USING OPTOGENETICS AND BIOCHEMISTRY TO UNCOVER HOW AUTOPHAGY REGULATES CELL DEATH

by

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A thesis submitted to the Faculty of the Graduate School of the University of Colorado in partial fulfillment of the requirements for the degree of Doctor of Philosophy Pharmacology Program 2018
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Macroautophagy (autophagy) is intimately linked with cell death and often allows cancer cells to evade apoptosis. This has prompted clinical trials to combine autophagy inhibitors with other drugs with the aim of reducing patient tumor burden by increasing the likelihood of cancer cells dying. However, the molecular basis for such effects is unknown. I describe a transcriptional mechanism that connects autophagy to apoptosis. The autophagy-regulating transcription factor, FOXO3a, is itself turned over by basal autophagy creating a potential homeostatic feedback loop. Increased FOXO3a upon autophagy inhibition stimulates transcription of the pro-apoptotic PUMA gene to cause apoptosis sensitization. This mechanism explains how autophagy inhibition can sensitize tumor cells to chemotherapy drugs and allows an autophagy inhibitor to change the action of an MDM2-targeted drug from growth inhibition to apoptosis, reducing tumor burden in vivo. Thus, a link between two processes—autophagy and apoptosis—mediated via a single transcription factor binding site in the genome can be leveraged to improve anti-cancer therapies. Also, I provide insight into FOXO3a regulation by autophagy, which is mechanistically distinct from its canonical regulation by the proteasome. Lastly, I developed an optogenetic tool to inhibit autophagy in a
rapid, transient, and spatial manner, allowing us to uncover other mechanisms that link autophagy to cell death.

The form and content of this abstract are approved. I recommend its publication.

Approved: Andrew Thorburn
I dedicate this Thesis to my entire family. You’ve inspired me to be the best person and scientist possible. This includes the family’s newest member, Marshall Theodore Labath; he should know that he lives in a world where the possibilities are infinite.
ACKNOWLEDGMENTS

I would like to thank many people for sharing their scientific expertise and guidance with me as a Ph.D. candidate; these people were instrumental in my success.

With the highest respect, I thank Christie G. Towers for her invaluable support as a scientist, colleague and dear friend. Her support of my scientific progress is extraordinary. She has provided both personal and scientific encouragement well beyond what I can name here. My wish is that her talent as a scientist will continue to be recognized, and result in an independent research position where she can attempt to satisfy her insatiable scientific curiosity.

My advisor Andrew Thorburn has been a pivotal figure in my scientific career. I believe that joining his laboratory has been the best professional decision I've made to date, and would not change a single aspect of my experience. He will remain as one of most scientifically inspirational mentors one could ask for. He has taught me to think as an independent researcher and has been supportive in every aspect of my development. I consider him a mentor, colleague and friend.

Jackie Thorburn has been a central part of my progress as a Ph.D. student. She has provided excellent scientific advice, reagents, and has taught me how to properly conduct experiments. In addition to being an exceptional colleague, she has led to my personal development with advice and support for every situation. I value her wisdom and talent both inside and outside the laboratory.

I would like to thank Tatiana Kutateladze for her interest in my success and making my move into Andrew’s laboratory a smooth transition. I would also like to thank Zdenek Andrysik for his scientific help on the 2018 Developmental Cell publication and...
his scientific advice. I would also like to thank Soheel Khan, PharmD for sparking my curiosity in the biology of drug targets during my time as a pharmacy technician, which ultimately led me to choose scientific research over pharmacy school. I would also like to thank Senthilnath (Sid) Lakshmana Chetty for his friendship and endless encouragement through graduate school.

I would like to thank Adeline Matschulat for her unwavering support; she helped me rehearse nearly every talk and poster presentation, and often reassured me of my talent as a scientist. She listened and gave advice on the daily happenings in the laboratory in regard to experimental results and remained a positive voice in my ear during difficult times.

I would like to acknowledge the support from my peers in the Pharmacology Graduate Program, as well as my thesis committee members for their commitment to my professional development.

Lastly, and most importantly, I would like to acknowledge my entire family for their encouragement and support over the past few years. They have shown tremendous interest in my research, and instilled determination in me to be the best scientist and person possible.

The brief acknowledgements above do not measure up to my appreciation—thank you.
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Overview of autophagy and cell death

Macroautophagy (hereafter autophagy) is an evolutionarily-ancient mechanism by which cellular material is delivered to lysosomes for degradation. Autophagy is best understood in the context of nutrient deprivation, and is often viewed as a way to maintain cellular homeostasis by recycling old or damaged organelles. Christian de Duve, a Belgian biochemist, first coined the term ‘autophagy’ during a lysosome conference in 1963, and since then the molecular mechanisms of autophagy have continued to emerge. The Nobel Prize for Physiology or Medicine was awarded to the Japanese cell biologist Yoshinori Ohsumi in 2016 for his ground-breaking studies elucidating the genetic components of autophagy in *Saccharomyces cerevisiae* (Mizushima et al., 1998; Takeshige et al., 1992; Tsukada and Ohsumi, 1993). Autophagy is conserved from yeast to humans, and is intimately intertwined with other cellular processes, including programmed cell death. Autophagy and cell death have some shared molecular machinery and recent work suggests that autophagy has great influence over a cell’s decision to live or die. Numerous studies demonstrate that autophagy plays a role in many physiological processes, and there are dozens of ongoing clinical trials aiming to pharmacologically manipulate autophagy in humans with

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the goal of mitigating disease. Understanding the cellular mechanisms by which autophagy regulates cell fate is critical for knowing how autophagy alteration might be beneficial in diseases where too much or too little cell death takes place e.g. neurodegenerative diseases and cancer, respectively.

As we consider how autophagy affects cell death, it is important to note that autophagy can determine whether or not cells die without directly affecting the cell death machinery. For example, it is well established that autophagy is critical for preventing cell death due to amino acid starvation, but this doesn’t necessarily mean that the apoptosis and necrosis mechanisms that cause amino acid starved cells to die are controlled by autophagy. Instead, autophagy can be protective here simply because autophagy degrades proteins, thereby providing the cell with the amino acids it needs to avoid activating those cell death mechanisms in the first place. Similarly, in mice genetic inactivation of autophagy in neurons leads to accumulation of aggregated proteins and eventual neuronal cell loss (Hara et al., 2006; Komatsu et al., 2006); however, this doesn’t mean that autophagy was directly controlling the activity of the cell death machinery in those neurons. Instead, in this case, it is thought that autophagy is removing the toxic protein aggregates so that a death signal was never activated. In general, I will ignore this type of indirect mechanism whereby autophagy removes a toxic signal to focus on recent findings that relate to more direct autophagy control of distinct programmed cell death pathways. My work in this thesis will reveal some of these direct mechanisms.

Autophagy’s influence over cell death decision making is complicated by the fact that autophagy’s role in determining whether a cell should live or die goes both ways—
autophagy inhibition can result in more or less cell death. Autophagy may also differentially affect different types of cell death. In this thesis, I will discuss literature and present work that helps make sense of this seemingly inconsistent role of autophagy in influencing a cell to live or die.

The direct mechanisms by which autophagy positively regulates cell death are still being uncovered. We arguably have better evidence for autophagy promoting cell death under physiological conditions than we do for autophagy providing a general protective effect. As I will discuss below, there are numerous examples in development and under normal physiological conditions where autophagy is required for cell death (Nelson and Baehrecke, 2014). This cell death depends on autophagy because deletion of core components of the autophagy machinery results in less death.

There are some direct molecular mechanisms explaining how autophagy prevents cell death. For example, in Chapter III, I describe a direct mechanism that explains how autophagy can protect cells from apoptosis by selectively degrading a transcription factor needed to promote cell death. Preventing the degradation of this transcription factor by blocking autophagy promotes apoptosis sensitization; this provides a specific mechanism whereby the autophagic degradation of a pro-apoptotic protein allows cells to evade apoptosis. I will discuss the practical implications of this mechanism in targeting autophagy in cancer cells.

It is now becoming clear that autophagy does in fact directly regulate the cell death machinery both positively and negatively depending on the death stimulus, cell type, and context. Autophagy can also influence different types of cell death (Figure 1.1) (Tait et al., 2014).
Figure 1.1
Cell death modes. Adopted from Hotchkiss et al 2009 NEJM
I aim to emphasize three important points: (1) Programmed cell death mechanisms and autophagy are indeed intimately linked. (2) The basis for autophagy influencing cell-fate decisions—whether to live or die—depends on which proteins are recruited to autophagosomes for lysosomal degradation. (3) Where and when the autophagy pathway—early versus late stages, before or after a death stimulus—is manipulated by genetic changes or pharmacological agents should be considered when trying to influence survival or cell death.

**Autophagy regulation**

Autophagy degrades cytoplasmic substrates to obtain energy and metabolic building blocks, especially when the cell is subjected to nutrient deprivation (Mizushima, 2007). Autophagy is also necessary for the homeostasis of organelle integrity, clearance of aggregated proteins, tissue remodeling during development, and resistance to infectious agents like bacteria and viruses. The signaling associated with autophagy is best understood in the context of coping with nutritional stress and maintaining cellular fitness. Many studies have concentrated on amino acid and insulin-dependent signaling involving mTOR signaling. Upstream of mTOR, the 5' AMP-activated protein kinase (AMPK) phosphorylates TSC2 in response to a low ATP/AMP ratio, leading to mTOR inactivation and autophagy induction. Other important complexes include the ULK1 initiation complex and the Beclin1-VPS34 PI3 Kinase complex, which are important for nucleation of the membrane that will eventually form the autophagosome (Figure 1.2) (Mizushima, 2007). In response to such signaling, coordinated actions of more than 30 ATG (autophagy-related genes) proteins promote the nucleation and elongation of an isolation membrane for engulfing cytosolic...
Figure 1.2
substrates into an autophagosome. Two ubiquitin-like conjugation systems cause elongation of the membrane and formation of the autophagosome. One of these systems is the covalent conjugation of ATG12 to ATG5, which interacts with ATG16L to form the ATG5-ATG12-ATG16L complex. The second system conjugates a lipid, phosphatidylethanolamine, to LC3 (and the other family members, LC3A, B, C, GABARAPL1, and GABARAPL2) resulting in the autophagosome-associated LC3-II protein, which is commonly monitored as a measure of autophagic activity in cells. At this stage, cellular material including proteins and organelles are either specifically recruited to the autophagosomal structures or, in the case of bulk non-specific autophagy, randomly captured. After fusion of the membranes to make an intact vesicle containing the cargos that will eventually be degraded, the resulting double-membrane vesicle fuses with a lysosome to form an autolysosome. Then, lysosomal enzymes hydrolyze the proteins, lipids and nucleic acids contained in the autophagosomes. The completion of this process is termed autophagic flux, and the magnitude of this flux can be measured on western blots by the amount of lipidated autophagosome-associated protein LC3-II that accumulates with or without blockade of the lysosomal fusion step. Alternatively, fluorescent labeling of LC3 can allow for tracking the redistribution of LC3 to autophagosomes. By using fluorescent tags with different pH sensitivities, it is possible to measure both the formation of autophagosomes and their fusion with lysosomes. Autophagy substrates are often recruited to autophagosomes by ubiquitylation, and recognized by adaptor proteins such as SQSTM1 (p62), which contain an LC3-interacting region (LIR). These adaptor proteins are degraded along
with the cargo, and their turnover can therefore also be used to measure autophagic flux.

When I discuss “early” steps in the autophagy process, I am referring to the activities of ATG proteins and signaling complexes necessary for autophagosomal nucleation, elongation, and maturation but not fusion with the lysosome and degradation of the cargo. For example, inhibition of early autophagy could be achieved by pharmacologically inhibiting the phosphatidylinositol 3-kinase (VPS34) with a kinase inhibitor, or the genetic knock down of ATG7 thus preventing the protein conjugations necessary for autophagosomal membrane elongation. Conversely, when I refer to “late” steps in autophagy, I mean the degradation of the autophagosome and its cargo by the lysosome. As I will discuss, interference with autophagy at these different steps may sometimes have quite different effects on the cell fate decision.

**Apoptosis regulation**

Caspase-dependent apoptosis is by far the best understood mode of programmed cell death. We usually think of apoptosis as being driven by either intrinsic or extrinsic pathways (Figure 1.3). Extrinsic apoptosis occurs through ligand binding to cell surface receptors such as CD95, also known as Fas, and tumor necrosis factor-related apoptosis inducing ligand (TRAIL). Activation of these death receptors causes formation of various protein complexes that ultimately serve to aggregate an initiator caspase (caspase-8). In some cells, this occurs efficiently enough to activate sufficient effector caspase (e.g. caspase-3) activity to kill the cell. However, in most cells, the active caspase-8 causes cleavage of the BH3-only protein BID to activate the so-called “intrinsic” apoptosis pathway and this is needed to induce enough caspase activity to
Figure 1.3
The intrinsic and extrinsic apoptosis pathway. Adopted from BiologyDictionary.net. (https://biologydictionary.net/apoptosis/#prettyPhoto)
cause apoptosis. The intrinsic pathway is also activated by apoptosis stimuli that do not work through death receptors.

Intrinsic apoptosis (Figure 1.3) is characterized by the summation of pro- and anti-death signals converging at mitochondrial membranes that become permeabilized (MOMP—mitochondrial outer membrane permeabilization), leading to the release of mitochondrial intermembrane proteins. The key proteins include cytochrome c and Smac/Diablo, an inhibitor of caspase inhibitors that normally keeps caspases in check. The release of cytochrome c interacts with Apaf1 to form a scaffold called the apoptosome, leading to the activation of the initiator caspase-9. MOMP is followed by rapid cell death because the combination of caspase activation on the apopotosome along with simultaneous block of caspase inhibitors starts a cascade of active caspases that cleave hundreds of cellular substrates to cause cellular demise.

Bcl-2 family proteins control MOMP through protein-protein interactions. The anti-apoptotic BCL-2 proteins Bcl-2, Bcl-xL, and Mcl-1 prevent activation of the pro-apoptotic proteins Bax and Bak, which form the pores in the mitochondrial membrane that defines MOMP. However, upon the accumulation of cellular stress, there is a synchronized effort by BH3-only members, like BID, BIM, BAD, PUMA, and NOXA, that inhibit the Bcl-2 proteins, or directly activate Bax and Bak to promote MOMP. A ~26 residue amphipathic helix comprises the BH3 domain that binds with high affinity to a hydrophobic groove of BCL-2 proteins to inhibit their anti-apoptotic activity.

MOMP is commonly thought of as a ‘point of no return’ that defines the cell’s commitment to apoptosis. However, new evidence questions this dogma by demonstrating that a small population of mitochondria in the cell may actually undergo
MOMP, yet not result in the death of the cell. This has been termed “Incomplete” (Tait et al., 2010) or “Minority” MOMP (Ichim et al., 2015). These effects whereby a cell activates incomplete MOMP and then recovers can have profound biological effects. Indeed, it was recently demonstrated that minority MOMP and caspase activation is sufficient to promote pro-tumorigenic effects in cells (Ichim et al., 2015; Liu et al., 2015). Moreover, bursts of caspase activity that are not sufficient to cause the cell to die occur during development (Tang et al., 2015), suggesting that these effects may be important in normal biological processes. Thus, complete MOMP causes enough caspase activation to cleave sufficient caspase substrates that the cell is destined to die with the morphology associated with canonical apoptosis, like plasma membrane blebbing and nuclear condensation etc. However, incomplete MOMP allows cells to recover after activating an insufficient level of caspases to cause death.

