, and then visualized by fluorescent confocal microscopy. Casp3 inhibits cell adhesion and spreading through a mechanism that is independent from its catalytic activity.
Figure 36. Caspase-3-deficient MEFs secrete and produce increased levels of fibronectin. Cells were collected and then seeded in tubes in serum free media and incubated at 37°C for 4 hours. After, cells were plated onto uncoated glass coverslips or culture dishes in HBSS and allowed to adhere and spread for 1 hour. (A) Adherent cells were then fixed, stained with anti-fibronectin, phalloidin and DAPI, and visualized by fluorescent confocal microscopy. (B) Supernatants were collected for ELISA detection of fibronectin. Brefeldin A (BA) was used to inhibit ER/Golgi transport. (C, D) Cells were collected and lysed for detection of fibronectin by western blot or ELISA. Casp3 regulates secretion and production of fibronectin. Data representative of at least 2 independent experiments and presented as mean ± SEM. (*p<0.01)
and ELISA show that Casp3−/− MEFs display increased levels of fibronectin within the cell when treated with brefeldin A (Figure 36C and D). Transcriptional regulation of fibronectin and its receptor integrin β-1 were measured by qRT-PCR, which shows that there are no significant differences between the mRNA levels of fibronectin or integrin β-1 in WT and Casp3−/− MEFs (Figure 37A and B). Taken together, these data indicate that caspase-3 is regulating the production and secretion of fibronectin.

G. Fibronectin protects WT MEFs from serum withdrawal-induced cell death

We found that Casp3−/− MEFs gain an additional survival advantage during serum withdrawal-induced cell death from increased adhesion. Furthermore, we showed an increase in production and secretion of fibronectin in Casp3−/− MEFs, which may increase adhesion. Therefore, we wanted to determine if WT MEFs are protected from serum withdrawal-induced cell death in the presence of supplied exogenous fibronectin. 6-well plates were coated with increasing concentrations of fibronectin and WT MEFs were seeded into the plates in serum free medium (-FBS). After 24 hours, cell death was analyzed by annexin V staining and flow cytometry. WT MEFs display 37.2 % ± 4.0 % cell death in the absence of a supplied ECM, however WT MEFs display significantly less cell death when supplied with fibronectin concentrations of ≥ 1.2 µg/cm² (Figure 38). Interestingly, the highest concentration of fibronectin tested, 10 µg/cm², displays 18.0% ± 2.2 % cell death, which is not statistically different than a fibronectin
Figure 3. Caspase-3-deficient MEFs display comparable levels of fibronectin and integrin-β1 mRNA message. (A, B) mRNA was collected from cells after an adhesion assay experiment and qRT-PCR was used to determine message levels of fibronectin and integrin-β1. Caspase-3 does not regulate fibronectin or integrin-β1 message levels. Data presented as mean ± SEM of 3 independent experiments.
Figure 38. Fibronectin protects WT MEFs from serum withdrawal-induced cell death. WT MEFs were seeded into 6-well plates coated with increasing concentrations of fibronectin in serum-free medium and after 24 hours, cell death was analyzed by annexin V staining and flow cytometry. Data presented as mean ± SEM of at least 3 independent experiments. (*p<0.5, **p<0.01)
concentration of 1.2 µg/cm², which displays 22.0% ± 2.6 % cell death. These data suggest that a minimal concentration of fibronectin is needed to confer protection from serum withdrawal-induced cell death (Figure 38). Taken together, these data demonstrate that fibronectin protects WT MEFs from serum withdrawal-induced cell and that Casp3⁻/‐ MEFs gain an additional survival advantage through increased production and secretion of fibronectin.
Chapter IV: Characterization of the role of caspase-3 in the motility of cancer cells

A. Caspase-3 regulates migration of MCF-7 breast cancer cells through a mechanism that is independent of its catalytic activity

We found that caspase-3 regulates migration of MEFs by controlling the average velocity and meandering of cells. Therefore, we wanted to determine the role of caspase-3 in migration of cancer cells by using the MCF-7 breast cancer cell line, which has a deletion in exon 3 of the CASP-3 gene resulting in no caspase-3 expression (181,182). MCF-7 cells reconstituted with murine caspase-3 (C3) or catalytically inactive-murine caspase-3 (C3\textsuperscript{C163S}) were made by retroviral transduction and expression levels were determined by western blot (empty pBabe vector was used as a control). Western blot analysis shows that murine caspase-3 or catalytically inactive-murine caspase-3 are successfully expressed in MCF-7 cells, although catalytically inactive-murine caspase-3 is expressed at lower levels (Figure 39A). In vitro wound healing assays were performed and analyzed by time-lapse microscopy to determine the affect of caspase-3 expression on the migration of MCF-7 cells, including average velocity and meandering. MCF-7 pBabe cells have an average velocity of 0.011 ± 0.001 µm/s, while MCF-7 C3 cells have a significantly increased velocity of 0.018 ± 0.0003 µm/s. Consistent with our previous data in MEFs, MCF-7 cells reconstituted with catalytically inactive-caspase-3 (MCF-7 C3\textsuperscript{C163S}) also display an increase in velocity (0.015 ± 0.001 µm/s) compared to MCF-7 pBabe cells (Figure 39B). Expression of murine caspase-3 or catalytically inactive-murine caspase-3 in MCF-7 cells does not have a significant effect on
Figure 39. Expression of caspase-3 or catalytically inactive-caspase-3 in MCF-7 cells increases their average velocity during migration. (A) MCF-7 cells were infected with Casp3 or Casp3<sup>C163S</sup> and caspase-3 expression was determined by western blot. (B) In vitro wound healing assays were performed and single cell tracking was used to determine average velocity and meandering. Caspase-3 regulates migration in MCF-7 breast carcinoma through a mechanism that is independent of its catalytic activity. Data are representative of 3 independent experiments. (*p=0.03, **p=0.001) (Jessica Tepe and David Weir)
the meandering of cells, although there is a trend toward increased directional migration in MCF-7 cells reconstituted with C3 and C3^{C163S}, more experiments need to be completed to determine biological significance (Figure 39C). Taken together, these data suggest that caspase-3 can regulate migration in a cancer cell line.
Chapter V: Discussion