Understanding canonical apoptosis execution and what determines apoptosis threshold is still an active area of research in cancer therapy. Cytotoxic chemotherapies (e.g. microtubule-targeted agents and DNA damaging agents) have cured millions of patients with otherwise fatal disease. That is, a patient presents in the clinic with a fatal cancer, and goes into complete remission following a defined anti-cancer regimen (Letai, 2015). Conventional chemotherapies take advantage of the differences in mitochondrial priming to apoptosis between ‘normal’ tissue and cancer cells; numerous studies have demonstrated that differential priming to apoptosis can predict clinical responses to cytotoxic chemotherapies (Ni Chonghaile et al., 2011). Understanding the mechanisms that regulate the distance that cancer cells are from the proverbial ‘cliff’—their likelihood of undergoing apoptosis—will be critical for the evolution of cytotoxic
chemotherapies. My work in this thesis will argue that autophagy levels in cancer cells dictate their apoptotic threshold.

One way in which autophagy controls cell death is by regulating apoptotic proteins, like PUMA. PUMA, or \textit{p53-upregulated modulator of apoptosis}, is a pro-apoptotic BH3-only protein that has the ability to bind to all Bcl-2 proteins, localizes to mitochondria, and promotes cytochrome c release (Nakano and Vousden, 2001; Yu and Zhang, 2008). PUMA contains three coding exons (exons 2-4) and two non-coding exons (1a and 1b), all of which (except 1b) are conserved in mice (Yu and Zhang, 2008). The PUMA protein has two functional domains; the BH3 domain and the mitochondrial localization signal. PUMA indirectly affects MOMP by directly blocking anti-apoptotic Bcl-2 proteins, which sustain inhibition on Bax and Bak (Yu and Zhang, 2008). PUMA expression is normally kept low, but can be increased by transcription factors FOXO3, E2F1, p53, and p73 following a range of stress stimuli (Hershko and Ginsberg, 2004; Melino et al., 2004; Nakano and Vousden, 2001; You et al., 2006; Yu et al., 2001). PUMA has also been shown to be regulated by phosphorylation at serine 10, which leads to increased proteasomal turnover (Fricker et al., 2010). These data show that PUMA can be regulated in response to a range of cellular stresses.

\textbf{Forkhead Box transcription factors in cell death and autophagy}

Forkhead Box (FOX) transcription factors are increasingly recognized as players in many different signaling pathways, including DNA damage, metabolism, apoptosis, cell cycle, oxidative stress, and cell differentiation (Figure 1.4) (Huang and Tindall, 2007). In addition, their activity has been shown to influence programmed cell death, autophagy, and ageing. There are numerous studies providing evidence that the
**Figure 1.4**
The biological roles of FOXO transcription factors. Adopted from Calnan and Brunet *et al* 2008 *Oncogene*
ortholog of FOXO, *daf-16*, is necessary to mediate the life-extension properties of insulin growth factor receptor mutations (*daf-2*) in *Caenorhabditis elegans* (Henderson and Johnson, 2001; Lin et al., 1997; Ogg et al., 1997). In humans, there are associations between genetic variation in FOXO3a and longevity (Donlon et al., 2012; Willcox et al., 2008). The first Forkhead protein was identified as a homeotic *Drosophila melanogaster* gene, and since then over 100 forkhead genes, with 19 human subgroups ranging from FOXA to FOXS, have been identified (Weigel et al., 1989). Here, I discuss the significance of the FOXO subclass of the forkhead box transcription factors, which are conserved from *Caenorhabditis elegans* to humans, are expressed in all human tissues, and consist of FOXO1, FOXO3, FOXO4, and FOXO6. The DNA binding domain of these proteins, known as the Forkhead domain appears as “butterfly-like” in X-ray crystallography studies, which is created by three α-helices and three β-sheets with two wing-like loops (Maiese, 2015). Their protein structure is described as a “winged helix” with 14 protein-DNA contact points, with the primary recognition region at α-helix H3 (Clark et al., 1993). FOXO transcription factors often act as transcriptional activators and bind to DNA at the core consensus sequence of TTGTTTAC (Furuyama et al., 2000; Xuan and Zhang, 2005). There are many post-translational modifications (PTMs) that can occur in the Forkhead domain that regulate this DNA binding ability.

FOXO transcription factor activity is primarily regulated through post-translational modifications, including phosphorylation, mono-ubiquitination, poly-ubiquitination, and acetylation (Brunet et al., 1999; Brunet et al., 2001; Li et al., 2016; van der Horst et al., 2004; Yuan et al., 2008) (Figure 1.5). The effect of these modifications can also influence FOXO localization, binding partners, and DNA affinity.
Figure 1.5
Post-translational modifications of FOXO transcription factors. Adopted from Calnan and Brunet 2008 *Oncogene*
(Calnan and Brunet, 2008). Phosphorylation events often regulate the nuclear/cytoplasmic shuttling acting as effectors in many signaling pathways intended to alter transcription. The PI3K-Akt pathway and the serum and glucocorticoid-induced kinase (SGK) are known to phosphorylate FOXOs on conserved residues (T32 and S253 in FOXO3) to promote cytoplasmic sequestration via binding to the chaperone protein 14-3-3 (Brunet et al., 1999; Li et al., 2007; Obsilova et al., 2005). Further regulation of FOXO transcription factors includes protein stability regulation by the proteasome pathway in response to insulin and growth factors (Aoki et al., 2004; Huang et al., 2005; Matsuzaki et al., 2003; Plas and Thompson, 2003). Taken together, the PI3K-Akt signaling node leads to both the cytoplasmic sequestration and proteasome-mediated degradation of FOXO transcription factors in response to insulin signaling. The RAS-ERK signaling pathway has also been shown to regulate FOXO3a by phosphorylation at Ser 294, Ser 344, and Ser 425, leading to increased Mouse double minute 2 homolog (MDM2)-dependent proteasomal degradation (Yang et al., 2008).

There is mounting literature that FOXO transcription factors play a role in autophagy regulation. The most compelling of these studies involves FOXO3a orchestrating a protective gene program in hematopoietic stem cells (HSC) by upregulating core autophagy genes and autophagy regulators in response to cytokine withdraw (Warr et al., 2013a). The autophagy induction mediated by FOXO3a activation poised HSCs for adaptation to this by this insult, protected from metabolic crisis, and allowed for HSC survival (Warr et al., 2013a). Another study found that FOXO1 was important for autophagy induction by eliciting its actions in the cytoplasm, independent of its transcriptional activity. In response to starvation or oxidative stress, FOXO1 was
determined to be acetylated and bound to the autophagy-related E1 conjugation enzyme ATG7, allowing for autophagy induction and eventually cancer cell death (Zhao et al., 2010).

The transcriptional and epigenetic mechanisms that regulate autophagy are becoming increasingly appreciated. A recent study implicated FOXO3a in a novel signaling axis involving AMPK-SKP2-CARM1 (AMP-activated protein kinase--S-phase kinase associated protein 2--Coactivator-associated arginine methyltransferase 1) in regulating autophagy following starvation. This seemingly complex mechanism suggested that following starvation, AMPK phosphorylates FOXO3a in the nucleus, leading to the transcriptional repression of SKP2. This repression then leads to CARM1 stabilization, and increased histone H3 Arg17 dimethylation. CARM1 mediates co-activator functions through the transcription factor EB (TFEB), leading to autophagy and lysosomal-related gene activation (Shin et al., 2016). These studies point to the need for integrating both transcriptional and epigenetic datasets to understand the outcome of transactivation of transcription factors like FOXOs. Of the thousands of FOXO transcriptional targets, which are important for mediating the primary effects on cell death or cellular stresses? Are there modifications or other unknown regulatory mechanisms that orient FOXO activity toward certain targets upon autophagy manipulation? Which kinds of FOXO transcriptional targets are regulated early and late in response to stress or other stimuli? With the advent of a novel optogenetic system to inhibit autophagy that I describe in Chapter VI, I will be able to tackle some of these questions.

A recent study (Li et al., 2016) determined that FOXO3a is oriented toward
specific targets by a phosphorylation event at S574 by c-Jun N-terminal Kinase (JNK)—phosphorylated FOXO3a bound at pro-apoptotic promoters as measured by ChIP in response to ethanol or lipopolysaccharide. This study demonstrates that a post-translational modification can determine the specificity of a transcriptional program toward a specific cell fate decision. Future studies should examine these questions to determine their role in diseases and biological processes where FOXO transcription factors are believed to be involved.

How autophagy regulates apoptosis

The Thorburn laboratory recently described a mechanism by which autophagy controls apoptosis; autophagy regulates the PUMA protein to govern the timing and extent of MOMP, and subsequently regulates the timing and efficiency of apoptosis (Thorburn et al., 2014). This study found that as compared to autophagy competent cells, autophagy deficient cells are highly sensitized to Tumor Necrosis Factor-Related Apoptosis Inducing Ligand (TRAIL)-induced apoptosis. Importantly, the majority of autophagy’s ability to inhibit apoptosis in this context was due to this mechanism because PUMA knockdown was sufficient to avoid sensitization to apoptosis when autophagy is inhibited. This indicates that autophagy is providing protection from TRAIL through the ability of autophagy to regulate constitutive levels of PUMA. This example defines a mechanism by which autophagy can protect cells from a canonical apoptotic stimulus by controlling the level of a key regulator that makes the rate limiting step in apoptosis—i.e. MOMP—work more efficiently. In Chapter III, I take these studies a step further by uncovering a transcriptional mechanism that explains how autophagy
regulates PUMA levels, and show in Chapter IV that this mechanism can be leveraged
to improve anti-cancer drugs (Fitzwalter et al., 2018).

The Thorburn laboratory also recently reported (Gump et al., 2014) that in a
population of cells, there are transient cell-to-cell variations in basal autophagy flux that
dictates cell fate in both a cell-type-specific and stimulus-specific fashion. For example,
distinct populations of cells with high or low autophagy flux respond very differently to
death receptor activation. Fas ligand and TRAIL act in similar and well-understood
ways, utilizing many of the same pathway components to elicit apoptosis. So, it was
surprising when cell sorting to isolate cells that were undergoing high or low levels of
autophagy, and treating these with Fas or TRAIL produced completely opposite results.
High autophagy cells were sensitized to Fas ligand-induced apoptosis, but resistant to
TRAIL treatment. As explained above, the protective effect of autophagy on TRAIL-
induced apoptosis can be largely explained by PUMA being lower when autophagy is
high, thus making MOMP more efficient. What explains the opposite effect when the
apoptotic stimulus is the very similar death agonist Fas Ligand?

The answer was that the differential responses to two canonical death ligands is
a result of what the high autophagy cells are or are not degrading. The key was the
selective degradation of a phosphatase, FAP-1, which is a negative regulator of FAS
signaling, but does not affect TRAIL signaling. This degradation of a negative regulator
of FAS signaling was sufficient and necessary to explain how high autophagy can
promote Fas-induced apoptosis. And, since FAP-1 has no effect on TRAIL receptors,
this also explains why only autophagy’s protective effects were seen with TRAIL-treated
cells in this case. This example demonstrates how autophagy can both positively and
negatively influence cell fate. The degradation of a negative apoptosis regulator results in more death in high autophagy cells, while autophagy regulation of a positive regulator of apoptosis leads to less death in high autophagy cells. This mechanism of apoptosis promotion by autophagy is conceptually similar to another study performed in *Drosophila* nurse cells during oogenesis; apoptosis is promoted by autophagic degradation of a negative regulator (dBruce) that controls the apoptosis machinery (Nezis et al., 2010). The difference here is that the FAP-1 mechanism affects only apoptosis induced by Fas Ligand, whereas the dBruce degradation in the nurse cells removes an inhibitor of a caspase inhibitor, sensitizing them to any stimulus that activates caspases.

I just discussed how autophagy influences cell death by selective degradation of pro- or anti-apoptotic proteins. An intriguing story where autophagosomal membranes serve as platforms for death signaling complexes offers another mechanistically distinct example of how the autophagy machinery can influence cell fate. Experiments with the sphingosine kinase inhibitor (SKI-I), which promotes cell death through the suppression of sphingosine 1-phosphate, showed caspase-dependent cell death occurred only in the presence of functional autophagy (Young et al., 2012). Further, SKI-I was demonstrated to promote the translocation of caspase-8 homocomplex and Fas-associated protein with death domain (FADD) to ATG5-positive autophagosomal membranes, forming a scaffold for the efficient formation of an intracellular death-inducing signaling complex (iDISC). This death was abrogated by ATG5 depletion, which resulted in lower activation of mitochondrial amplification loop that is initiated by caspase-8. A follow-up study proposes that ATG2A/B deletion causes an accumulation of immature
autophagosomal membranes, and promotes the recruitment of the iDISC signaling complex (Tang et al., 2017). The authors argue that targeting autophagosomal closure may be a novel target to switch cytoprotective autophagy to apoptosis. Overall, these studies show a mechanism of apoptosis that is dependent on autophagosomal structures but not necessarily the whole process of autophagy whereby the material sequestered in the autophagosome is degraded.

Two important implications of this mechanism whereby the autophagy machinery alters signaling by serving as a scaffold should be considered. First, there may be different effects on the outcome of the cell depending on whether basal autophagy or stimulus-induced autophagy is manipulated. For the anti- and pro-apoptotic effects that are ultimately due to altering levels of apoptosis regulators such as PUMA, dBruce or FAP-1, autophagy's effects must be mediated prior to the initiation of the death signal if they are to alter the likelihood that the subsequent apoptosis stimulus will be sufficient to cause the cells to die. Indeed, when cells were separated into high and low autophagy from a population, the study found that this predicted their likelihood to undergo apoptosis that wasn’t even initiated until several hours after the separation of the cells into the two groups (Gump et al., 2014). This is a case whereby the effects of basal autophagy that occur before the apoptosis signal are critical for deciding whether or not cells will die at a later time. Not surprisingly then, manipulations that affect such basal autophagy can influence these mechanisms (Yonekawa et al., 2015). When autophagic structures are working by forming a scaffold for efficient formation of death inducing protein complexes as in the case with the intracellular DISC formed after SKI-I inhibitor treatment, autophagy is having its effects after initiation of the death stimulus.
These kinds of effects are, therefore, more likely to be influenced not by the basal autophagy that occurred before the death stimulus, but instead by the induced autophagy that occurs in response to the stimulus. Since many apoptotic stimuli (e.g. drugs that kill cells as well as pure apoptosis inducers like the death receptor agonists) also induce autophagy, it has been assumed that this induced autophagy is doing the same things to the cell death machinery as the ongoing basal autophagy does. That’s not necessarily true. To date, it has been impossible to test such ideas because we usually design experiments to determine how autophagy affects cell death by inhibiting autophagy using genetic approaches (knockout or knockdown of essential ATGs, expression of dominant negatives etc.) that take several days to become effective (Staskiewicz et al., 2013). Technical advances will be needed to address these questions because one needs a way to very rapidly, selectively and reversibly target stimulus-induced autophagy without affecting basal autophagy in order to test if both types of autophagy have the same effects, or not. To tackle this problem, I describe in Chapter VI the development of an optogenetic system to inhibit autophagy with light.

Second, interference with autophagy at different steps in the process may sometimes have the same effects or different effects on a cell’s likelihood of dying. If the important mechanism is regulation of proteins like FAP-1 or PUMA, it most likely doesn’t matter how autophagy is blocked—if you block formation upstream of autophagosomal structures or degradation of cargo, similar effects are expected. Indeed, that’s what we saw with FAP-1– genetic inhibition of autophagosome formation gave similar effects to blocking lysosomal function. However, if autophagy’s role in regulating apoptosis is by providing scaffolding structures as discussed above, one might expect that inhibiting
formation of autophagosomal structures would block cell death, while inhibiting their degradation (e.g. by blocking the lysosome) might promote these effects by causing more scaffolding.

There are hints in the literature (Tang et al., 2017) where apoptosis inducing anti-cancer drugs are affected oppositely depending on whether autophagosome formation versus degradation are targeted. Do these kinds of mechanistic differences explain such effects? The answer to this question is important because although we are already treating cancer patients with lysosomal inhibitors, other autophagy inhibitors that affect early steps in the process are being developed (Bago et al., 2014; Dowdle et al., 2014; Egan et al., 2015; Ronan et al., 2014). For example, the VPS34 kinase inhibitor, SAR405, is a potent ($K_D=1.5$ nM) and selective compound targeting the ATP binding cleft. SAR405 was shown to inhibit starvation-induced autophagy by limiting the formation of lipidated LC3, reducing GFP-LC3 puncta, and causing the accumulation of a receptor protein p62 (Ronan et al., 2014). Compounds like SAR405 that target the initiation of autophagy may prove to be efficacious in clinical trials as independent therapies, or useful alongside late-stage inhibitors like chloroquine. One could also imagine a situation in which these drugs might have the opposite effect that is hoped for (i.e. inhibit rather than promote tumor cell death) e.g. by interfering with scaffolding activities and this might be highly context dependent. Namely, if a cell death complex requires an autophagosomal scaffold then blocking autophagosome formation may mitigate death, whereas blocking autophagosome-lysosome fusion could potentiate death. As autophagy inhibitors increasingly become a part of clinical development, understanding the consequences of blocking each step in the autophagy pathway will
be important. Below, I will discuss how another type of cell death, programmed necrosis, may be reliant on autophagosomes as signaling scaffolds.

**How apoptosis regulates autophagy**

Apoptosis can directly influence autophagy. For example, Beclin 1 is a substrate for cleavage by caspase-3. Two caspase cleavage sites in Beclin 1 were discovered (Zhu et al., 2010), resulting in reduced affinity for Bcl-2, a reduction in autophagy, and an enhancement of apoptosis in HeLa cells. The cleavage of Beclin 1 may be a way to help the cell fully commit to apoptosis by putting the brake on autophagy induction, reducing the likelihood of cellular recovery. Other essential autophagy proteins are also targeted by caspases. The autophagosomal biogenesis regulatory protein ATG3 is a substrate for caspase-8 cleavage following the activation of extrinsic apoptosis (Oral et al., 2012). This cleavage site in ATG3 is conserved in humans, mice, rats, zebra fish, frogs, worms, and baker’s yeast suggesting a conserved regulatory role. Cleavage of ATG3 following a death receptor signal inactivated autophagy, but the expression of a non-cleavable ATG3 could re-establish autophagic activity. Further, the autophagy protein AMBRA1 (activating molecule in BECN1-regulated autophagy) is degraded by calpains and caspases, and expressing a cleavage-resistant mutant leads to apoptosis avoidance by prolonging autophagy induction (Gu et al., 2014).

BCL family proteins regulate autophagy initiation, as well as control the balance of pro- and anti-apoptotic signals. One of the best examples of this regulation is the ability of BH3 (BCL-2 homology 3)-only proteins to neutralize BCL-2 anti-apoptotic proteins, stimulate pro-apoptotic proteins, and displace protein interactions that put the brake on autophagy induction. The subcellular localization and phosphorylation status
of these BH3-only proteins dictate the direction toward survival or undergoing apoptosis, and the tendency to promote autophagy. BH3-only proteins, like PUMA, NOXA, NIX, BID, and BNIP3, disrupt inhibitory interactions between the BCL-2 proteins (Bcl-2, Bcl-X<sub>L</sub>, Mcl-1) and the autophagy regulator Beclin 1 (BECN1) (Pattingre et al., 2005). Relieving the inhibition of BECN1 by Bcl-2 or Bcl-X<sub>L</sub> displacement allows for the activation of a PI3 Kinase called vacuolar protein sorting 34 (VPS34), resulting in the nucleation of an isolation membrane. This regulation of Bcl-2 protein and subsequent displacement from BECN1 is also controlled by phosphorylation, e.g. by the stress-activated c-Jun N-terminal protein kinase 1 (JNK1) (Wei et al., 2008). Although understanding how apoptosis influences autophagy is important, the work presented in this Thesis mainly focuses on how autophagy regulates apoptosis.

**Autophagic cell death—autophagy as a direct cause of cell death**

The mechanisms described above involve autophagy interplay with apoptosis; however, autophagy may also regulate non-apoptotic cell death mechanisms where there is no need for caspase activation in order for the cell to die. For example, it was shown that acute expression of oncogenic H-RAS can cause caspase-independent death with characteristics of autophagy (Elgendy et al., 2011). This autophagic cell death was mediated by and dependent on Ras-induced expression of the pro-apoptotic protein, Noxa, and Beclin 1. These findings provided the first unequivocal genetic evidence for autophagy-dependent death in mammalian cells in response to a specific signaling event and although seen in an artificially controlled system, suggest that autophagic cell death may, under some circumstances, provide a mechanism to limit the oncogenic potential of dysregulated RAS signaling.
A requirement for functional autophagy for programmed cell death has been most clearly shown in vivo in developmental systems that are genetically tractable, such as Drosophila (Nelson and Baehrecke, 2014). For example, the first clear genetic evidence that autophagy is required for "physiological" cell death in vivo came from the demonstration that autophagy is induced just before salivary gland cell death, and salivary glands are not properly degraded in ATG mutants (Berry and Baehrecke, 2007). During salivary gland degradation, autophagic cell death is thought to take place alongside caspase-dependent apoptosis. Conversely, as mentioned above, during Drosophila oogenesis, autophagy controls cell death by promoting caspase activation and subsequent apoptosis (Nezis et al., 2010). In other contexts, developmental cell death in Drosophila can be shown to be autophagy-dependent but independent of caspases (Denton et al., 2009). These examples show that developmentally programmed cell deaths can involve autophagy working alongside apoptosis, autophagy controlling apoptosis and autophagy working on its own with no involvement of the apoptosis machinery.

Another example of a morphologically distinct form of autophagy-dependent cell death was identified called “autosis” (Liu et al., 2013), which was activated by an autophagy-inducing peptide and is modulated by widely used cardiac glycosides that target the Na⁺/K⁺-ATPase. This type of death may also occur in response to physiological signals because autosis was demonstrated to occur in a small population of cells (~1%) during nutrient starvation conditions and in vivo during hypoxic-ischemic injury in neonatal rats. The morphological features of autosis include nuclear convolution, increased autophagosomes, nuclear shrinkage, and focal perinuclear
swelling. Key experiments demonstrated that the inhibition of early autophagy abrogated Tat-Beclin 1-mediated cell death, and that this death does not require the canonical apoptosis or necroptotis machinery. Interestingly, blocking autophagosome/lysosomal fusion with a late-stage autophagy inhibitor, bafilomycin A1, did not reduce the Tat-Beclin 1 death, suggesting that only the early steps of autophagy are required for this cell death mode. These sorts of distinctions—death modalities requiring early or late stage autophagy machinery—require consideration when developing autophagy inhibitors for patient use. It should be noted that there have been no subsequent studies describing autosis as a mode of cell death; more studies to understand the mechanisms and biological relevance of autosis are needed.

**Necrosis and autophagy**

For many years, necrosis was regarded as an “accidental” process, but it is now understood that necrosis is often highly regulated and an intentional programmed mechanism. The most extensively studied form of programmed necrosis is called necroptosis, which is a form of programmed cell death dependent on receptor-interacting Ser/Thr protein kinase 1 (RIPK1), RIPK3 and the pseudokinase MLKL. Necroptosis is best characterized by the stimulation of the TNFR1 (Tumor Necrosis Factor Receptor 1) by TNF resulting in the formation of different signaling complexes that create a “switch” leading to cell survival, apoptosis or necroptosis. The ubiquitin-editing system and initiator caspases dictate the response to TNF ligand binding to TNFR. Complex I formation upon TNF binding consists of TNFR-associated death domain (TRADD), RIPK1, cellular inhibitor of apoptosis 1 (cIAP1), cIAP2, TNFR-associated factor 2 (TRAF2) and TRAF5, leading to cell survival. Conversely, the
internalization of the TNFR1 and deubiquitylation of RIPK1 by the deubiquitylating enzyme cylindromatosis results in the formation of complex II, promoting either apoptosis or necroptosis. This complex consists of RIPK1, RIPK3, TRADD, caspase-8, and FAS-associated protein with a death domain (FADD). One result of complex II formation is the proteolytic cleavage of RIPK1 and RIPK3 by caspase-8, generating a pro-apoptotic caspase activation cascade. However, when caspase-8 is deleted or inhibited, complex II cannot produce apoptotic signals. Instead, RIPK1 and RIPK3 phosphorylate each other, and aggregate in a complex called the necrosome. These autophosphorylation and transphosphorylation events recruit the mixed-lineage kinase domain-like (MLKL), which is itself phosphorylated by RIPK3. MLKL is then responsible for the permeabilization of the plasma membrane and death of the cell (Fuchs and Steller, 2015; Vandenabeele et al., 2010). A recent study found that the BH3-only pro-apoptotic protein PUMA is transcriptionally activated, mediated by autocrine TNFα and enhanced NF-κB activity, in a RIP3/MLKL-dependent manner upon necroptosis induction. Upon induction, PUMA mediates the cytosolic release of mitochondrial DNA and activation of the cytosolic sensors DAI/Zbp1 and STING, resulting in enhanced phosphorylation of RIP3 and MLKL and a positive feedback loop (Chen et al., 2018).

The molecular determinants of a cell’s commitment to necroptosis are still being uncovered. A surprising study recently found that cancer cells can be targeted by activating caspase-independent cells death (CICD) (Giampazolias et al., 2017). When caspases are disengaged, permeabilization of the mitochondria does not lead to apoptosis but leads to the transcriptional upregulation of TNFα by NF-κB allowing for CICD/necroptosis to be activated. Consistent with previous literature, induction of
necroptosis in this work was induced by the production of TNFα in a manner that was dependent on activation of the transcription factor NF-κB. NF-κB was activated through the release of mitochondrial proteins such as SMAC (which accompany cytochrome c on MOMP), leading to the degradation of inhibitor of apoptosis proteins (IAPs) by the proteasome. IAP inhibition is known to cause stabilization of another kinase called NF-κB-inducing kinase (NIK) and demonstrated that, when caspase activation is prevented, this mechanism occurs upon mitochondrial permeabilization leading to increased activity of NF-κB and subsequent increased expression of TNFα (Giampazolias et al., 2017). It is important to delineate whether cancer cells die by necroptosis or apoptosis, as the type of cell death specifies whether or not the immune system is activated. Apoptosis is generally thought to be non-inflammatory, whereas necrotic types of cell death activate inflammatory responses (Green et al., 2009). This study provides a glimpse into how activating a specific type of cell death may have different outcomes in cancer therapy and human disease generally.

Little is known about how autophagy is intertwined with necroptosis. However, some of the first evidence to show that autophagy could promote cell death came from a system that has gone on to become the best understood necroptosis pathway (Yu et al., 2004) and in this case, autophagy was shown to modulate these effects by selectively degrading the reactive oxygen species (ROS) scavenger enzyme, catalase (Yu et al., 2006). Bray et al. provided another example of the coordination between necroptosis and autophagy (Bray et al., 2012). They found that concurrent mTOR inhibition by CCI-779 and inhibiting autophagosome maturation with chloroquine lead to the accumulation of autophagosomes that induced RIPK3-dependent and ROS-
dependent necroptosis in renal cell carcinoma lines. Some evidence also implies that RIPK1 might be degraded by autophagy (Bray et al., 2012).

As with formation of an apoptosis-inducing scaffold described above, evidence points to autophagosomal membranes acting as platforms for necrosome assembly, and serving as key sites to mediate necroptosis. Obatoclax, or GX15-070, is an indole bipyrrrole compound that antagonizes Bcl-2, Bcl-XL, Bcl-w, and Mcl-1, and has been shown to activate autophagy and elicit non-apoptotic cell death in rhabdomyosarcoma cells. GX15-070-stimulated autophagy was linked to the assembly of the necrosome—i.e. a complex involving FADD, RIPK1, and RIPK3—on autophagosomal membranes. ATG5 or ATG7 silencing mitigated this cell death, and co-immunoprecipitation studies suggest that GX15-070 stimulates an interaction between ATG5 and necrosome components. Further, RIPK1 knockdown or pharmacological inhibition of RIPK1 with necrostatin-1 blocked death by GX15-070 (Basit et al., 2013). Together, these data point to the formation of autophagosomes as key mediators for achieving efficient necrosome formation, resulting in necroptotic cell death by GX15-070.

Studies from the Thorburn and Cramer laboratory (Goodall et al., 2016) show that mouse prostate cancer cells harboring a Map3k7 deletion and treated with TRAIL undergo necroptosis, not apoptosis, which is dependent on the ability of p62/SQSTM1 to mediate necrosome assembly by RIPK1 recruitment. In the case where the assembly of the necrosome by p62/SQSTM1 is blocked, these cells will instead undergo apoptosis (Goodall et al., 2016). This provides additional evidence that autophagy can act as a scaffold to control cell death in addition to acting to degrade cargo. One of the implications of this study is that blocking autophagy at early steps of the pathway (e.g.
the formation of the autophagosome) versus blocking autophagy at later stages (e.g.
the autophagosome-lysosome fusion step) can have differential effects on the cell death
mode (Goodall et al., 2016). Overall, the above examples support the notion that
autophagy can influence the fate of cells treated with compounds that induce
necroptosis. While these examples are suggestive of important interactions between
autophagy and the necroptosis machinery, more work is needed to uncover the
mechanistic ties and to work out how these processes are controlled
CHAPTER II

METHODS ²

Experimental model and subject details

Cell lines were maintained at 37°C and 5% CO₂ with media indicated in table below. The cell lines were authenticated at the Barbara Davis Center at University of Colorado—Anschutz Medical Campus. Six week old athymic nude mice were purchased and housed according to IACUC guidelines. See Table 2.1 for cell culture conditions.

Clonogenic assay

HCT116 cells were plated at 2000 cells per well of a 12-well plate. The next day, cells were treated with Nutlin-3a 20μM and/or chloroquine 40μM for 48 hours. These reagents were replenished after 24hrs. After 48 hours of exposure to the above reagents, full media was used to allow grow back for 7 days, replenishing media every 72 hours. Cells were then fixed and stained with crystal violet.

Chromatin immunoprecipitation

HCT116 cells were plated at 40,000 cells/cm² of a 150mm plate—40,000 X 152 cm²= 6,080,000 per plate (3 plates per condition). The next day, cells were treated with bafilomycin 10 nM, Nutlin-3a 20μM, or chloroquine 40μM for 24 hours. Cells were then washed with PBS, fixed with a 1% formaldehyde/1x PBS solution for 15 minutes at

² Portions of this chapter were published with permission from Developmental Cell: Autophagy Inhibition Mediates Apoptosis Sensitization in Cancer Therapy by Relieving FOXO3a Turnover. Fitzwalter et al., 2018, Developmental Cell 44, 555–565 March 12, 2018, Elsevier Inc. https://doi.org/10.1016/j.devcel.2018.02.014
room temperature. The fixing reaction was stopped by the addition of 1 mL of 2.5 M glycine (0.125 M final). Cells were then washed 2X with cold PBS and harvested with RIPA buffer (150 mM NaCl, 1% v/v, Nonidet P-40, 0.5% w/v, deoxycholate, 0.1% w/v, SDS, 50mM EDTA, protease inhibitor cocktail, phosphatase inhibitor). Samples were then sonicated to generate <500 base pair DNA fragments. One mL of 1 mg/mL of protein lysate was precleared for 2 hours with 30μL of Protein A/G beads. FOXO3a CST 75D8 (10 μL per IP) and RNA Polymerase II Clone H5 (20μL per IP) were used with 60μL of Protein A/G beads, and inverted overnight at 4°C. Beads were pre-washed twice with PBS, then twice with RIPA buffer from above. For phosphorylated CTD of polymerase, immunocomplexes were recovered using anti-mouse IgM/protein A/G beads. Beads were washed twice with RIPA buffer, 4 times with IP buffer (100mM Tris HCl pH 8.5, 500mM LiCl, 1% v/v, Nonidet P-40, 1% w/v, deoxycholic acid), twice again with RIPA buffer, then twice with TE. Immunocomplexes were eluted at 65°C for 10 minutes with 1% SDS. Reverse crosslinking was performed by adjusting to 200mM NaCl and incubated for 5hrs at 65°C. DNA was purified and then used in QPCR reaction. PCR primers used in these reactions are listed in the attached table.