A. ROS production in effector caspase-deficient MEFs during serum withdrawal-induced cell death

The effector caspases -7 and -3 were thought of as redundant caspases because they have a 49.2 % amino acid sequence homology, a 62.0 % amino acid sequence similarity and share the same preferred cleavage recognition site (DEVD) (175,183). However, recent studies have determined that caspase-7 and caspase-3 are distinct proteases that have differential activity toward multiple substrates, including Bid, gelsolin and XIAP, which suggests a non-redundant role for these related caspases (173,184). Also, our lab has found that blocking caspase activation downstream of MOMP during IL-3 withdrawal of FL5.12 cells resulted in a partial block in the loss of mitochondrial membrane potential ($\Delta \Psi_m$). Furthermore, we showed that there was an increase in ROS production in the absence of effector caspase activity (174). These studies suggest that caspases have distinct and sequential effects on the mitochondria during apoptosis stimulation. However, the exact roles of caspase-7 and caspase-3 were not determined because effector caspase inhibition was achieved with the use of BocD-fmk, which inhibited all DEVDase activity and we could not discriminate between inhibition of caspase-7 and caspase-3. We hypothesized that caspase-7 and caspase-3 have distinct functions during cell death stimulation and used a genetic approach to study the effects of effector caspase knockout during apoptosis stimulation.
Our data indicate that during serum withdrawal-induced cell death of WT MEFs, a minimal amount ROS is produced and this is increased with the addition of BocD-fmk, suggesting that overall ROS production is inhibited by an effector caspase. In the absence of caspase-3, ROS production is increased to levels comparable with the addition of BocD-fmk, suggesting that caspase-3 is responsible for the inhibition of ROS production during serum withdrawal-induced cell death (Figure 18). In contrast, in the absence of caspase-7 or both caspase-3 and caspase-7, there is no ROS production, even in the presence of BocD-fmk, suggesting that caspase-7 contributes to the accumulation of ROS (Figure 18). We have previously reported that in the presence of effector caspase inhibition, cells release cytochrome c from their mitochondria, but maintain a ΔΨ_m. This ΔΨ_m allows the import of electron transport chain (ETC) substrates and the continued shuttling of electrons through complexes I-IV (174). Also, it has been shown that in the absence of cytochrome c, electrons will be lost to oxygen between complexes III and IV in the mitochondria, resulting in ROS production (185). Therefore, caspase-3 may feedback on the mitochondria to inhibit electron transport through the ETC and/or cause a complete loss of ΔΨ_m, which inhibits the production of ROS, but the exact mechanism remains to be determined. Additionally, the mechanism by which caspase-7 accumulates ROS is unknown, however previous data from the lab show that the inhibition of caspase-9 also prevents ROS production. Caspase-9 was found to initiate ROS production through the cleavage of Bid into tBid, which remodeled the mitochondria and blocked the ability of cytochrome c to participate in electron transport.
transport (186). However, more research needs to be completed to determine the possible role of caspase-7 in this process or a similar mechanism. Since overall ROS production is inhibited by caspase-3 in a dying cell, ROS production and accumulation may be detrimental to apoptosis and previous work has shown that accumulation of ROS will change an apoptotic death to a necrotic death (187-189). Therefore, caspase-3 is needed to ensure the integrity of apoptotic cell death in vivo and prevent an inflammatory response caused by necrosis (190). However, in the absence of caspase-3, caspase-7 causes an accumulation of ROS and presumably a necrotic cell death. However, our data indicate that ROS induced-necrotic cell death is no more efficient than cell death in the absence of both caspase-3 and caspase-7 (Figure 19).

B. Serum withdrawal-induced cell death in effector caspase-deficient MEFs

Our results show that caspase-7-deficient MEFs die at the same rate as WT MEFs, while caspase-3-deficient MEFs have a delay in cell death during serum withdrawal (Figure 20). However, the cells are not completely resistant to cell death and we propose that this is due to caspase-9 activation. Interestingly, MEFs deficient in both caspase-3 and caspase-7 display the same sensitivity to serum withdrawal-induced cell death as MEFs deficient in caspase-3 alone, suggesting that caspase-3 is the dominant executioner caspase and that caspase-7 is neither necessary nor sufficient for cell death (Figure 20). These findings are currently being investigated further by reconstituting caspase-3-deficient MEFs with wild-type caspase-3 or one of three different
caspase-3 / caspase-7 fusion constructs that swap each of the caspase-3 regions (pro / p20 / p10) with the same region of caspase-7 (Figure 40 and 41A). These studies will allow for a better understanding of the caspase-3 protein as a whole and how the prodomain and p10 subunit regulate the catalytic activity of the p20 subunit during apoptosis. Preliminary data show that the p10 subunit is necessary for full caspase-3 apoptotic activity and interestingly the prodomain of caspase-3 has a negative regulatory effect on apoptotic activity (Figure 41B). The negative regulatory activity of the prodomain of caspase-3 may be an evolutionarily conserved function, because amino acid sequence analysis shows that the prodomain of caspase-3 is highly conserved between species. Also, two potential phosphorylation sites are conserved, which may be a mechanism by which the prodomain of caspase-3 functions as a negative regulator of apoptotic activity (Figure 42). Interestingly, the prodomain of caspase-7 is also highly conserved between species, however the prodomains of murine caspase-3 and murine caspase-7 share only 19.4 % amino acid sequence homology, which suggests that the prodomains of these caspases evolved separately and may have unique roles in regulating the function of these caspases (Figure 42, 17).

These data also suggest that MOMP and caspase-9 activation are the ‘point-of-no-return’ for the cell and even though cell death is more efficient in the presence of caspase-3, the cell will still eventually die in an effector caspase-independent manner. However, previous work has shown that under some circumstances and only in certain organisms, cells will die in the absence of all caspase activity through a caspase-independent cell death (CICD).
Figure 40. Caspase-3 and caspase-7 fusion constructs. Fusion constructs were designed to swap each of the regions of caspase-3 and -7. (Oskar Laur Ph.D., Emory University Custom Cloning Core)
Figure 41. The prodomain and p10 region of caspase-3 regulate its apoptotic activity. (A) Caspase-3/7 fusion constructs were expressed in C3/−/− MEFs by retroviral transduction and expression was determined by western blot. (B) A serum withdrawal time course was performed and cell death was measured by annexin V staining and flow cytometry. Data presented as mean ± SEM of at least 3 independent experiments. (Jessica Tepe)
Figure 42. Homology of the prodomain of caspase-3 and -7 is conserved between species. EMBOSS stretcher pair wise alignment tool was used to analyze the inter-species homology of the prodomain of caspase-3 and -7 and phosphosite (Cell Signaling) was used to determine potential phosphorylation sites. The prodomain of caspase-3 and -7 is conserved between species, but not within caspases of the same species.
Therefore, to understand the role of caspase-9 on cell death in our system and the ability of MEFs to undergo CICD, we would need to develop MEFs that are deficient in caspase-3, caspase-7 and caspase-9 by knocking down caspase-9 in Casp3−/−7−/− MEFs using a lentiviral system with shRNA specific to caspase-9.

Although caspase-7 does not function in sensitizing cells to serum withdrawal-induced cell death, our data indicate that it has other roles during the demolition phase of apoptosis. We show that caspase-7 functions to detach cells from the ECM, which suggests that caspase-7 functions to aid in the removal of apoptotic cells (Figure 19). In vivo, apoptotic cells have a profound effect on the microenvironment and it is necessary to regulate these processes and efficiently remove dead cells. Caspase-7 may contribute to this removal process by hastening the detachment of cells from the ECM. Caspases are known to cleave a variety of actin and cytoskeleton components, but the specific components important for detachment and cleaved by caspase-7 are yet to be determined. Interestingly, an early report demonstrated that FAK is an apoptotic substrate that is preferentially cleaved by caspase-7. However, this study was performed in non-adherent cells and it was difficult to fully appreciate the significance of these data. Therefore, it will be important to examine the caspase-7 specific-cleavage of actin and cytoskeleton components, including FAK, to determine the mechanism by which caspase-7 is detaching cells from the ECM during serum withdrawal-induced cell death.
Our data also indicate another interesting finding about the role of caspase-3 and caspase-7 in cell death. Caspase-deficient MEFs reconstituted with either caspase-7 or catalytically inactive caspase-7 displayed expression levels that were much higher than the endogenous levels of caspase-7 in WT MEFs. However, caspase-deficient MEFs reconstituted with caspase-3 or catalytically inactive caspase-3 displayed expression levels comparable to endogenous levels of caspase-3 in WT MEFs (Figure 25). These data suggest that high levels of caspase-3 are unfavorable in a cell because of its ‘day job’ functions in regulating adhesion and fibronectin and/or its high apoptotic activity. In contrast, there is no selective pressure against high levels of caspase-7. Interestingly, our data also indicate that cell death is increased in caspase-3-deficient MEFs that are reconstituted with catalytically inactive caspase-3, however there is no increase in cell death in MEFs deficient in both caspase-3 and caspase-7 that are reconstituted with catalytically inactive caspase-3 (Figure 26). These data suggest that caspase-7 has a role in cell death under certain circumstances. In this circumstance catalytically inactive caspase-3 may function as an XIAP sink or regulate survival signaling through adhesion, which increases the activity of caspase-7 and lowers the apoptotic threshold of cells, but this needs to be explored further.

Taken together, our data indicate that caspase-9, caspase-7 and caspase-3 have distinct functions during apoptosis. Caspase-9 functions to remodel the mitochondria, which we hypothesize is through the cleavage and activation of Bid into tBid. In the presence of caspase-7, this mitochondrial
remodeling causes favorable conditions for ROS production and caspase-7 functions to allow for ROS accumulation and causes cell detachment. In contrast, caspase-3 is the dominant apoptotic caspase and we hypothesize it functions to decrease ROS by inhibiting electron transport through the ETC by blocking the import of substrates into the ETC and/or lowering the $\Delta \Psi_m$ (Figure 43).

C. Caspase-7 and caspase-3 regulate morphology and actin-cytoskeleton organization

We made the empiric observation that double knockout MEFs were harder to trypsinize off of cell culture dishes and that caspase-deficient MEFs display differences in morphology (data not shown). Fluorescent microscopy shows that caspase-7-deficient MEFs display an elongated and large cell footprint with multiple long protrusions and no well-defined leading edge-lamellipodia structure. Caspase-3-deficient MEFs display an elongated morphology and no distinct leading edge (Figure 20). Also, we show that both of these phenotypes can be reverted with the introduction of the appropriate wild-type caspase or catalytically inactive caspase (Figure 27). These data suggest that effector caspases regulate actin and cytoskeleton organization independent of their catalytic function. Previous work has shown that actin and cytoskeleton organization are regulated by the Rho family of GTPases and the downstream targets, including coflin and Arp2/3 (195-197). Cofilin regulates actin polymerization and is required for the formation of lamellipodia (198). Preliminary data demonstrate that caspase-7-deficient MEFs display lower expression of phosphorylated coflin
Figure 43. Schematic of the functions of caspase-9, caspase-7 and caspase-3 during intrinsic apoptosis. After cytochrome c release and apoptosome formation, caspase-9 functions to increase ROS production. Caspase-7 functions to cause ROS accumulation and is responsible for cell detachment, while caspase-3 inhibits ROS production and is the dominant apoptotic caspase.
(inactive) and lower expression of phosphorylated cofilin when migrating compared to WT (Figure 44). However, we show that caspase-7-deficient MEFs appear to lack lamellipodia structures and cofilin localization to the leading edge of the cell is lost (Figure 45). These data suggest that in caspase-7-deficient MEFs, although cofilin is not phosphorylated, it has no activity and it is not localized to the leading edge of the cell where it is needed for proper lamellipodia formation. Therefore, caspase-7 may be required for cofilin activity and localization. Arp2 also regulates formation of the lamellipodia and its localization to a leading edge is lost in caspase-7-deficient MEFs (Figure 45) (158,199). Taken together, these data indicate that caspase-7 is required for the formation of lamellipodia through proper activity and localization of cofilin and Arp2 (Figure 46). Binding of caspase-7 to these proteins may be needed for full activity, localization and the formation of the lamellipodia, which is currently under investigation through the use of co-immunoprecipitation.

D. Caspase-3 regulates the apoptotic threshold upstream of the mitochondria by a mechanism that is independent from its catalytic activity