**RT-QPCR**

RNA was isolated using Qiagen RNeasy kit following the manufacturer’s instructions. Reverse transcription reaction was performed using the Qiagen Quantitect RT kit according to manufacturer’s instructions. SYBR green CFX from Applied Biosystems was used in QPCR reactions with a standard curve ranging from 0.04 ng to 50ng of cDNA. Quantities were calculated relative to this standard curve, and
normalized to 18s rRNA or GAPDH as housekeep gene control. Primers are listed in Table 2.2.

**Expression constructs, shRNAs, and transduction**

Protein depletion by shRNA was achieved by using the pLKO.1 system. These plasmids were obtained from the University of Colorado Functional Genomics Core. Lentiviruses were obtained by cotransfection of pLKO.1 shRNA plasmids with the lentiviral plasmids pMD2G, pRRE, and pRSV into HEK293FT using Mirus Transit LT1 transfection reagent. Media was harvested at 48 and 72 hours post-transfection and stored at -80°C. Cells were plated at 30,000 cells per well of a 6-well plate and 1mL of virus used. Puromycin was used for selection (1 μg/mL). Sequences are listed in Table 2.2.

**CRISPR/Cas9-mediated knock out**

For CRISPR/Cas9 knockout of ATG5 and ATG7, lentiviral constructs containing gRNAs targeting ATG5 and ATG7 (Table S1) were transduced into HCT116 cells as previously published (O’Prey et al., 2017). To avoid complications caused by clonal variation, ATG5 or ATG7 knockout cells from polyclonal populations were assayed by Western Blot for complete loss of protein expression and the polyclonal population used for functional tests. For CRISPR/Cas9 mediated knockout of the forkhead response element (FHRE), ribonuclear protein complexes (RNPs) were transfected into HCT116 cells with Lipofectamine CRISPR Max based on previously published methods (Liang et al., 2015). Single cell clones were isolated and genomic DNA analyzed to determine whether the FHRE was mutated, then to minimize clonal variation, clones with 6
different mutated FHRE sequences were pooled and used for functional tests. Sequences of gRNAs are listed in Table 2.2.

**Immunoblots**

Western blotting was performed using standard methods with antibodies in 5% milk or 5% bovine serum albumin fraction V in TBST (0.1% Tween-20). Proteins were separated on SDS-PAGE 1.0mm mini gels and transferred to nitrocellulose or PVDF membranes. Blots were then probed with antibodies diluted as above. Semi-dry transfer apparatus was run at 15V for 70 minutes. Antibodies are listed in Table 2.3.

**Fluorescence microscopy**

Lentiviral plasmid pBABE puro mCherry-eGFP-HA-FOXO3a was cloned by digesting pBABE puro mCherry-eGFP-LC3 with MfeI and Sall, and ligating FOXO3a cDNA generated from AddGene plasmid #1787 (HA-FOXO3a). Sequences are listed in Table 2.2. Lentiviral transduction of this construct into MCF7, MCF10a, and HCT116 was performed, expressed for 72hrs to reach steady-state, and imaged. Live cells were plated at 2,000 cells and imaged in MatTek 35mm glass bottom culture dishes using a confocal laser scanning Olympus FV1000 with a 60X objective. LysoTracker was used at concentration of 0.5μM.

**Incucyte cell imaging**

Cells were plated in 96-well plates at 3,000 cells/well. After cell attachment (48hrs), cells were treated with indicated concentrations of Nutlin-3a, chloroquine, bafilomycin A1, or staurosporine. CellEvent Green Caspase-3/7 reagent was from Invitrogen C10423 (used at 5μM final concentration). Sytox Green (Invitrogen S7020) cell dye reagent was used at 50nM. Images were taken using the Incucyte Zoom
system every 4hrs using a 4X objective. Results are displayed normalized to the cell number and the initial time point (time point zero).

**In vivo tumor studies**

Six week old athymic nude mice were purchased and housed according to IACUC guidelines. Two million HCT116 parental or ΔFHRE cells were counted using the Vi-cell (Beckman Coulter) resuspended, on ice, in 100μL of 0.2% matrigel:PBS (1:1) and injected into each flank using 28g needles. Mice were anesthetized using isoflurane. Dosing of Nutlin-3a and chloroquine began when 80% of the mice had tumors that were ≥100mm³. Mice were given oral gavage (28g needles, 38mm long) twice daily of Nutlin-3a (Selleckchem—S1061) at 200 mg/kg—a 20 mg/mL stock was formulated in 2% Klucel (Hydroxypropyl cellulose—AlfaAesar) and 0.5% Tween 80 (Fischer BP338-500) in PBS. Mice were also given intraperitoneal injections once daily of chloroquine diphosphate (MPBio—193919) at 60 mg/kg—a 6 mg/mL stock was formulated in PBS. Tumor volume was measured and calculated regularly (roughly every other day) using a Sylvac-Fowler Bluetooth S_cal EVO caliper. Mice were sacrificed when tumor volume reached 200mm³, or a combined tumor burden per mouse of 300mm³. Mice were sacrificed by CO₂ followed by cervical dislocation according to IACUC protocol and tumors were harvested for RNA and protein. Tumor samples were homogenized and processed for RNA using above methods for RT-QPCR analysis.

**Optogenetic autophagy manipulation**

Experiments were performed with a LofTek ultraviolet 395nm LED handheld flashlight mounted in an aluminum foil-lined cardboard box. Cells were exposed to the
LED for 5 or 10 minutes with continuous light, and all experiments were controlled to account for any light-associated cell toxicity (all experiments were normalized to toxicity associated with parental cells not expressing the ASAP construct, or to an ASAP control plasmid—these control conditions were exposed to the same amount of light as the experimental condition). Cells were centered within the LED box to allow for direct incidence with the LED; each plate was photoactivated separately to ensure equal and proper LED exposure. Following LED exposure, cells were immediately returned to the cell culture incubator and incubated for indicated durations, followed by collection with stringent RIPA buffer (150 mM NaCl, 1% v/v, Nonidet P-40, 0.5% w/v, deoxycholate, 0.1% w/v, SDS, 50mM EDTA, protease inhibitor cocktail, phosphatase inhibitor).

**Quantification and statistical analysis**

Data are presented as means ± standard errors of the mean (SEMs) as indicated within each figure legend. Independently prepared samples from at least two experiments, displayed as circles, squares or triangles, are displayed on column graphs. One-way analyses of variance (ANOVA), two-way analyses of variance, or unpaired Student’s t-tests were performed where indicated in figure legends using Prism/Graphpad. *P < 0.05; **P < 0.01; ***P < 0.001.
Table 2.1

Cell culture conditions—All cell lines authenticated via University of Colorado—

Anschutz Medical Campus Barbara Davis Center

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<th>Line</th>
<th>Medium</th>
<th>Serum</th>
<th>Additives</th>
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<tr>
<td>MCF7</td>
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<td>10% FBS</td>
<td>L-glutamine, insulin, Pen/Strep</td>
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## Table 2.2

**ChIP-QPCR, RT-QPCR, QPCR primers, gRNAs, and shRNAs**

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<td>BBC3 +9,137 RV</td>
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Table 2.2 Continued.

**ChIP-QPCR, RT-QPCR, QPCR primers, gRNAs, and shRNAs**

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**Key Resources Table**

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CHAPTER III

AUTOPHAGY INHIBITION INCREASES PUMA TRANSCRIPTION VIA FOXO3A

Introduction

Autophagy is the mechanism by which cellular material is delivered to lysosomes via double membrane vesicles called autophagosomes. Although autophagy’s ability to protect against apoptosis is well established, the molecular machinery that links autophagy and apoptosis to govern cell-fate decisions is poorly understood (Fitzwalter and Thorburn, 2015a).

The Thorburn laboratory previously reported (Thorburn et al., 2014) that the pro-apoptotic protein PUMA (p53-upregulated modulator of apoptosis, also known as BBC3) is increased upon autophagy inhibition. This increase is not sufficient to cause cells to die on their own but can sensitize them to an apoptosis inducer. Here, I investigated the underlying mechanism by which this occurs revealing a transcriptional feedback loop that links basal autophagy homeostasis to apoptosis sensitivity. I found that the ability of the Forkhead Box transcription factor FOXO3a to bind to the PUMA gene is necessary to upregulate PUMA levels upon genetic or pharmacological autophagy inhibition.

3 Portions of this chapter were published with permission from Developmental Cell: Autophagy Inhibition Mediates Apoptosis Sensitization in Cancer Therapy by Relieving FOXO3a Turnover. Fitzwalter et al., 2018, Developmental Cell 44, 555–565 March 12, 2018, Elsevier Inc. https://doi.org/10.1016/j.devcel.2018.02.014

4 Portions of this chapter were published with permission from Autophagy: FOXO3 links autophagy to apoptosis, Autophagy, 14:8, 1467-1468, DOI: 10.1080/15548627.2018.1475819
Brent E. Fitzwalter & Andrew Thorburn
Further, I show that FOXO3a is selectively regulated by autophagic turnover to regulate its transcriptional activity.

Results

Previous work demonstrated that shRNA knockdown of essential autophagy regulators or pharmacological inhibition of autophagy caused higher PUMA protein levels (Thorburn et al., 2014). A simple explanation for this effect would be that PUMA protein is degraded by autophagy. However, inhibition of autophagy by knockdown of multiple autophagy regulators including, ATG7, PIK3C3/Vps34, and ULK1 (Unc-51-like Autophagy Activating Kinase 1) caused increased PUMA mRNA levels in HCT116 colorectal cancer cells (Figures 3.1a-c) as well as other cancer cell lines including HeLa and MCF7 (Figures 3.1d-e). An increase in PUMA mRNA levels was also observed when two essential autophagy regulators ATG7 or ATG5 were knocked out using CRISPR/Cas9 (Figure 3.2a). Treatment with bafilomycin A1, an inhibitor of vacuolar-type H+-ATPase that blocks autophagy by preventing lysosomal acidification also caused increases in PUMA mRNA (Figure 3.2b), and this was abolished following drug washout (Figure 3.2c). PUMA is a well-known target gene for p53 however, basal autophagy inhibition in HCT116 cells that lack p53 displayed similar increases in PUMA mRNA compared to HCT116 wild-type cells (Figure 3.2d). To test if autophagy inhibition causes increased PUMA gene transcription, chromatin immunoprecipitation (ChIP) was performed at the PUMA locus using an antibody that recognizes elongating RNA Polymerase II (Komarnitsky et al., 2000). Autophagy inhibition with bafilomycin A1 (Figure 3.3a) or chloroquine (Figure 3.3b) caused enrichment of active RNA Pol II occupancy at the PUMA locus that was comparable to other stimuli that activate PUMA
Figure 3.1
Autophagy inhibition increases PUMA mRNA levels in multiple cancer cell lines
Figure 3.1
Autophagy inhibition increases PUMA mRNA levels in multiple cancer cell lines (a-e) HCT116, HeLa, or MCF7 cells were transduced with lentiviral shRNAs targeting autophagy regulators (shATG7, shVps34, and shULK1) or shCtrl, and the resulting BBC3 (PUMA) mRNA levels were measured relative to 18S rRNA control
Figure 3.2
Autophagy gene knockout or pharmacological autophagy inhibition increases PUMA mRNA levels, independent of p53.
(a) HCT116 cells were transduced with a lentiviral CRISPR/Cas9 plasmid targeting ATG5, ATG7, or a non-targeting control, and BBC3 (PUMA) mRNA levels were measured relative to 18S rRNA control.
(b) HCT116 cells were treated with vehicle or bafilomycin A1 10 nM, an autophagy inhibitor, for 24 hr and BBC3 (PUMA) mRNA levels were measured relative to 18S rRNA control.
(c) HCT116 cells treated with bafilomycin 10 nM for 24 hr, then washed out for 16 and 24 hr. BBC3 (PUMA) mRNA levels were measured relative to 18S rRNA control.
(d) HCT116 cells or HCT116 p53 -/- cells were treated with vehicle or bafilomycin A1 10 nM, an autophagy inhibitor, for 24 hr, and BBC3 (PUMA) mRNA levels were measured relative to 18S rRNA control.
Figure 3.3
Autophagy inhibition causes increased PUMA transcription similar to other stimuli that active PUMA.
(a) Chromatin immunoprecipitation (ChIP) analysis in HCT116 cells of RNA polymerase II phosphoserine 2 (indicative of active, elongating RNA Pol II) occupancy at the BBC3/PUMA genomic locus relative to IgM control with vehicle or bafilomycin A1 10 nM treatment, an autophagy inhibitor, for 24 hr.
(b) Chromatin immunoprecipitation analysis of RNA polymerase II phospho serine 2 (indicative of active, elongating RNA pol II) occupancy in HCT116 cells at the BBC3 (PUMA) genomic locus relative to IgM control with vehicle or chloroquine 40μM treatment for 24hrs.
(c) Chromatin immunoprecipitation analysis in HCT116 cells of RNA polymerase II phospho serine 2 (indicative of active, elongating RNA pol II) occupancy at the BBC3 genomic locus relative to IgM control with vehicle or Nutlin-3a 20μM treatment for 24hrs.
transcription (Figure 3.3c). Taken together, these data indicate that basal autophagy inhibition leads to increased PUMA mRNA transcription in a p53-independent manner.

The basal rate of autophagy is regulated by several transcription factors (Feng et al., 2015; Füllgrabe et al., 2014) including FOXO3a, which is critical for maintenance of a gene expression program that allows autophagy to protect hematopoietic stem cells from cytokine deprivation (Warr et al., 2013b). Additionally, FOXO3a can regulate PUMA expression (Amente et al., 2011; You et al., 2006) leading us to hypothesize that FOXO3a might be responsible for increased PUMA expression upon autophagy inhibition. In support of this idea, bafilomycin A1 failed to increase PUMA mRNA when FOXO3a was knocked down via shRNA (Figure 3.4a) or knocked out with CRISPR/Cas9 (Figures 3.4b). ChIP analysis revealed that FOXO3a displays a constitutive level of occupancy at the PUMA locus in an intronic region containing a Forkhead Response Element (FHRE) +1,902 basepairs downstream of the transcriptional start site as was previously reported (Eijkelenboom et al., 2013). Upon basal autophagy inhibition with bafilomycin A1, FOXO3a became enriched approximately 20-fold over IgG control at this site (Figures 3.4c). Mutation of the FOXO3a-binding FHRE in the PUMA locus using CRISPR/Cas9 gene editing to create a set of three pooled HCT116 clones each with deletions in the FHRE (Figure 3.4d) was sufficient to block the increase in PUMA expression upon pharmacological (Figure 3.4e). These data indicate that inhibition of basal autophagy results in increased FOXO3a-driven transcription of the PUMA gene through a single transcription factor binding site in the genome.
Figure 3.4
FOXO3a binding to the BBC3/PUMA locus is necessary for PUMA transcriptional activation.
Figure 3.4
FOXO3a binding to the BBC3/PUMA locus is necessary for PUMA transcriptional activation.