Previous work has demonstrated that caspase-3-deficient MEFs are resistant to cell death and display a delay in the mitochondrial events of apoptosis, including bax activation, cytochrome c release and the loss of ΔΨm (172). These data suggest that effector caspases feedback on the mitochondria or have functions upstream of the mitochondria. Our data indicate that caspase-3-deficient MEFs are less sensitive to cell death stimulation and display
Figure 44. Caspase-7-deficient MEFs display lower expression levels of P-cofilin compared to WT MEFs. (A) MEFs were collected, lysed and protein expression levels were measured by western blot. (B) MEFs were grown to confluency then scratched multiple times to stimulate migration and allowed to migrate for the indicated time. After, cells were collected, lysed and protein expression levels were measured by western blot. (Bethany Bray)
Figure 45. Caspase-7-deficient MEFs display altered localization of cofilin and Arp2. (A, B) Cells were seeded on to fibronectin coated glass coverslips and allowed to adhere for 24 hours. Then fluorescent microscopy was used to determine the localization of cofilin and Arp2. Caspase-7 regulates the localization of cofilin and Arp2. Data representative of at least 3 independent experiments. (Bethany Bray)
Figure 46. Schematic of the role of caspase-7 in the formation of the lamellipodia. Caspase-7 is needed for the activity and localization of cofilin and Arp2 to form the lamellipodia.
changes in cell morphology and increased adhesion over time compared to WT MEFs (Figure 19, 20, 23). Taken together, our data indicate that effector caspases have functions upstream of the mitochondria that regulate the apoptotic threshold of cells and allow for the efficient activation of apoptosis. Reports have shown that adhesion signaling through integrins and FAK upregulates survival signaling, including the PI3K / Akt and Ras / MAPK pathways (200,201). The survival signaling from these pathways inhibits the activity of proteins involved in the initiation of apoptosis and over activation of this signaling leads to apoptosis resistance (202). These data led to the hypothesis that caspase-3-deficient MEFs gain an additional survival advantage through increased adhesion, which increases the apoptotic threshold of the cell and causes the previously mentioned delay in the mitochondrial events of apoptosis. Consistent with this, caspase-3-deficient MEFs are protected from serum-withdrawal induced cell death, however they are significantly less resistant to cell death induced by anoikis (Figure 24). Therefore, the additional adhesion signaling in caspase-3-deficient MEFs provides upregulated survival signaling, a higher apoptotic threshold, and resistance to cell death stimulation. Currently, we have demonstrated that resistance to cell death stimulation in caspase-3-deficient MEFs is reverted under anoikis conditions, however we also want to determine a reversion in the delay of the mitochondrial events of apoptosis, including MOMP. Therefore, MEFs expressing a cytochrome c-GFP
fusion protein or an Omi-mCherry fusion protein were made by retroviral transduction to be used in future studies (fusion proteins provided by Dr. Douglas R. Green) (Figure 47) (203).

Interestingly, we find that while caspase-3-deficient MEFs are sensitive to anoikis, they are initially protected from death induced by anoikis when there is also no serum present, however this protection is lost at later time points (Figure 24). These data suggest that there is activation of a survival signaling pathway under conditions where cells cannot adhere and are not provided with exogenous serum. We hypothesize that this survival signaling and initial protection from cell death is attributed to the activation of the autophagy pathway. Previous work demonstrated that autophagy is negatively regulated by serum and attachment to the ECM and therefore is induced under conditions of serum-withdrawal and detachment (118). These findings are currently under further investigation using a mCherry-GFP-LC3 fusion construct that was provided by Dr. Jayanta Debnath, and allows for the measurement of autophagy activation and autophagic flux (204,205). MEFs expressing the fusion protein will be monitored for autophagy activation in the presence and absence of serum and/or adhesion to determine the role of autophagy in protecting MEFs from cell death (Figure 48).

Since adhesion is regulating sensitivity to cell death stimulation, the survival signaling pathway that is responsible for resistance to serum withdrawal in caspase-3-deficient MEFs is likely centered on the adhesome and FAK signaling. In the absence of serum and exogenous ECM factors,
Figure 47. MEFs expressing cytochrome c-GFP or Omi-mCherry fusion proteins. MEFs expressing cytochrome c-GFP or Omi-mCherry fusion proteins were made by retroviral transduction and expression was analyzed by either flow cytometry or fluorescent microscopy. (Constructs provided by Douglas R. Green Ph.D.)
Figure 48. MEFs expressing the mCherry-GFP-LC3 fusion protein. MEFs expressing mCherry-GFP-LC3 fusion protein were made by retroviral transduction and expression was analyzed by flow cytometry. (Constructs provided by Jayanta Debnath M.D.)
caspase-3-deficient MEFs adhere to and spread on uncoated glass coverslips more quickly than WT MEFs and appear to have increased P-FAK expression and therefore signaling (Figure 35). However, increased P-FAK expression could not be verified by western blot due to lack of proper reagents, so the increase in other signaling pathways should be explored, including PI3K / Akt and Ras / MAPK, and an increase in anti-apoptotic signaling. Although the signaling pathway responsible for the increased apoptotic threshold in caspase-3-deficient MEFs is yet to be determined, the data show that in the presence of a supplied ECM, WT and caspase-3-deficient MEFs adhere and spread at the same rate (Figure 35). These findings suggest that ECM production and secretion play a role in the ability of caspase-3-deficient MEFs to adhere to and spread on uncoated glass coverslips. Our data indicate that caspase-3-deficient MEFs display increased fibronectin secretion and an increase in fibronectin within the cell when treated with the ER/Golgi transport inhibitor Brefeldin A, which blocks fibronectin secretion (Figure 36). Taken together, these data suggest that caspase-3 is regulating the production and secretion of fibronectin. However, mRNA levels of fibronectin are comparable in WT and caspase-3-deficient MEFs, suggesting that upregulation of fibronectin expression is not at the level of transcription (Figure 37). Therefore, fibronectin expression is regulated at the level of translation, however the regulation of fibronectin production may be an indirect effect of caspase-3 caused by a feedback loop. Previous work has shown that attachment to fibronectin increases overall translation and translation of fibronectin may be upregulated in caspase-3-deficient MEFs merely because
they are secreting more fibronectin (206). Preliminary data shows that caspase-3-deficient MEFs display an increase in total protein secretion, suggesting that caspase-3 functions to regulate protein secretion (Figure 49). Therefore, in the absence of caspase-3, cells secrete more protein, including fibronectin, which signals for the upregulation of fibronectin translation.

Taken together, our data suggest that caspase-3-deficient MEFs gain an additional survival advantage through increased adhesion and a higher apoptotic threshold from increased fibronectin production and secretion. Therefore, we wanted to determine if WT MEFs gain a similar survival advantage when provided with exogenous fibronectin. Our data show that small concentrations of fibronectin (≥ 1.2 µg/cm²) protect WT MEFs from serum withdrawal-induced cell death, suggesting that an increase in fibronectin production and secretion protects cells from cell death stimulation (Figure 38). Therefore, we are currently investigating if the knockdown of fibronectin by shRNA in caspase-3-deficient MEFs sensitizes them to serum withdrawal-induced cell death by lowering the apoptotic threshold (Figure 50).

Overall, we have shown that caspase-3 regulates survival signaling through a mechanism that affects cell morphology, cell adhesion and protein secretion. Caspase-3 is regulating protein secretion, including fibronectin, which leads to downstream survival signaling and controls the apoptotic threshold of the cell (Figure 51). Moreover, caspase-3 may be regulating actin organization and microtubule stabilization, which are needed for changes in morphology and ER/Golgi transport. The transport of proteins through the ER/Golgi relies on the
Figure 49. Caspase-3-deficient MEFs display an increase in total protein secretion. MEFs were serum starved for 4 hours and then plated on petri dishes for 1 hour. After, medium was collected and analyzed for total protein secretion through a TCA protein precipitation and silver staining of a SDS-page gel. Data representative of at least 3 independent experiments.
Figure 50. Knockdown of fibronectin by shRNA in caspase-3-deficient MEFs. (A, B) Knockdown of fibronectin in caspase-3-deficient MEFs was performed using a lentiviral system and shRNA to fibronectin. Expression of fibronectin was measured by western blot and mRNA levels were determined by qRT-PCR. (David Weir)
Figure 51. Schematic of the role of caspase-3 in regulating the apoptotic threshold of the cell. Caspase-3 regulates the secretion of fibronectin, which leads to changes in the downstream survival signaling and the apoptotic threshold of the cell. In the presence of caspase-3 apoptosis happens quickly and efficiently upon cell death stimulation.
movement of proteins along microtubules using kinesin motors. In this study, we concentrated on fibronectin because of the cell survival and adhesion phenotypes that we observed. However, the use of proteomics on the supernatants of WT and caspase-3-deficient MEFs would allow for the determination of all proteins whose secretion is regulated by caspase-3 and this would give us a better understanding of how and which specific secretion process caspase-3 regulates.

E. Caspase-7 and caspase-3 regulate cell motility through a mechanism that is independent from their catalytic activity

Our results demonstrate that effector caspase-deficient MEFs display changes in morphology and adhesion and that caspase-3-deficient MEFs display an increase in fibronectin production and secretion. Previous studies have shown that these processes are involved in the regulation of cell migration and therefore we examined the role of effector caspases in cell motility (152,164,207). *In vitro* wound healing assays and time-lapse microscopy show that caspase-3-deficient MEFs have a defect in wound closure that can be attributed to a decrease in average velocity and a decrease in directional migration (Figure 28, 31). Also, reconstituting caspase-3-deficient MEFs with wild-type caspase-3 or catalytically inactive caspase-3 was sufficient to revert their motility phenotype to a WT phenotype, suggesting that the regulation was not through a cleavage event (Figure 33, 34). Since caspase-3-deficient MEFs display increased fibronectin production and secretion, the role of fibronectin in this process needs to be
determined. Previous work has shown that fibronectin promotes cell migration and overexpression of FAK, the kinase downstream of fibronectin signaling, increases migration (208-210). However, it is yet to be determined how overexpression and increased secretion of fibronectin affects cell migration. Interestingly, previous work has shown that nascent adhesions are involved in cell motility, while mature focal adhesions are rare in migrating cells and adversely affect migration (211,212). Therefore, an optimal amount of fibronectin may be necessary for efficient migration and too little or too much may have adverse effects on motility. Also, caspase-3-deficient MEFs do not overexpress Integrin-β1, which is the receptor for fibronectin and is necessary for proper binding and organization of fibronectin into the matrix.

These studies also demonstrate that caspase-7-deficient MEFs display an increase in average velocity and a decrease in directional migration (Figure 31). Also, similar to caspase-3-deficient MEFs, a catalytically inactive caspase-7 is sufficient to revert the motility phenotype back to a WT phenotype (Figure 34). Previously discussed data indicated that caspase-7-deficient MEFs lack the formation of a distinct leading edge due to altered activity and localization of cofilin and Arp2, which would coincide with a decrease in directional migration. Consistent with this, recent work on Arp3 knockout cells show that Arp3 is required for formation of the lamellipodia and another study demonstrated that cofilin is necessary for directional migration (199,213-216). Taken together, these data indicate that effector caspases are required for efficient cell migration by regulating cell velocity and polarity for directional migration. Also, the regulation
of these processes by caspase-3 and caspase-7 is independent from their catalytic activity. In order to better understand the function of these caspases in regulating motility, we reconstituted the caspase-deficient MEFs with the previously mentioned caspase-3 / caspase-7 fusion constructs. These reconstituted MEFs may provide insight into the regions of caspase-3 or caspase-7 that are responsible for regulating velocity and directional migration during motility. Since these are novel functions of the proenzymes of these caspases, we hypothesize that the prodomains may be implicated in the regulation of these functions. Moreover, the prodomains of caspase-7 and caspase-3 are the least similar regions between the proteins, suggesting that there was evolutionary pressure for the divergence of the homology of these regions.

F. Relevance to cancer

We have demonstrated that caspase-3 and caspase-7 regulate morphology, adhesion and motility and previous work has shown that these processes are all differentially regulated in cancer during tumorigenesis (217-220). Therefore, we examined the role of caspase-3 in the migration of the breast cancer cell line, MCF-7. Most cancers do not have mutations or deletions of caspase-3 or caspase-7, however MCF-7 cells are a minimally invasive cancer cell line that has a natural deletion of caspase-3 (181,182,221,222). Reconstitution of MCF-7 cells with murine caspase-3 or catalytically inactive-murine caspase-3 increases the average velocity of cell migration during
in vitro wound healing assays (Figure 39). These data suggest that caspase-3 regulates the migration of a cancer line independent from its catalytic activity. Interestingly, they also indicate that the expression of caspase-3 may be beneficial for tumorigenesis. Previous work has suggested that effector caspases are rarely deleted or mutated in cancers and it is speculated that this is because these caspases are past the ‘point-of-no-return’ in the process of cell death and there is no selective pressure for loss of effector caspase activity (222). However, our data indicate that there is a selective advantage to retain effector caspase expression because of their ‘day job’ functions in regulating cell morphology, adhesion and motility. Caspase-3 expression regulates cell velocity possibly through the secretion of fibronectin, while caspase-7 regulates morphology and directional migration possibly through the activity and localization of Cofilin and Arp2. Both of these novel functions of effector caspases would be beneficial to a cell during the process of tumorigenesis and this may be why these caspases are rarely deleted or mutated in cancers.

G. Concluding remarks

Taken together, our data indicate that caspase-3 and caspase-7 are non-redundant caspases that also have novel non-apoptotic functions or ‘day jobs’. We have demonstrated that these caspases have distinct functions during cell death and that caspase-3 is the dominant executioner caspase, while caspase-7 may function more in the demolition phase of apoptosis and insure the apoptotic integrity of the cell (Figure 43). Even though caspase-3 and caspase-7
have been shown to prefer the same substrate recognition site, multiple studies have indicated that they have differential cleavage of a variety of proteins and sequence alone obviously does not determine cleavage. Therefore, the cause of this specificity is still in need of further investigation.

Interestingly, we also have demonstrated that the non-apoptotic functions of caspase-3 and -7 are through mechanisms that are independent of their catalytic activity. Caspase-3 regulates morphology, adhesion and motility possibly in part through controlling the secretion of fibronectin and we show that a catalytically inactive caspase was sufficient for these functions. Similarly, caspase-7 is regulating morphology and motility, possibly through the localization of proteins involved in actin organization, including cofilin and Arp2, and a catalytically inactive caspase was sufficient for these functions. Some studies have suggested that there is controlled or localized caspase activation within the cell at times, however our data would argue that these caspases are acting more in the manner of a scaffolding protein or through a protein-protein interaction, not cleavage. More work has to be done to determine how these caspases function in these processes, if not with the use of their caspase activity.