(a) Left: HCT116 p53-/- cells were transduced with two different lentiviral shRNAs targeting FOXO3a transcripts. After 24 hr of vehicle or bafilomycin A1 10 nM treatment, PUMA mRNA levels were measured relative to 18S rRNA control. Right: western blot showing FOXO3a protein levels after treatment with two independent shRNAs targeting FOXO3a or shCtrl.

(b) Parental or FOXO3a-/- pooled HCT116 cells were treated with bafilomycin A1 10 nM, an autophagy inhibitor, for 24 hr, and BBC3 (PUMA) mRNA levels were measured relative to GAPDH control.

(c) ChIP analysis in HCT116 cells of FOXO3a occupancy at the BBC3/PUMA genomic locus relative to IgG control or treatment with vehicle or 10 nM bafilomycin A1, an autophagy inhibitor, for 24 hr. The x axis indicates the probed position along the BBC3/PUMA locus relative to the transcriptional start site. The BBC3/ PUMA locus schematic is not to scale; stars indicate the approximate position of each amplicon.

(d) Sequencing alignments showing ΔForkhead Response Element (ΔFHRE) deletions in HCT116 cells at the BBC3/PUMA locus at position +1,902 relative to the transcriptional start site using CRISPR/Cas9 targeting. Blue and green base pairs indicate FHRE. Multiple clones were obtained with homozygous indels and were pooled to limit clonal variation.

(e) HCT116 parental or ΔFHRE cells were treated for 24 hr with vehicle or 10 nM bafilomycin A1, an autophagy inhibitor, and BBC3 (PUMA) mRNA levels were measured relative to 18S rRNA control.
Multiple FOXO3a-/- clones were obtained by CRISPR/Cas9-mediated knockout (Figure 3.5a). FOXO3a occupancy at the PUMA locus had a basal level of approximately 10-fold over an IgG control, but increased to 15-fold upon treatment with chloroquine (Figure 3.5b). HCT116 cells lacking a FHRE were unable to upregulate PUMA mRNA levels upon genetic knockdown of ATG7 (Figure 3.5c), but were still able to upregulate PUMA upon treatment with Nutlin-3a (Figure 3.5d). FOXO3a had a constitutive level of occupancy at the BIM locus that increased nearly two-fold with bafilomycin A1 treatment for 24hrs (Figure 3.5e), but FOXO3a occupancy at non-apoptotic proteins like SOD2 did not change with autophagy inhibition at that time point (Figure 3.5f). Pharmacological or genetic autophagy inhibition by CRISPR/Cas9 knockout of essential autophagy genes resulted in higher BIM mRNA levels (Figures 3.5g-h). Several other known FOXO3a target genes including non-apoptotic genes were also activated (albeit with varying kinetics) upon bafilomycin A1 treatment (Figure 3.5i and Figures 3.6a-d). These data suggest that FOXO3a increases its transcriptional activity at multiple gene targets upon genetic or pharmacological autophagy inhibition.

FOXO3a is degraded by the proteasome in response to various signaling events (Huang and Tindall, 2011) but it is not known if autophagy also regulates FOXO3a. To test this and determine if autophagy-mediated turnover of FOXO3a explains how autophagy inhibition can enhance FOXO3a transcriptional activity, HCT116 cells were treated with bafilomycin A1 (Figures 3.7a), resulting in a time-dependent increase in the amount of FOXO3a protein indicating that basal FOXO3a turnover can occur via the lysosome. Consistent with this turnover being due to (macro)autophagy, HCT116 cells that lack the essential autophagy genes ATG5 or ATG7 by CRISPR/Cas9-mediated
Figure 3.5
FOXO3a and the Forkhead Response Element are necessary for PUMA upregulation following autophagy inhibition, and FOXO3a binds to other FOXO3a targets upon autophagy inhibition.
Figure 3.5
FOXO3a and the Forkhead Response Element are necessary for PUMA upregulation following autophagy inhibition, and FOXO3a binds to other FOXO3a targets upon autophagy inhibition.

(a) Western blot analysis of FOXO3a protein levels in HCT116 clones targeted with CRISPR/Cas9 using recombinant Cas9 and gRNAs. These clones were pooled to limit monoclonal variation.

(b) Chromatin immunoprecipitation analysis in HCT116 cells of FOXO3a occupancy at the BBC3 (PUMA) genomic locus relative to IgG control with vehicle or chloroquine 40μM for 24hrs.

(c) Parental or ∆FHRE HCT116 cells were transduced with lentiviral shCtrl or shATG7 and PUMA mRNA levels were measured relative to an 18s rRNA control.

(d) HCT116 ∆FHRE cells that lack the ability to bind FOXO3a in the BBC3 (PUMA) locus were treated with Nutlin-3a 20μM for 24hrs and PUMA mRNA levels were measured relative to 18S rRNA control.

(e) Chromatin immunoprecipitation analysis in HCT116 cells of FOXO3a occupancy at the BIM genomic locus relative to IgG control with vehicle or bafilomycin A1 10nM for 24hrs.

(f) Chromatin immunoprecipitation analysis in HCT116 cells of FOXO3a occupancy at the SOD2 genomic locus relative to IgG control with vehicle or bafilomycin A1 10nM for 24hrs.

(g) HCT116 cells treated with bafilomycin A1 10nM for indicated duration and BIM mRNA levels were measured relative to 18S rRNA control.

(h) HCT116 cells were transduced with a lentiviral CRISPR/Cas9 plasmid targeting ATG5, ATG7, or a non-targeting control and BIM mRNA levels were measured relative to 18s rRNA control.

(i) HCT116 cells treated with bafilomycin A1 10nM for indicated duration and SOD2 mRNA levels were measured relative to 18S rRNA loading control.
Figure 3.6
Other FOXO3a targets are upregulated upon pharmacological autophagy inhibition

(a) HCT116 cells treated with bafilomycin A1 10nM for indicated duration and SESN1 mRNA levels were measured relative to 18S rRNA loading control.
(b) HCT116 cells treated with bafilomycin A1 10nM for indicated duration and Prkaa2 mRNA levels were measured relative to 18S rRNA loading control.
(c) HCT116 cells treated with bafilomycin A1 10nM for indicated duration and BNIP3 mRNA levels were measured relative to 18S rRNA loading control.
(d) HCT116 cells treated with bafilomycin A1 10nM for indicated duration and BNIP3L mRNA levels were measured relative to 18S rRNA loading control.
Figure 3.7
FOXO3a is a substrate for basal autophagy
Figure 3.7
FOXO3a is a substrate for basal autophagy
(a) HCT116 cells treated with 10 nM bafilomycin A1, an autophagy inhibitor, for the indicated duration. Whole-cell lysates were probed for FOXO3a protein levels.
(b) HCT116 cells were transduced with lentiviral constructs containing Cas9 and gRNA targeting ATG5, ATG7, or a non-targeting control, and whole-cell lysates were probed for indicated proteins.
(c) HCT116 cells were transduced with lentiviral shRNAs targeting p62/SQSTM1 or shCtrl and probed for indicated proteins.
(d) Western blot analysis on whole-cell lysates of HCT116 cells expressing mCherry-GFP-FOXO3a treated with 10 nM bafilomycin A1, an autophagy inhibitor, for 8 hr.
(e) Western blot analysis of whole-cell lysates of HCT116 cells expressing mCherry-GFP-FOXO3a transduced with shCtrl or shp62/SQSTM1 lentivirus.
(f) Schematic representation of expression construct and how it was used to understand the environment of FOXO3a localization.
(g) Confocal images of HCT116 cells expressing mCherry-GFP-FOXO3a and treated with a lysosomal dye. White arrows indicate regions of interest where FOXO3a resides in an acidic compartment.
(h) Confocal images of HCT116 cells expressing mCherry-GFP-FOXO3a transduced with shRNA targeting ATG5.
(i) Confocal images of HCT116 cells expressing mCherry-GFP-FOXO3a transduced with shRNA targeting ATG7.
(j) Confocal images of HCT116 cells expressing mCherry-GFP-FOXO3a treated with 10 nM bafilomycin A1, an autophagy inhibitor, for 8 hr.
Scale bars, 10 µm.
knockout showed a robust increase in FOXO3a protein compared to control cells (Figure 3.7b). Additionally, shRNA knockdown targeting p62/SQSTM1, a protein that recruits substrates to the autophagosome, also increased FOXO3a protein HCT116 cells (Figures 3.7c). Exogenously expressed FOXO3a protein also increased when cells were treated with bafilomycin A1 (Figure 3.7d) or p62/SQSTM1 shRNA (Figure 3.7e).

If FOXO3a is a substrate for autophagy, it should localize to autophagosomes and lysosomes. To test this, I expressed a construct expressing mCherry-eGFP-FOXO3a (Figure 3.7f). eGFP fluorescence, but not mCherry, is sensitive to decreased pH and this provides a basis to monitor autophagosome and autolysosome targeting of autophagy markers such as LC3B (Gump et al., 2014; Kimura et al., 2007) and autophagy cargos (Dou et al., 2015). If FOXO3a is in a pH-neutral environment, such as the cytoplasm or nucleus, both eGFP and mCherry should fluoresce resulting in a diffuse yellow signal from the overlayed mCherry and eGFP. If FOXO3a is concentrated into a small pH neutral environment, like an autophagosome, I expect a punctate yellow signal and, if FOXO3a resides in an acidic environment, such as an autolysosome, I should see punctate red signals from the overlayed mCherry and the quenched eGFP fluorescent signals (Figure 3.7f). HCT116 cells expressing the mCherry-eGFP-FOXO3a protein under basal conditions showed a significant amount of FOXO3a localized to acidic compartments that were also positively stained by a lysosomal marker (Figures 3.7g). Upon shRNA knockdown of ATG5 or ATG7 (Figures 3.7h-i) or blocking lysosomal acidification with bafilomycin A1 (Figure 3.7j), the predominance of punctate red signal was reverted to yellow and FOXO3a accumulated in the nucleus consistent with the
data above showing that FOXO3a binds to and transactivates the PUMA locus and causes new transcription upon autophagy inhibition.

The above results were also observed in other cell lines that are tumorigenic and non-tumorigenic. MCF7 cells treated with bafilomycin A1 (Figure 3.8a) or chloroquine (Figure 3.8b-c) showed an increase in FOXO3a protein levels. FOXO3a mRNA levels did not change upon genetic deletion of the autophagy genes ATG5 or ATG7 (Figure 3.8d). ATG7 shRNA knockdown also caused a robust increase in FOXO3a protein compared to shCtrl cells (Figure 3.8e) and with p62/SQSTM1 shRNA knockdown (Figure 3.8f-g), without increased FOXO3a mRNA (Figure 3.8h). Importantly, shRNA targeting of another autophagy receptor that recruits cargo to the autophagosome, NBR1, did not cause an increase in FOXO3a protein (Figure 3.8i).

Using the construct described above, I determined that FOXO3a is a substrate for basal autophagy in multiple cells lines, including MCF7 (Figure 3.9a) and MCF10a (Figure 3.9b), and quantified the amount of lysosomal-targeted FOXO3a puncta (Figures 3.9c-d).

These data indicate that FOXO3a is targeted to autolysosomes allowing FOXO3a levels to be controlled by basal autophagy in addition to the proteasome as previously identified (Huang and Tindall, 2011). These data support a model whereby a transcription factor that controls autophagy is itself degraded by autophagy and, when this is blocked, the elevated FOXO3a causes an increase PUMA transcription.
Figure 3.8
Autophagy regulates FOXO3a protein levels, but not mRNA levels in different tumor cells
Figure 3.8
Autophagy regulates FOXO3a protein levels, but not mRNA levels in different tumor cells
(a) MCF7 cells were treated with vehicle or bafilomycin A1 10nM and probed for FOXO3a protein levels.
(b) MCF7 cells were treated with vehicle or chloroquine 40μM and probed for FOXO3a protein levels.
(c) MCF7 or MCF10a cells were treated with vehicle or chloroquine 40μM for 2 or 4 hours and probed for FOXO3a protein levels.
(d) HCT116 cells transduced with lentiviral constructs containing Cas9 and gRNA targeting ATG5, ATG7, or a non-targeting control and FOXO3a mRNA levels were measured relative to 18s rRNA control.
(e) MCF7 cells were transduced with lentiviral shRNAs targeting autophagy transcript shATG7 or shCtrl scrambles. FOXO3a protein levels are shown in the presence or absence of ATG7 shRNA knockdown.
(f) MCF7 cells were transduced with lentiviral shRNAs targeting autophagy transcript shp62/SQSTM1 or shCtrl scrambles. FOXO3a protein levels are shown in the presence or absence of p62/SQSTM1 shRNA knockdown.
(g) MCF10a cells were transduced with lentiviral shRNAs targeting autophagy transcript shp62/SQSTM1 or shCtrl scrambles. FOXO3a protein levels are shown in the presence or absence of shp62/SQSTM1 shRNA knockdown.
(h) HCT116 cells were transduced with lentiviral shRNAs targeting p62/SQSTM1 or shCtrl, and FOXO3a mRNA levels were measured relative to 18s rRNA control.
(i) HCT116 cells were transduced with lentiviral shRNAs targeting NBR1 or shCtrl scramble. FOXO3a protein levels are shown in the presence or absence of NBR1 shRNA knockdown.
Figure 3.9
FOXO3a is substrate for basal autophagy in both cancer cells and non-tumorigenic cells
**Figure 3.9**  
FOXO3a is substrate for basal autophagy in both cancer cells and non-tumorigenic cells  
(a) Confocal images of MCF7 cells expressing mCherry-GFP-FOXO3a treated with a lysosomal dye.  
(b) Confocal images of MCF10a cells expressing mCherry-GFP-FOXO3a treated with a lysosomal dye.  
(c) Quantitation of lysosomal FOXO3a puncta per cell in MCF7 cells.  
(d) Quantitation of lysosomal FOXO3a puncta per cell in MCF10a cells.  
Scale bars= $20\mu m$. 
Discussion

Upon genetic or pharmacological autophagy inhibition, the levels of the pro-apoptotic protein PUMA increase. Surprisingly, the obvious mechanism for such an effect – i.e., PUMA protein is degraded by selective autophagy – does not apply. Instead, PUMA is actively transcribed upon autophagy inhibition by the Forkhead Box transcription factor FOXO3a, which upregulates the BBC3 gene via a single forkhead response element (FHRE) in an intron. CRISPR/Cas9-mediated deletion of this FHRE in the endogenous BBC3 locus is sufficient to block PUMA upregulation upon autophagy inhibition. As I will show in the next Chapter, mutating a single transcription factor binding site in the intron of the PUMA gene is able to completely reverse the apoptosis sensitization conferred by inhibiting autophagy.