Conventional thought would categorize caspase-3 and -7 as tumor suppressor genes because of their major function in apoptosis and their obstructive behavior in cells. However, our data indicate that these caspases may actually be beneficial for cells to have during the process of tumorigenesis. Caspase-3 and -7 are rarely mutated or deleted in cancers and this may not only be because they are past the ‘point-of-no-return’ in the process of apoptosis. Its
true that there is no selective pressure for a tumor cell to lose these caspases because they will still eventually die if a death pathway has reached this point, however there may be selective pressure to keep caspase-3 and -7 because their regulatory effects on adhesion and motility may be useful for tumor cells in vivo.
Chapter VI: Materials and Methods

Characterization of the phenotypic differences between wild-type and effector caspase-deficient MEFs during serum withdrawal-induced cell death

These experiments were used in determining the phenotypic differences between wild-type (WT) and caspase-7, caspase-3, or double caspase-7 and -3 deficient MEFs during serum withdrawal-induced cell death. Phenotypic changes were examined by death assays with detachment and ROS determination.

Cell Culture

Mouse embryonic fibroblasts (MEFs) were grown in Dulbecco’s Modification of Eagle’s Medium (DMEM, Cellgro) supplemented with 10% fetal bovine serum (Cellgro), 1% Penicillin - Streptomycin (Cellgro), 1% L-Glutamine (Cellgro), 1% non-essential amino acids (Cellgro), 1% sodium pyruvate (Cellgro) and 0.001% 2-Mercaptoethanol (Gibco) at 37°C in a humid 5% CO₂ incubator. Serum free medium for death assays was made as described without 10% fetal bovine serum. Splitting and harvesting of adherent cells was conducted by washing cells with phosphate buffered saline (PBS, Cellgro) and applying 0.25% Trypsin (Cellgro) for 5-10 minutes. WT, Casp7⁻/⁻, Casp3⁻/⁻ and Casp3⁻/⁻ 7⁻/⁻ MEFs were a gift from the Richard Flavell, Ph.D. laboratory.
**Death assays with detachment and ROS production determination**

Cells were seeded in 6-well plates and allowed to recover for 24-48 hours. Then cells were washed with PBS, medium was replaced with serum free medium and plates were returned to the incubator. After designated time points, medium and adherent cells were harvested, washed with PBS and stained with annexin V-FITC (Biovision) and propidium iodide (PI, Sigma) in annexin binding buffer (10 mM HEPES, 0.1 M NaCl, 2.5 mM CaCl$_2$). Cell death was determined by flow cytometry on a BD FACSCanto II system with FACSDiva software and data was analyzed with FlowJo. ROS production was determined by staining with H2DCFDA for 30 minutes, treatment with antimycin A for 30 minutes was used as a positive control. Percent detachment was determined by separating detached from adherent cells and counting on a hemocytometer.

**Characterization of the non-apoptotic functions of effector caspases.**

These experiments were used in determining the phenotypic differences between wild-type (WT) and caspase-7, caspase-3, or double caspase-7 and -3 deficient MEFs to examine possible non-apoptotic functions of effector caspases. Phenotypic changes were determined in regards to morphology, adhesion and motility. Cell volume analysis was used to show equal volume between cells in order to verify significance of differences in morphology. Doubling time determination and cell cycle analysis, with and without release from contact inhibition, was used to rule out proliferation differences being responsible for changes in motility.
Morphological studies

Cells were grown on glass coverslips (Fisher) coated with 5 µg/cm² fibronectin (Chemicon International) in a 24-well plate and then fixed for 10 minutes with PHEMO buffer (68 mM PIPES, 25 mM HEPES, 15 mM EGTANa₂, 3 mM MgCl₂·6H₂O, 10% DMSO, pH 6.8) supplemented with 3.7% formaldehyde (Fisher), 0.05% glutaraldehyde (Fisher) and 0.5% Triton X-100 (Fisher). Cells were washed with PBS and blocked for 10-15 minutes in 10% goat serum (Cellgro). Actin was labeled by staining with Alexa Fluor 555 phalloidin (Invitrogen) at 1:40 in PBS and DNA was stained with 300 nM 4′-6-diamidino-2-phenylinodle, dilactate (DAPI, Invitrogen) for 5-10 minutes in dH₂O. Coverslips were then mounted on microslides (Fisher) using 20-30 µl polyvinyl alcohol mounting medium with DABCO anti-fade (Fluka) and allowed to dry over night at room temperature in the dark. Cell morphology was determined by visualizing cells using a point scanning laser confocal microscope (LSM 510 META) and analyzing cell length and cell footprint area using Ziess Image Browser’s region of interest tool (ROI).

Cell volume analysis

Cells were harvested by trypsinization, resuspended in FACS buffer (PBS with 1% BSA and 0.01% Sodium Azide) and cell volume was measured by forward scatter (FSC) on a FACSCanto II flow cytometer and analyzed with FlowJo software.
**Cell adhesion**

5 x 10^3 cells were seeded in 96-well plates in triplicate and allowed to adhere for 30 minutes, 1, 2, and 4 hours. Cells were then fixed with PHEMO fixative and stained with crystal violet (Fisher Scientific) in 2% ethanol for 10 minutes. Plates were washed 3x in H_2O and then 2% SDS was added for 30 minutes to reconstitute the remaining crystal violet. Absorbance was measured on a 96-well plate reader at 590 nM.

**Anoikis-induced cell death assays**

Cells were seeded in 6-well plates coated with polyHEMA, to block adhesion, in full medium or cells were seeded in 6-well plates in serum free medium or a combination of both polyHEMA coated plates in serum free medium. After the indicated time points, medium and adherent cells were harvested, washed with PBS and stained with annexin V-FITC (Biovision) and propidium iodide (PI, Sigma) in annexin binding buffer (10 mM HEPES, 0.1 M NaCl, 2.5 mM CaCl_2). Cell death was determined by flow cytometry on a BD FACSCanto II system with FACSDiva software and data was analyzed with FlowJo.

**Motility analysis**

Cells were seeded into 35 mm x 10 mm cell culture dishes (Corning) and allowed to grow till confluence. Initiation of migration was achieved by scratching confluent cells with a p10 pipette tip. Motility was measured on a Ziess Axiovert
200m microscope mounted with a Perkin Elmer Ultraview ERS enclosed in a heated chamber (37°C) with 5% CO₂ injection. Images were acquired every 10 minutes for at least 15 hours. After image acquisition, Volocity software was used to determine percent wound closure at designated time points and to analyze single cell tracks over time. Cell tracking data included average velocity (distance/time) and meandering (displacement/distance).

**Cell cycle analysis**

Cells were seeded in 6-well plates, allowed to grow for 24 hours and then harvested by trypsinization. Cells were fixed in PBS:ethanol (1:3) and stored at 4°C overnight. Cell cycle analysis was performed on a FACSCanto II flow cytometer after staining with PI/RNase staining buffer (BD Pharmingen) for 30 minutes at room temperature.

**Motility cell cycle analysis**

Cells were seeded in 6-well plates and allowed to grow to confluency. Then a p10 pipette tip was used to make 8 parallel scratches or an 8 by 8 scratch grid in order to mimic motility assay conditions and the release of cells from contact inhibition. After 12 hours of migration was allowed to occur in a 37°C incubator, the cells were harvested and cell cycle was analyzed as above.
Doubling time analysis

5 x 10^4 cells were seeded in 6-well plates and then cells were trypsinized and counted on a hemocytometer at indicated time points.

Cloning of effector caspases and development of reconstituted cell lines used to show rescue of WT phenotype and determination of independence from apoptotic catalytic activity

These methods were used to create and clone Casp7 or Casp7^{c186s} (catalytically inactive) and Casp3 or Casp3^{c163s} (catalytically inactive) into pBabe-puro, so they could be reconstituted into their respective knockout cell line or the double knockout. Retroviral transduction was used to introduce the caspase containing plasmids and empty pBabe-puro plasmid back into the MEFs as needed. Expression of the plasmids was checked by western blot after selection with puromycin. The experiments that followed were designed to determine if the phenotypic changes seen in the caspase knockout MEFs could be reverted to a WT phenotype and to determine if the catalytic activity of each caspase was needed for reversion. Once the new cell lines were created, previous experiments including morphology and motility analyses were performed.

Caspase-7 site directed mutagenesis and cloning

Casp7 pCMV-SPORT6 (Open Biosystems) was used for transforming JM109 High Efficiency Competent Cells (Promega) and plasmid DNA was
isolated using HiSpeed Plasma Midi Kit (Qiagen). QuickChange XL Site-Directed Mutagenesis Kit (Stratagene) was used to mutate the catalytic cysteine (C\textsuperscript{186}) of Casp7 pCMV-SPORT6 to a serine (S). SDM primers were used during the mutant strand synthesis reaction step. After transformation and isolation of Casp7\textsuperscript{c186s} pCMV-SPORT6, the plasmid was sent to Macrogen USA for sequencing to verify Casp7 sequence and catalytic site mutation.

SDM primers were used with TaKaRa LA PCR kit Ver.2.1 (Takara Bio Inc.) to amplify Casp7 and Casp7\textsuperscript{c186s} inserts from Casp7 pCMV-SPORT6 and Casp7\textsuperscript{c186s} pCMV-SPORT6, respectively. PCR amplification products were separated on a 1% agarose (EMD Chemicals) gel supplemented with 0.5 µg/ml ethidium bromide (Sigma). Bands were visualized by UV light, excised from the gel and amplification products were isolated by MinElute Gel Extraction Kit (Qiagen). TA cloning was performed with Casp7 or Casp7\textsuperscript{c186s} PCR amplification products using the pTargeT Mammalian Expression Vector System (Promega) in order to introduce BamHI and SalI cloning sites for later use. Transformations and plasmid DNA isolation were performed as above. Ligation efficiency and success was assessed by restriction enzyme digestion of Casp7 pTargeT or Casp7\textsuperscript{c186s} pTargeT with BamHI and SalI (New England BioLabs), and separation and visualization of inserts on a 1% agarose gel, as described. Plasmids were sent to Macrogen USA for Casp7 and Casp7\textsuperscript{c186s} sequence verification.

Cloning of Casp7 pTargeT and Casp7\textsuperscript{c186s} pTargeT inserts into pBabe-puro was performed by restriction enzyme digestion of all plasmids with
BamHI and Sall. After separation and extraction of digested products, as described above, ligation was performed using T4 DNA ligase and buffer from pTargetT Mammalian Expression Vector System (Promega). Ligation efficiency and success was assessed as above.

**Caspase-3 cloning**

Casp3 pGEM and Casp3<sup>c163s</sup> pGEM were made by Bryan W. Johnson, Ph.D, a previous student in the laboratory. The constructs were given to Oskar Laur, Ph.D, at the Custom Cloning Core facility at Emory University, and Casp3 pBabe-puro and Casp3<sup>c163s</sup> pBabe-puro were made using subcloning. After receiving the constructs, JM109 High Efficiency Competent Cells (Promega) were used during transformations and HiSpeed Plasma Midi Kit (Qiagen) was used to isolate plasmid DNA.

**Cell culture**

Parental cell lines were grown and maintained as above. New cell lines created through retroviral transduction were maintained as above, but with medium supplemented with 2.5 µg/ml puromycin. ΦNX-Ecotropic packaging cell lines (Nolan lab, Stanford University) were grown in DMEM medium (Cellgro) supplemented with 10% fetal bovine serum (Cellgro), 1% Penicillin - Streptomycin (Cellgro), 1% L-Glutamine (Cellgro), 1% non-essential amino acids (Cellgro) and 1% sodium pyruvate (Cellgro) at 37°C in a humid 5% CO<sub>2</sub> incubator. Splitting and harvesting of cell lines was performed as above.
**Retroviral transduction**

ΦNX-Ecotropic packaging cell lines (Nolan lab, Stanford University) were transfected with a plasmid (pBabe-puro, Casp7 pBabe-puro, Casp7 \(^{c186s}\) pBabe-puro, Casp3 pBabe-puro or Casp3\(^{c163s}\) pBabe-puro) in separate T25 flasks using Lipofectamine (Invitrogen). Target MEFs were seeded in 6-well plates and allowed to grow for 24 hours. Viral supernatants were collected and filtered through 0.45 µm syringe filters (Pall) at 24, 28, and 32 hours and then replaced with fresh medium. At each time point, viral supernatants were applied directly on target cells for infection using Polybrene Infection / Transfection Reagent (Millipore). After 24 hours viral supernatants were removed from the target cells and replaced with fresh medium for 24-72 hours. Once cells recovered from infection they were selected with 2.5 µg/ml puromycin (Sigma).