FOXO3a poises cells for rapid induction of autophagy following cytokine deprivation (Warr et al., 2013a). And, like some other transcription factors, it does so by transactivating autophagy-related genes. I wondered how the FOXO3a transcription factor is regulated so as to increase its activity upon autophagy inhibition. I found that FOXO3a is itself a substrate for basal autophagic degradation, and when this is blocked by either pharmacological autophagy inhibitors or by inactivation of autophagy regulators such as ATG5 or ATG7, then FOXO3a translocates to the nucleus where it can activate target genes. This finding adds another interesting feature to the mechanism I uncovered; FOXO3a – a transcription factor that regulates autophagy – is itself regulated by autophagic degradation. This suggests that FOXO3a is at the center of a homeostatic feedback loop to correct autophagy perturbations by transactivating autophagy targets when basal autophagy is inhibited. However, if autophagy inhibition
persists, then the cell also activates pro-apoptotic genes such as PUMA via the same transcription factor and thus becomes sensitized to undergo cell death. I therefore propose that basal autophagic turnover of FOXO3a serves as a surveillance mechanism to detect and correct autophagy flux disruptions. As I will show in the next Chapter, this mechanism is important ensure that those cells where autophagy is deficient, are primed to undergo apoptosis.

Acknowledgments

I would also like to acknowledge Christina G. Towers for making the ∆FHRE cell lines in this Chapter. I would also like to thank Zdenek Andrysik for his instrumental help with the chromatin immunoprecipitation studies in this Chapter. Lastly, I would also like to thank Michael J. Morgan for his help in experimental design and troubleshooting.
CHAPTER IV

AUTOPHAGY REGULATION OF PUMA EXPRESSION VIA FOXO3A CAN BE LEVERAGED TO IMPROVE ANTI-CANCER DRUGS

Introduction

Understanding the mechanistic links between autophagy and apoptosis will aid in future therapies aimed at influencing cell fate. There are currently dozens of clinical trials using autophagy inhibitors such as chloroquine or hydroxychloroquine, but there is little mechanistic insight into how autophagy inhibition kills more cancer cells when combined with chemotherapeutic agents or targeted therapies (Levy et al., 2017b; Towers and Thorburn, 2016). Here, I expand on the mechanism described in Chapter III to explain why autophagy inhibition can increase the effectiveness of anti-cancer drugs, such as doxorubicin and etoposide, by causing more apoptosis. Moreover, this mechanism also allows autophagy inhibitors to change the mode of action of an MDM2 inhibitor from growth arrest to apoptosis, even though MDM2 inhibitors alone are often known to merely cause growth inhibition and fail to induce tumor cell apoptosis (Burgess et al., 2016). Thus, by capitalizing on a mechanism that connects autophagy

5 Portions of this chapter were published with permission from Developmental Cell: Autophagy Inhibition Mediates Apoptosis Sensitization in Cancer Therapy by Relieving FOXO3a Turnover. Fitzwalter et al., 2018, Developmental Cell 44, 555–565 March 12, 2018, Elsevier Inc. https://doi.org/10.1016/j.devcel.2018.02.014

6 Portions of this chapter were published with permission from Oncotarget: Autophagy inhibition improves anti-cancer drugs via FOXO3a activation. Brent E. Fitzwalter & Andrew Thorburn, 2018, Oncotarget, Vol. 9, (No. 39), pp: 25384-25385.
to apoptosis, it is feasible to improve and even change the mode of action of an anti-cancer drug.

**Results**

I tested if the FOXO3a mediated PUMA activation mechanism described in Chapter III is important for sensitizing cells to anti-cancer drugs upon autophagy inhibition. Consistent with previous studies using anti-cancer drugs, etoposide (Figure 4.1a-b) or doxorubicin (Figure 4.1c-d) caused greater caspase 3/7 activity when combined with autophagy inhibition by chloroquine compared to the individual agents. However, this ability of autophagy inhibition to sensitize parental HCT116 tumor cells to drug-induced apoptosis was abolished in isogenic cells that either lack PUMA (PUMA-/-) or merely lack the ability to bind FOXO3a at the single site in the PUMA locus (ΔFHRE) (Figures 4.2a-d). These data suggest that the ability of autophagy inhibitors like chloroquine to sensitize tumor cells to apoptosis by standard chemotherapy is via the FOXO3a-dependent mechanism described above. Moreover, although autophagy inhibition can lead to increased transcription of other FOXO3a target genes (Figure 3.6a-d), including other pro-apoptotic genes including BIM (Figures 3.5g and 3.5h), BNIP3 (Figure 3.6c), and BNIP3L (Figure 3.6d) the increased drug-induced apoptosis observed when autophagy is blocked is mediated largely through a single binding site in the PUMA gene.

I next asked if this idea could be taken a step further and change the mode of action of a drug. Nutlin is an inhibitor of MDM2 that activates the p53 transcriptional program including PUMA but only results in growth inhibition rather than tumor cell death in most cancer cell types.
**Figure 4.1**
The combination of autophagy inhibition and anti-cancer agents induces more cancer cell apoptosis
(a) HCT116 cells were treated with vehicle, chloroquine 40 µM, etoposide 2.5 µM, or etoposide + chloroquine. Caspase-3/7 activation (an indicator of apoptosis execution) was measured and normalized to cell number per mm².
(b) Representative images of caspase-3/7 activation from (a) treated with vehicle, chloroquine 40 µM, etoposide 2.5 µM, or etoposide + chloroquine.
(c) HCT116 cells were treated with vehicle, chloroquine 40 µM, doxorubicin 2.5 µM, or doxorubicin + chloroquine. Caspase-3/7 activation (an indicator of apoptosis execution) was measured and normalized to cell number per mm².
(d) Representative images of caspase-3/7 activation from (c) treated with vehicle, chloroquine 40 µM, doxorubicin 2.5 µM, or doxorubicin + chloroquine.
Figure 4.2
The combination effect of autophagy inhibition and anti-cancer agents requires FOXO3a binding the FHRE to induce apoptosis
(a) HCT116 parental cells, PUMA-/- cells, or ΔFHRE cells lacking the endogenous Forkhead Response Element in the BBC3/PUMA locus, were treated with etoposide 2.5 µM + chloroquine 40 µM. Caspase-3/7 activation was measured and normalized to cell number per mm².

(b) Representative images of caspase-3/7 activation from (a) in HCT116 parental cells, PUMA-/- cells, or ΔFHRE cells lacking the Forkhead Response Element in the endogenous BBC3/PUMA locus treated with etoposide 2.5 µM + chloroquine 40 µM.

(c) HCT116 parental cells, PUMA-/- cells, or ΔFHRE cells lacking the endogenous Forkhead Response Element in the BBC3/PUMA locus were treated with doxorubicin 2.5 µM + chloroquine 40 µM. Caspase-3/7 activation was measured and normalized to cell number per mm².

(d) Representative images of caspase-3/7 activation from (c) in HCT116 parental cells, PUMA-/- cells, or ΔFHRE cells lacking the Forkhead Response Element in the endogenous BBC3/PUMA locus treated with doxorubicin 2.5 µM + chloroquine 40 µM.
(Huang et al., 2009; Paris et al., 2008; Tovar et al., 2006). I used publicly available datasets (Garnett et al., 2012) to identify determinants of Nutlin response and found that PUMA levels, but not other BH3-only proteins, is positively correlated with Nutlin sensitivity ($IC_{50} < 5\mu M$) in different cell types (Figures 4.3a-d). This led us to hypothesize that by combining MDM2 inhibition with autophagy inhibition, both of which increase PUMA levels but through different transcription factors (i.e. p53 and FOXO3a, respectively), it might be possible to enhance PUMA transcription more than with MDM2 inhibition alone and thus cause cancer cells that would otherwise only undergo growth inhibition with Nutlin to commit to apoptosis instead.

Consistent with this hypothesis, Nutlin plus bafilomycin A1 or chloroquine caused a greater increase in PUMA mRNA levels compared to either compound alone (Figure 4.4a). Previous reports (Paris et al., 2008; Tovar et al., 2006) indicate that HCT116 cells undergo growth arrest upon treatment with Nutlin, but no apoptosis. Consistent with this, the combination of Nutlin and chloroquine was much more effective at reducing colony formation than either drug alone (Figure 4.4b). As expected, Nutlin alone caused a robust cytostatic effect, however, Nutlin plus chloroquine resulted in a reduction in tumor cell number (Figure 4.4c) and resulted in more cell death compared to each agent alone as measured by a cell permeable dye (Figures 4.4d-e). Only the combination of Nutlin and either pharmacological or genetic autophagy inhibition was able to increase caspase 3/7 activity (Figures 4.4f-g, and 4.4h-j). Caspase activation was greatly reduced in HCT116 cells lacking either PUMA or containing deletions in the single FOXO3a-binding FHRE in the PUMA gene locus (Figures 4.4k-m). However, cells that lack the FOXO3a binding element were still able to activate caspases similar to parental
Figure 4.3
PUMA levels, but not other BH3-only proteins, are positively correlated with Nutlin sensitivity
(a) Publicly available gene expression dataset of cancer cell lines from multiple tumor types via Oncomine shows that cell lines sensitive to Nutlin (median inhibitory concentration <5 µM) have increased expression of BBC3/PUMA.
(b) Publicly available gene expression data set of cancer cell lines from multiple tumor types via Oncomine shows Nutlin resistant (IC50 > 5 µM) or Nutlin sensitive (IC50 < 5 µM) cell lines and their relative BAD expression levels.
(c) Publicly available gene expression data set of cancer cell lines from multiple tumor types via Oncomine shows Nutlin resistant (IC50 > 5 µM) or Nutlin sensitive (IC50 < 5 µM) cell lines and their relative BIM/BCL2L11 expression levels.
(d) Publicly available gene expression data set of cancer cell lines from multiple tumor types via Oncomine shows Nutlin resistant (IC50 > 5 µM) or Nutlin sensitive (IC50 < 5 µM) cell lines and their relative BID/BCL2L13 expression levels.

Values were Log2 transformed and the median values were scaled to zero according to Garnett et al., (2012).
**Figure 4.4**
FOXO3a-mediated upregulation of PUMA changes the mode of action of an anti-cancer drug
Figure 4.4
FOXO3a-mediated upregulation of PUMA changes the mode of action of an anticancer drug
(a) HCT116 cells were treated with vehicle, bafilomycin A1 10 nM, chloroquine 40 µM, Nutlin 20 µM (an MDM2 inhibitor that activates p53), Nutlin + bafilomycin A1, or Nutlin + chloroquine) for 24 hr, and the resulting BBC3/PUMA mRNA levels were measured relative to 18S rRNA control.
(b) Clonogenic survival assay using HCT116 cells treated for 72 hr with vehicle, chloroquine 40 µM, Nutlin 20 µM, or Nutlin + chloroquine. Cells were then allowed to grow back for 7 days in full media.
(c) HCT116 cells were treated with vehicle, chloroquine 40µM, Nutlin-3a 20µM—an MDM2 inhibitor that activates p53, or Nutlin-3a + chloroquine for 24 hrs and normalized cell count was measured per mm².
(d) HCT116 cells were treated with vehicle, chloroquine 40 µM, Nutlin 20 µM, or Nutlin + chloroquine. Cell permeability by SYTOX green (an indicator of cell death) was measured and normalized to cell number per mm².
(e) Representative images from (d) of cell permeability by SYTOX green in HCT116 parental cells treated with vehicle, chloroquine 40 mM, Nutlin 20 mM, or Nutlin + chloroquine.
(f) HCT116 cells were treated with vehicle, chloroquine 40 µM, Nutlin 20 µM, or Nutlin + chloroquine. Caspase-3/7 activation (an indicator of apoptosis execution) was measured and normalized to cell number per mm².
(g) Representative images of caspase-3/7 activation from (f) in HCT116 parental cells treated with vehicle, chloroquine 40 µM, Nutlin 20 µM, or Nutlin + chloroquine.
(h) HCT116 cells were treated with bafilomycin 10nM, an autophagy inhibitor, Nutlin-3a 20µM—an MDM2 inhibitor that activates p53, or Nutlin-3a + bafilomycin. Caspase 3/7 activation (an indicator of apoptosis execution) was measured over the indicated time points.
(i) HCT116 cells were transduced with lentiviral shCtrl, shATG5, or shATG7 and the level of knockdown is shown for panel j.
(j) HCT116 cells were transduced with lentiviral shCtrl, shATG5, or shATG7 and treated with vehicle or Nutlin-3a 20µM for 24hrs. Caspase 3/7 activation was measured and normalized to cell number per mm².
(k) HCT116 parental cells or ΔFHRE cells lacking the endogenous Forkhead Response Element in the BBC3/PUMA locus were treated with Nutlin 20 µM + chloroquine 40 µM. Caspase-3/7 activation was measured and normalized to cell number per mm².

(l) Representative images of caspase-3/7 activation from (k) in HCT116 parental cells or ΔFHRE cells lacking the Forkhead Response Element in the endogenous BBC3/PUMA locus treated with Nutlin 20 µM + chloroquine 40 µM.

(m) HCT116 parental or PUMA-/- cells were treated with Nutlin-3a + bafilomycin or vehicle for indicated duration. Caspase 3/7 activation (an indicator of apoptosis execution) was measured over the indicated time points.

(n) HCT116 parental cells, PUMA-/- cells, or ΔFHRE cells lacking the endogenous Forkhead Response Element in the BBC3 locus were treated with staurosporine 200nM. Caspase 3/7 activation (an indicator of apoptosis) was measured and normalized to cell number per mm².
cells when treated with the broad apoptotic stimulus, staurosporine (Figure 4.4n). Taken together, these data indicate that the mechanism whereby autophagy inhibition promotes FOXO3a-driven PUMA expression is necessary for the combination effect of Nutlin and autophagy inhibitor that leads to tumor cell apoptosis rather than growth arrest.

A similar combinatorial effect of Nutlin and autophagy inhibition on gene activation of BIM, or other BH3 proteins, was not observed (Figure 4.5a). And although HCT116 cells had low apoptosis sensitivity upon BIM shRNA knockdown (Figure 4.5b), this occurred independent of BIM transcriptional regulation by Nutlin and autophagy inhibition. Thus, the primary mechanism by which the drug combination causes the switch to apoptosis seems to be mediated through the PUMA gene suggesting that the ability to activate two transcription factors (p53 and FOXO3a) that target the same gene (i.e. PUMA) is important for mediating apoptosis ability by the p53-activating drug upon autophagy inhibition.

Consistent with the in vitro studies, xenografted tumors grown from HCT116 parental cells responded to the combination of Nutlin and chloroquine by enhancing PUMA mRNA levels better than either drug alone (Figure 4.5c). However, tumors from HCT116 cells lacking the endogenous Forkhead Response Element (ΔFHRE) treated with the combination of Nutlin and chloroquine did not show this effect. As expected for a compound that activates p53, other p53 target genes, including BAX and p21, were also upregulated (Figure 4.5d). Importantly, mice with tumors grown from the parental HCT116 cells had markedly improved survival due to tumor burden by the drug combination compared with either drug alone. However, mice with tumors that lacked
Figure 4.5
FOXO3a-mediated upregulation of PUMA changes the mode of action of an anti-cancer drug in vivo
Figure 4.5  
FOXO3a-mediated upregulation of PUMA changes the mode of action of an anti-cancer drug in vivo

(a) HCT116 cells were treated with vehicle, chloroquine 40μM, Nutlin-3a 20μM—an MDM2 inhibitor that activates p53, or Nutlin-3a + chloroquine for 24 hrs and the resulting BBC3 (PUMA), BIM, BNIP3, and BNIP3L mRNA levels were measured relative to 18s rRNA control.

(b) (left) Parental or ∆FHRE HCT116 cells were transduced with lentiviral shCtrl or shBIM shRNAs and treated with Nutlin-3a 20μM and chloroquine 40 μM or vehicle for indicated duration. Caspase 3/7 activation was measured and normalized to cell number per mm$^2$. (right) mRNA levels of BIM upon lentiviral shCtrl or shBIM.

(c) Athymic nude mice given subcutaneous parental or ∆FHRE HCT116 tumors were treated with vehicle, Nutlin, chloroquine, or Nutlin + chloroquine, and PUMA mRNA levels in the tumors were measured relative to 18S rRNA control.

(d) Harvested tumor tissue QPCR analysis of Bax, and p21 mRNA in tumors treated with vehicle, chloroquine, Nutlin-3a, or Nutlin-3a + chloroquine normalized to 18S rRNA control.

(e) Kaplan-Meier survival curves based on tumor burden of athymic nude mice given subcutaneous parental or ∆FHRE HCT116 tumors and treated with vehicle, Nutlin, chloroquine, or Nutlin + chloroquine over indicated duration.

(f) (left) Normalized tumor volumes of athymic nude mice subcutaneously injected with either HCT116 parental or HCT116 ∆FHRE cells and treated with vehicle, chloroquine, Nutlin-3a, or Nutlin-3a + chloroquine over indicated duration. Treatment began when 80% of the tumors were 100 mm$^3$. (right) Day 21 tumor volume measurement from left panel comparing treatment groups.

(g) Normalized mouse body weights of Parental and ∆FHRE HCT116 tumors treated with Vehicle, Nutlin, CQ, or Nutlin + CQ normalized to day 1 of drug treatments.
the single FHRE in the PUMA locus displayed no better survival due to tumor burden with the combination treatment than was seen with Nutlin alone in the HCT116 parental cell tumors (Figure 4.5e). Tumor growth rates were slowest with the drug combination in parental HCT116 tumors while tumor growth rate with the drug combination in tumors lacking the single FHRE in the PUMA locus were similar to that seen with Nutlin alone in the HCT116 parental tumors (Figure 4.5f). In contrast to the improvement in survival due to tumor burden (Figure 4.5e), the robust cytostatic effect of Nutlin alone meant that the study did not achieve statistical significance (p=0.16) when comparing tumor volume of Nutlin treated mice to Nutlin and chloroquine treated mice (Figure 4.5f). Additionally, I noted increased toxicity (e.g. as determined by weight loss, Figure 4.5g) in animals treated with the drug combination that led some animals to be removed from the study irrespective of their tumor burden. This is consistent with recent clinical studies where it has been found that autophagy inhibition with chloroquine or hydroxychloroquine can increase the toxicities that are known to be caused by the other drug that is used in combination (Levy et al., 2017a).

Discussion

There are many ongoing clinical trials to inhibit autophagy for cancer therapy (Levy et al., 2017a; Towers and Thorburn, 2016). The underlying idea is to enhance tumor cell apoptosis and thus make other drugs work more effectively but the molecular mechanism by which this occurs has been unclear, making it difficult to determine which drugs might benefit from autophagy inhibition. The regulatory mechanism identified in Chapter III begins to solve these problems and my data indicate that the ability of an autophagy inhibitor to increase tumor cell apoptosis by standard chemotherapy drugs...
like etoposide or doxorubicin requires that FOXO3a be able to increase PUMA gene expression. Moreover, I also show that it is possible to exploit the mechanistic link between autophagy homeostasis and apoptosis sensitivity to improve the therapeutic efficacy of an anti-cancer drug like Nutlin that often only causes growth inhibition of cancer cells (Huang et al., 2009; Paris et al., 2008; Tovar et al., 2006) into one that kills cancer cells. Thus, pharmacological manipulation of the autophagic turnover of a transcription factor can work through a single genomic binding site in one gene to improve the ability of cancer drugs to cause tumor cell apoptosis and can even change the mode of action of a highly specific anti-cancer drug. Since there are several MDM2 inhibitors in clinical development (Burgess et al., 2016), my work suggests that they could be good candidates for combination with autophagy inhibition in the clinic (Sullivan et al., 2015). However, the increased general toxicity that I observed with the drug combination means that as with other attempts to widen the therapeutic window for a given cancer drug by inhibiting autophagy, it will be important to optimize doses of both the MDM2 inhibitor and the autophagy inhibitor in clinical studies.

**Acknowledgments**

I would like to acknowledge Maria Hoh and Mike Ludwig for their excellent help while conducting the mouse studies for this Chapter. I would also like to acknowledge Christina G. Towers for analyzing Incucyte data in this Chapter, and providing invaluable help during the *in vivo* tumor studies. Lastly, I would like to thank Kelly D. Sullivan for overseeing the experimental design and progress of the *in vivo* tumor studies.
CHAPTER V

FOXO3A DEGRADATION BY AUTOPHAGY, BUT NOT THE PROTEASOME, CONFERS APOPTOSIS SENSITIZATION

Introduction

In Chapters III and IV, I discovered one of the first reports of a transcription factor being selectively targeted for autophagic degradation, and ascribe a physiological role for this regulation—namely, dictating apoptosis threshold in cancer cells. FOXO3a is also regulated by the proteasome in response to various stimuli. I wondered if this proteasomal regulation also influences the likelihood of a cell undergoing apoptosis in response to anti-cancer therapies. Previous studies show that growth factor stimulation results in increased ERK and Akt signaling, leading to FOXO phosphorylation and sequestration in the cytoplasm where the E3 ubiquitin ligase SKP2 induces ubiquitination and subsequent degradation by the proteasome (Huang and Tindall, 2011). Similarly, ERK activation via the Ras-Raf-MEK pathway leads to FOXO3a phosphorylation and recognition by the E3 ubiquitin ligase MDM2, leading to proteasomal degradation (Huang and Tindall, 2011; Yang et al., 2008). These studies point to FOXO regulation in response to physiological signaling, and prompted me to test the contribution of proteasomal degradation to FOXO3a stability in cancer cells.

Results

I began by testing the relative contributions of both the proteasome and lysosome to FOXO3a degradation. Bortezomib, a proteasome inhibitor, bafilomycin A1, a lysosome inhibitor, and the combination caused a time-dependent accumulation of the FOXO3a protein in HCT116 cells that peaked at 8 hours following treatment (Figure
5.1a). Bortezomib is known to active the p53 transcriptional program by preventing the proteasomal degradation of p53 (Williams and McConkey, 2003), so the protein levels of the p53 protein are displayed as a positive control. These data indicate that both the proteasome and autophagy pathways contribute to regulating FOXO3a protein levels. Next, I tested the effect of these increases in FOXO3a levels on PUMA mRNA levels in HCT116 cells. Consistent with data presented in Chapter III, bafilomycin A1 treatment led to an increase in PUMA mRNA levels at both 8 and 24 hours (Figure 5.1b).

Bortezomib treatment also caused an increase in PUMA mRNA levels (Figure 5.1b), but this increase was dependent on p53—performing these experiment in p53-/- HCT116 cells abolished the effects of bortezomib without affecting bafilomycin A1’s ability to increase PUMA mRNA (Figure 5.1c). These data show that despite both proteasome and lysosomal turnover, FOXO3a only increases PUMA mRNA levels when treated with bafilomycin A1.

In Chapter IV, I demonstrated that HCT116 cells undergo growth arrest when treated with Nutlin, but commit to apoptosis if they are treated with a lysosome inhibitor like chloroquine. Also, I showed that this combinatorial effect was dependent on the ability of FOXO3a to bind to the PUMA gene at the FHRE—deletion of this binding site reversed these effects. Next, I compared the amount of caspase 3/7 activity in both parental and ∆FHRE HCT116 cells treated with Nutlin and bortezomib, and found that there was no difference in apoptosis between these cell lines (Figure 5.2a). Though the combination of Nutlin and bortezomib caused apoptosis, this effect was not dependent on FOXO3a binding to the FHRE. Consistent with these data, the treatment of HCT116
Figure 5.1
FOXO3a levels increase upon autophagy and proteasome inhibition, but transactivate PUMA via different transcription factors
(a) Western blot analysis of HCT116 cells treated with vehicle, bafilomycin A1 10nM, bortezomib 50nM, or the combination for indicated duration
(b) HCT116 cells were treated with vehicle, bafilomycin A1 10 nM, bortezomib 50nM for 8 hr or 24 hr and the resulting BBC3/PUMA mRNA levels were measured relative to 18S rRNA control.
(c) HCT116 p53-/- cells were treated with vehicle, bafilomycin A1 10 nM, bortezomib 50nM for 8 hr or 24 hr and the resulting BBC3/PUMA mRNA levels were measured relative to 18S rRNA control.
Figure 5.2
Autophagy-directed, but not proteasome, FOXO3a turnover confers apoptosis sensitization
(a) Parental or ΔFHRE HCT116 cells were treated with vehicle, bortezomib 50nM, Nutlin 20 µM, or Nutlin + bortezomib. Caspase-3/7 activation (an indicator of apoptosis execution) was measured and normalized to cell number per mm².
(b) Confocal images of HCT116 cells expressing mCherry-GFP-FOXO3a treated with bafilomycin A1 10nM or bortezomib 50nM for 8 hr. Scale bars, 10 µm.
cells with bortezomib did not cause nuclear translocation of FOXO3a, but as expected bafilomycin A1 was capable of promoting nuclear enrichment of FOXO3a (Figure 5.2b). Taken together, FOXO3a is degraded by both the lysosomal and proteasome, but only lysosomal blockage was able to cause FOXO3a activation and subsequent increases in PUMA levels.

**Discussion**

The data in Chapter V create a very interesting set of findings; although both the proteasome and autophagy are responsible for turnover of FOXO3a, only the protein that is degraded by autophagy is able to activate PUMA transcription when its turnover is blocked. This may represent an example whereby the same protein is degraded by two distinct mechanisms but only one of the mechanisms is linked to another process (in this case apoptosis sensitization). Although I do not understand the molecular mechanism yet, this suggests that there may be different pools of FOXO3a that are degraded by the proteasome and the autophagosome and only blocking the one process leads directly to increased transcription of FOXO targets. Future studies should aim to understand the specific post-translational modifications that direct different FOXO3a protein pools to their respective regulation pathways. One approach for experimental testing may include mass spectrometry studies in vehicle, bafilomycin A1 (a lysosomal inhibitor), and bortezomib (a proteasome inhibitor) treated cells to determine the modifications of FOXO3a specific to each inhibitor. Once a critical modification necessary for autophagic targeting is determined, additional experiments should involve expressing a mutant version of that protein to show an inability to be recruited to autophagosomes. These experiments could reveal how the cell directs a
protein pool toward a specific function, and how differential modifications can influence cellular fate. This underscores the importance of knowing the specific molecular mechanism before targeting a cellular pathway.
CHAPTER VI
DEVELOPMENT OF AN OPTOGENETIC METHOD TO RAPIDLY BLOCK
AUTOPHAGY

Introduction

Current methods of autophagy inhibition include pharmacological or genetic manipulation by shRNA knockdown or CRISPR/Cas9-mediated knockout of essential autophagy regulators, but takes ~48hrs to achieve a substantial level of autophagy inhibition (Staskiewicz et al., 2013). Pharmacological autophagy inhibition can be achieved more quickly, with bafilomycin A1 working more rapidly than chloroquine, at blocking autophagosome-lysosome fusion (Collins et al., 2018; Mauthe et al., 2018), but both lack pathway specificity and promote off-target effects. If, for example, we were interested in testing the immediate effects of autophagy blockade on a particular signaling cascade, using these sluggish and non-specific methods would not allow for dissecting this biology.

In this Chapter, I will describe the development of an optogenetic system to achieve rapid and specific autophagy inhibition within a manner of minutes. I show that I can use light to block autophagy and cause accumulation of autophagy substrates, with no background activity in the dark. Further, I show that I can kill cancer cells with light that are dependent on autophagy for survival. Consistent with data shown in Chapter IV, I also demonstrate that I can change the mode of action of the anti-cancer drug Nutlin from growth arrest to apoptosis using autophagy inhibition with light. Using a pH-sensitive fluorophore tagged to an autophagy cargo protein, I provide preliminary data showing that this optogenetic system can block the delivery of new cargo to the
lysosome within a matter of minutes. I termed this optogenetic autophagy inhibition system Autophagic SNARE for Abrupt Photoinhibition (ASAP).

Results

Syntaxin 17 (STX17) is a SNARE protein that has been previously shown to be important for autophagosome-lysosome fusion to occur, and expressing a mutant lacking the N-terminus of the protein (STX17ΔNTD) acts as a dominant negative to inhibit fusion (Itakura et al., 2012; Uematsu et al., 2017). STX17 has been shown to localize to the mitochondrial surface (Arasaki et al., 2015), so I decided to target STX17ΔNTD to mitochondria using a TOM20 targeting sequence. In order to create a rapid optogenetic system whereby autophagy is functional in the dark but inhibited with incidence of light, I chose to use a photocleavable protein called PhoCL (Zhang et al., 2017). When cells expressing this plasmid (pBABE TOM20-PhoCL-myc-STX17ΔNTD) are kept in the dark, the STX17ΔNTD fragment remains tethered to mitochondria by PhoCL and is not able to inhibit autophagosome-lysosome fusion (Figure 6.1a). Conversely, upon 405nm light incidence, PhoCL is cleaved and the STX17ΔNTD fragment is released to inhibit autophagosome-lysosome fusion by acting as a dominant negative (Figure 6.1a). When PhoCL is full-length it is fluorescent and excited by 488nm, but upon 405nm light incidence, is cleaved and loses its fluorescence (Figure 6.1a). Upon expressing this system in MCF10a cells, as expected the ASAP system localizes to mitochondria (Figure 6.1b). In the dark, the ASAP system is fluorescent and localized to mitochondria, but upon 405nm stimulation is transiently photo-converted to a species that is excited by ~570nm (Zhang et al., 2017), and then becomes cleaved (Figure 6.1c).
Figure 6.1
**Autophagy SNARE for Abrupt Photoinhibition (ASAP) system**
(a) Schematic representation of how the ASAP optogenetic system works to block autophagy with 405nm light treatment, but maintains functional autophagy in the dark.
(b) Confocal images of MCF10a cell expressing TOM20-PhoCL-myc-STX17ΔNTD (ASAP) and treated with MitoTracker mitochondrial stain. Scale bars=10µm.
(c) Confocal images of MCF10a cell expressing TOM20-PhoCL-myc-STX17ΔNTD (ASAP) and treated with MitoTracker mitochondrial stain in the dark and after 405nm light stimulation.
To test if the ASAP system inhibits autophagy with 405nm light stimulation but not affecting autophagy in the dark, I monitored autophagy substrates by western blot analysis. BT549 cells expressing ASAP in the dark show an expected increase in the autophagy cargo receptor p62/SQSTM1 with the lysosome inhibitor bafilomycin A1, and a comparable increase in p62/SQSTM1 with 405nm light treatment. Further, the combination of autophagy inhibition by bafilomycin A1 and 405nm light did not cause a further increase in p62/SQSTM1 (Figure 6.2a). Autophagy inhibition with the ASAP system also caused an increase in other autophagy substrates FOXO3a and LC3-II in multiple cell lines that did not increase further with bafilomycin A1 treatment (Figure 6.2b-c). Taken together, these data indicate that the ASAP optogenetic system can inhibit autophagy following 405nm light stimulation causing established autophagy substrates to accumulate. Next, because the cleavage product resulting from 405nm light stimulation causes autophagy inhibition, I was interested in the stability of this cleaved fragment. I treated ASAP cells with 405nm light (or kept in the dark) and harvested 1 hour, 8 hours, and 12 hours after, then detected the amount of ASAP cleavage fragment and its effects on autophagy substrates FOXO3a and LC3-II (Figures 6.2d-e). Of note, cells expressing ASAP in the dark showed no photocleavage, suggesting that there is minimal background inhibition of autophagy.