**Western blot analysis**

Cells were harvested, washed with PBS and lysed with radioimmunopercipitation assay buffer (RIPA, 150 nM NaCl, 1% NP-40, 0.5% Na-deoxycholate, 0.1% SDS, 50ηM Tris, pH 7.4 with HCl) supplemented with 1 mM Phenylmethanesulfonyl fluoride (PMSF, Sigma), 0.1 µM Aprotinin (Sigma), 10 µM Leupeptin (Sigma). Protein concentrations were determined by Pierce BCA Protein Assay Kit (Thermo Scientific) using a Bio-Rad SmartSpec Plus Spectrophotometer. 30 µg of each sample was prepared by adding 4 µl 6x loading dye (0.375 M Tris-HCl, 24% SDS, 60% glycerol, 30% 2-mercaptoethanol, 0.012% bromophenol blue, pH 6.8) and bringing the final volume to 24 µl with
RIPA. Prior to loading, samples were boiled at 95°C for 5 minutes and then quickly spun in a microcentrifuge. Samples were loaded in a 4-20%, 10-well, Mini-Protean TGX gel (Bio-Rad) and ran at 150 V in Tris-Glycine-SDS buffer (TGS, 25 mM Tris, 200 mM glycine and 0.1% SDS). Gels were then transferred onto 0.2 µm nitrocellulose membranes (Whatman) in TGS buffer containing 20% methanol at 40 mA over night.

Following the transfer, membranes were blocked for 1 hour in 5% Bovine Serum Albumin (BSA) in Tris-buffered saline (TBS, 50 mM Tris, 150 mM NaCl, pH 7.4) with 0.1% Tween and 0.1% Sodium Azide. Primary antibodies for Casp7 or Casp3 (Cell Signaling) were applied 1:1000 shaking overnight at 4°C in BSA solution. Membranes were then washed 3x in TBS with 0.1% Tween (TBST) for 5 minutes and appropriate secondary antibodies (Amersham) were applied 1:2000 in 5% dried milk in TBST for 1 hour at room temperature. Membranes were then washed 3x in TBST for 5 minutes. Enhanced chemiluminescence (ECL, Amersham) was added to the blots and emitted light was detected by Hyperfilm (Amersham) in order to visualize and quantify proteins of interest. Actin was used as a loading control and was visualized as above, except the antibody (Sigma) was applied 1:3000 for 15 minutes and the appropriate secondary was applied 1:2000 for 15 minutes. In between primary antibodies, membranes were stripped for 30 minutes in stripping buffer (60 mM Tris, 5% SDS, 0.01% 2-mercaptoethanol) at 50°C.
Morphological studies

Morphological studies were repeated the same as above to look for reversion to a WT phenotype.

Motility analysis

Motility analysis were repeated the same as above to look for reversion to a WT phenotype.

Determination of the mechanisms responsible for phenotypic changes seen between WT and caspase-deficient cells

These methods were used to determine the mechanism by which effector caspases are regulating morphology, adhesion and motility.

Cell spreading

Cells were harvested, washed in PBS and counted with a hemocytometer. Then an equal number of cells were seeded into 14 ml polypropylene tubes (Becton Dickinson) in serum free medium and incubated at 37°C for 4 hours. Cells were collected by centrifugation and resuspended in Hanks’ Balanced Salt Solution (HBSS, Cellgro) and then seeded into 24-well plates onto glass coverslips with or without fibronectin coating. Plates were incubated at 37°C for 1 hour and then cells were fixed, stained with phalloidin, DAPI and either an antibody for P-FAK (Sigma) or fibronectin (Sigma). Cells were then mounted and visualized as described above.
Fibronectin secretion and production analysis

Cell spreading assays were performed as above. Then medium was collected for fibronectin secretion analysis by a fibronectin ELISA kit (Abcam) and cells were collected and lysed for fibronectin production by western blot, as above using an rabbit anti-fibronectin antibody (Sigma) and ELISA.

qRT-PCR

qRT-PCR was performed using Taqman Gene Expression (Applied Biosystems) and fibronectin and integrin-β1 probes (Applied Biosystems).

Fibronectin cell death rescue analysis

WT MEFs were seeded in serum free medium into 6-well plates coated with increasing concentrations of fibronectin (Millipore) from 0 µg/cm² to 10 µg/cm². After 24 hours, cell death was analyzed by annexin V-FITC staining (BD Biosciences) and flow cytometry as above.

Determination of the role of caspase-3 in the migration of MCF-7 cells

These methods were used to determine the function of caspase-3 in the motility of the MCF-7 breast carcinoma cell line. Average velocity and meandering were analyzed after in vitro wound healing assays.
Creation of cell lines and motility analysis

MCF-7 cells were reconstituted with caspase-3 or catalytically inactive caspase-3 by retroviral transduction as described above. Motility analysis was performed as above to determine the effects of caspase-3 on the migration of MCF-7 cells.

Preliminary data methods

Cell death activity of caspase-3 and -7 fusion constructs

Caspase-3 and -7 fusion constructs were made by Oskar Laur at the Emory University Custom Cloning Core. Caspase-3-deficient MEFs were reconstituted with fusion proteins by retroviral transduction as described above and cell death assays were performed at indicated time points as above.

P-cofilin and Arp2 analysis in caspase-7-deficient MEFs

WT and caspase-7-deficient MEFs were collected and lysed for western blot analysis as described above and blotted with an anti-P-cofilin or total cofilin antibody (Cell Signaling). Also, WT and caspase-7-deficient MEFs were grown to confluency and then scratched multiple times with a pipette tip. After being allowed to migrate 0, 2, 4, or 8 hours, cells were collected lysed and analyzed by western blot. Cells were also seeded on glass coverslips for 24 hours and fluorescent microscopy was performed as described above using an anti-cofilin antibody or an anti-Arp2 antibody (Cell Signaling).
Creation of MEFs expressing cytochrome c-GFP, Omi-mCherry, or mCherry-GFP-LC3 fusion proteins

Cytochrome c-GFP and Omi-mCherry constructs were a gift from Douglas R. Green Ph.D. and the mCherry-GFP-LC3 construct was a gift from Jayanta Debnath M.D. Fusion proteins were expressed in MEFs using retroviral transduction as described above. Expression of the fusion proteins was determined by flow cytometry or fluorescent microscopy.

Total protein secretion

Cell spreading assays were performed as described above and then medium was collected to determine protein secretion. TCA protein precipitation was performed to precipitate secreted protein. Protein was separated by gel electrophoresis and the gel was stained with a silver stain kit (BioRad) to visualize secreted protein.

shRNA knockdown of fibronectin

Knockdown of fibronectin was completed using a lentiviral system and shRNA for fibronectin (Open Biosystems). Expression of fibronectin after knockdown was determined by western blot as described and mRNA levels were determined by qRT-PCR as described using a fibronectin probe (Applied Biosystems).
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