Some cancer cells are more dependent on autophagy for survival than others (Maycotte et al., 2014). BT549 cells have been previously shown to be sensitive to autophagy inhibition; treatment with the lysosomal inhibitor chloroquine or bafilomycin A1 causes a decrease in growth (Figure 6.3a). Expression of the ASAP optogenetic system (in the dark) in these cells does not influence this effect on growth, suggesting
Figure 6.2

Autophagy SNARE for Abrupt Photoinhibition (ASAP) system allows for light-mediated autophagy inhibition in multiple cancer cell lines.

(a) Western blot analysis of BT549 cells treated with 405nm light or kept in the dark in the absence or presence of bafilomycin A1 10nM.

(b) Western blot analysis of BT549 cells treated with 405nm light or kept in the dark in the absence or presence of bafilomycin A1 10nM.

(c) Western blot analysis of MCF10a cells treated with 405nm light or kept in the dark in the absence or presence of bafilomycin A1 10nM.

(d) Western blot analysis of BT549 cells treated with 405nm light and harvested at indicated duration after 405nm light treatment.

(e) Western blot analysis of HCT116 cells treated with 405nm light and harvested at indicated duration after 405nm light treatment.
Figure 6.3
*Autophagy inhibition kills autophagy-dependent cancer cells*
(a) BT549 Parent cells were treated with vehicle, bafilomycin A1 10nM, or chloroquine 40µM for indicated duration and cell confluence was normalized to time point 0.
(b) BT549 Parent or ASAP (in the dark) cells were treated with vehicle, bafilomycin A1 10nM, or chloroquine 40µM for indicated duration and cell confluence was normalized to time point 0.
that the ASAP system has no background effects on autophagy or cell growth in the dark (Figure 6.3b). Next, I tested the hypothesis that the ASAP system could alter the growth of BT549 cells following 405nm light incidence compared to cells kept in the dark. Indeed, ASAP cells treated with 405nm light displayed less growth compared to ASAP cells kept in the dark (Figure 6.4a). ASAP cells treated with light causes a more rapid effect on cell growth than ASAP cells (in the dark) treated with the autophagy inhibitor bafilomycin A1 (Figure 6.4a). The ability of ASAP to affect cell growth upon treated with 405nm light is dose-dependent (Figure 6.4b), and is able to cause apoptosis, compared to parental cells treated with the same light dosage (Figure 6.4c). Because the ability of ASAP to inhibit autophagy is dependent on the amount of the photocleaved fragment, and previous results show the half-life of this fragment to be ~6 hours, this ASAP system has a transient effect on cell growth compared to chloroquine (Figure 6.4d). Taken together, these data show the ability of the ASAP system to rapidly and transiently inhibit autophagy, and to subsequently elicit biological effects on tumor cell survival in a cell line that is known to undergo apoptosis upon autophagy inhibition by other methods with no background activity in the dark.

Previous reports from the Thorburn laboratory have shown that inhibiting autophagy can sensitize cells to apoptosis (Fitzwalter et al., 2018; Thorburn et al., 2014). Treating some cancer cells with the anti-cancer drug Nutlin alone causes growth arrest, but when combined with an autophagy inhibitor can promote commitment to apoptosis. This demonstrates that autophagy inhibition can change the mode of action of a cancer therapy from growth arrest to cell death, like I showed in Chapter IV (Fitzwalter et al., 2018). To control for any light-mediated toxicity and control for any
**Figure 6.4**

Autophagy SNARE for Abrupt Photoinhibition (ASAP) system can kill autophagy-dependent cancer cells in a rapid and transient manner.

(a) BT549 ASAP cells were treated with vehicle, bafilomycin A1 10nM, or 405nm light for indicated duration and cell confluence was normalized to time point 0.

(b) BT549 Parent or ASAP cells were treated with two doses of 405nm light for indicated duration and cell confluence was normalized to time point 0.

(c) BT549 Parent or ASAP cells were treated with 405nm light for indicated duration and caspase 3/7 activity was measured per mm² and normalized to time point 0.

(d) BT549 ASAP cells were treated with vehicle, chloroquine 40µM, or 405nm light for indicated duration and cell confluence was normalized to time point 0.
effects of ASAP on mitochondrial biology, I made ASAP control cells that have a mitochondrial-targeted PhoCL that undergoes light-induced cleavage, but lacks the STX17∆NTD, and thereby does not inhibit autophagy with light (Figure 6.5a). I show that ASAP cells (in the dark) treated with the combination of Nutlin and the autophagy inhibitor chloroquine leads to a decrease in cell confluence (Figure 6.5b) and an increase in caspase 3/7, a marker of apoptosis (Figure 6.5c), compared to each drug alone. Importantly, the treatment of ASAP control or ASAP cells with 405nm light and Nutlin led to an abrupt decrease in cell confluence in the ASAP cells (Figure 6.5d) and an increase in apoptosis compared to ASAP control cells with the same treatment (Figure 6.5e). These data provide a rigorous test of the ability of the optogenetic method to inhibit autophagy in a rapid manner and cause a biological effect.

To determine how rapidly ASAP can block autophagy, I chose to develop a way to detect the delivery of autophagy substrates to the lysosome in real time. I achieved this using a pH-sensitive fluorophore called pHRed that has minimal fluorescence under physiological pH (pH~7), but strongly fluoresces in acidic compartments, such as lysosomes (pH~4) (Tantama et al., 2011). I tagged this fluorophore to the autophagy cargo receptor protein p62 to watch the kinetic delivery of p62 to lysosomes (Figure 6.6a). I observed robust colocalization of pHRed-p62 with lysotracker, indicating that this tool can detect delivery of p62 to lysosomal compartments (Figure 6.6b). To detect the dynamic delivery of new pHRed-p62 molecules to lysosomes, I performed localized photobleaching on a region of interest (ROI) and monitored the subsequent recovery of fluorescence after photobleaching (FRAP) of that ROI. Because photobleaching causes irreversible damage to the fluorophore, the restoration of a fluorescent signal represents
Figure 6.5  
**Autophagy SNARE for Abrupt Photoinhibition (ASAP)** system can kill autophagy-dependent cancer cells.
Figure 6.5
Autophagy SNARE for Abrupt Photoinhibition (ASAP) system can kill autophagy-dependent cancer cells.

(a) Schematic representation of the ASAP control and ASAP protein products used for experimentation.

(b) HCT116 ASAP control cells were treated with vehicle, Nutlin 20µM, chloroquine 40µM, or Nutlin + chloroquine for indicated duration. Percent confluence was measured and normalized to time point 0.

(c) HCT116 ASAP control cells were treated with vehicle, Nutlin 20µM, chloroquine 40µM, or Nutlin + chloroquine for indicated duration. Caspase-3/7 activation was measured and normalized to cell number per mm$^2$.

(d) HCT116 ASAP cells were treated with vehicle or Nutlin 20µM and 405nm light. Caspase-3/7 activation was measured and normalized to cell number per mm$^2$. 
Figure 6.6
pHRed-p62 reveals rapid, successive substrate delivery to lysosomes
(a) Schematic representation of how pHRed-p62 works to monitor the delivery of p62 to lysosomes. pHRed-p62 is fluorescent in cellular compartments with low pH, such as lysosomes, but is not fluorescent in pH-neutral environments.
(b) Representative confocal images of MEFs starved for 4hrs in EBSS expressing pHRed-p62 (red) and treated with lysotracker (green).
(c) Confocal image series of MEF starved for 4hrs in EBSS expressing pHRed-62 and photobleached at 561nm for 1ms at 50% laser power. Time is indicated in minutes.
(d) Quantitation of mean fluorescent intensity of ROI indicated following photobleaching of pHRed-p62 at 561nm for 1ms at 50% laser power.
newly delivered pHRed-p62 molecules to lysosomes. p62/- mouse embryonic fibroblasts (MEFs) were starved for 4 hours using EBSS, and subject to photobleaching in an ROI (Figure 6.6c). The fluorescent recovery over time of this ROI was then quantified (Figure 6.6d). These experiments reveal that under starvation conditions there is rapid, successive delivery of pHRed-p62 to lysosomes (Figure 6.6c-d). Next, I tested the hypothesis that the rapid delivery of pHRed-p62 in these experiments is due to autophagosome-lysosome fusion events. To visualize such events, I expressed a previously described (Itakura et al., 2012) marker of autophagosomes, GFP-STX17TM, along with pHRed-p62 and discovered that lysosomes can accommodate multiple autophagosome fusion events (Figure 6.7a).

In Figures 6.2-6.5, I validated the ability of ASAP to inhibit autophagy by functional and biochemical assays; however, these techniques are not able to determine how rapidly ASAP can block autophagosome-lysosome fusion. To measure this timescale, I combined the ASAP optogenetic system with pHRed-p62 to uncover how quickly ASAP can block the new delivery of pHRed-p62 to lysosomes. p62/- MEFs were starved using EBSS, subject to photobleaching at two ROIs, and the fluorescent recovery of these ROIs was monitored over time (Figure 6.8a). Next, using the same cell, I photoactivated ASAP using 405nm light to inhibit autophagosome-lysosome fusion and then photobleached three ROIs (Figure 6.8b). Of note, and consistent with previous studies, there is a transient burst of red fluorescence of PhoCL just prior to photocleavage (Zhang et al., 2017). Quantitation of these photobleached ROIs both before and after ASAP activation revealed that ASAP could block the delivery of new pHRed-p62 to lysosomes (Figure 6.8c). Although these experiments have not been
Figure 6.7
Lysosomes can accommodate multiple autophagosome fusion events
(a) Image series (in minutes) showing a single fusion event in U2OS cells expressing Syntaxin 17 Transmembrane domain to mark autophagosomal structures, and pHRed-p62. Cells were starved using EBSS.
Figure 6.8

ASAP blocks autophagic delivery of p62 to lysosomes
**Figure 6.8**

**ASAP blocks autophagic delivery of p62 to lysosomes**

(a) Confocal image series of EBSS-starved MEFs expressing pHRed-62 and ASAP (in the dark) and photobleached in ROIs at 561nm for 1ms at 50% laser power. Time is indicated in minutes.

(b) Confocal image series of EBSS-starved MEFs expressing pHRed-62 and ASAP (stimulated with 405nm laser line) and subsequently photobleached at indicated ROIs at 561nm for 1ms at 50% laser power. Time is indicated in minutes.

(c) Quantitation of mean fluorescent intensity of indicated ROIs following photobleaching in pHRed-p62-expressing and ASAP cells (in the dark or stimulated with 405nm laser line). Photobleaching of pHRed-p62 was performed at 561nm for 1ms at 50% laser power.
extensively repeated, these data provide preliminary evidence that ASAP can block the delivery of new pHRed-p62 molecules to lysosomes with 405nm light incidence within a short timescale. A lot more optimizing of this workflow using ASAP and pHRed-p62 is needed. To date, I am not aware of any other system to genetically block autophagy within a matter of minutes.

**Discussion**

In this Chapter, I show that I can combine a useful optogenetic protein PhoCL with a mutant SNARE protein STX17\(\Delta\)NTD to block the process of autophagy in a light-dependent manner, with negligible background in the dark. The advent of ASAP will allow us to understand the biological effects of blocking autophagy transiently and acutely using genetic means, rather than using current methods—pharmacological inhibitors that have off-target effects (bafilomycin A1 and chloroquine), and genetic manipulations that take a long time to work (shRNA and CRISPR/Cas9).

I show biochemically that ASAP can inhibit autophagy in a population of cells subject to 405nm light exposure, resulting in increased autophagy substrates. Additionally, I show proof-of-principle experiments that inhibiting autophagy with the ASAP system can kill cancer cells that are dependent on autophagy for survival and this death occurs more rapidly than pharmacological inhibitors like chloroquine, which take time to accumulate in the lysosome (Collins et al., 2018). ASAP is also able to change the mode of action of the anti-cancer drug Nutlin from growth arrest to apoptosis, confirming my previous reports (Fitzwalter et al., 2018).

To further validate the ASAP system, I sought to determine if I could achieve rapid and robust autophagy inhibition within a timescale of minutes. Current methods
are incapable of determining if autophagy is inhibited on this short of a timescale, so I decided to develop a technique to monitor the real-time delivery of the autophagy substrate p62/SQSTM1 to the lysosome. By tagging p62/SQSTM1 to the pH-sensitive fluorophore, pHRed, I could observe the population of p62/SQSTM1 that resides in the lysosome. Next, to visualize dynamic population of p62/SQSTM1 that was newly-delivered to the lysosome, I chose to photobleach a select region of the cell to watch the rate of recovery of pHRed-p62. I believe that the fluorescent recovery after photobleaching (FRAP) is indicative of pHRed-p62 molecules that are newly delivered to the lysosome. I am able to visualize autophagy flux in live cells in real time using this tool, and are able to test the hypothesis that the ASAP system is blocking autophagy on a rapid timescale. Below, I will discuss how future studies could harness the power of using light to block autophagy with the ASAP system.

**Acknowledgments**

I would like to acknowledge Chandra Tucker and Matthew Kennedy for their advice on troubleshooting the ASAP and pHRed-p62 systems. Also, I would like to acknowledge Ashely Bourke for sharing her protocol and expertise in photobleaching.
CHAPTER VII
DISCUSSION

FOXO3a links autophagy to apoptosis

The work in this Thesis builds on a previous report from the Thorburn laboratory showing that autophagy controls the timing and extent of apoptosis by regulating PUMA levels (Thorburn et al., 2014). This study demonstrated that autophagy inhibition led to increased PUMA protein levels, but the mechanism by which this occurs was not resolved. I describe in Chapter III that upon genetic or pharmacological autophagy inhibition, the mRNA levels of PUMA increase via new transcription, independent of its common transcriptional regulator p53 (Figures 3.1-3.3). This transcriptional activation of PUMA was dependent on the Forkhead Box transcription factor FOXO3a binding at a single Forkhead Response Element (FHRE) in an intron of the PUMA gene (Figures 3.4-3.5). Importantly, CRISPR/Cas9-mediated knockout of this endogenous FHRE completely blocked the ability of autophagy inhibition to upregulate PUMA mRNA levels, but still allowed for PUMA upregulation in other contexts (p53 activation for example) (Figures 3.4-3.5).

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7 Portions of this chapter were published with permission from Oncotarget: Autophagy inhibition improves anti-cancer drugs via FOXO3a activation. Brent E. Fitzwalter & Andrew Thorburn, 2018, Oncotarget, Vol. 9, (No. 39), pp: 25384-25385.

FOXO transcription factors are known to be regulated by post-translational modifications governing their localization and protein stability (Calnan and Brunet, 2008). In this Thesis, I uncovered a previously undescribed mechanism of FOXO regulation—constitutive basal autophagic degradation. FOXO3a is selectively degraded by basal autophagy, but this turnover is relieved upon autophagy inhibition, allowing for transactivation of PUMA and other gene targets (Figures 3.5-3.9).

I have discussed how autophagy and cell death are two evolutionarily conserved cellular processes that are intimately linked by their molecular machinery (Fitzwalter and Thorburn, 2015b). In this Thesis, I believe that I have uncovered a fundamental transcriptional mechanism showing how autophagy influences apoptosis. When autophagy is intact PUMA levels are kept low, but when autophagy is blocked, PUMA mRNA levels are increased by the transcription factor FOXO3a binding to the FHRE in the PUMA gene, leading to new transcription. Surprisingly, the FOXO3a protein is directly targeted for basal autophagic turnover under normal physiological conditions, but upon autophagy inhibition causes FOXO3a to become relieved from this regulation allowing for transactivation of numerous gene targets, but most importantly PUMA. This mechanism implies that there is a homeostatic feedback loop in place whereby a transcription factor that was previously shown to control autophagy (Warr et al., 2013a) is itself regulated by autophagic turnover (Fitzwalter and Thorburn, 2018). Further, the data from this Thesis also suggests that FOXO3a acts as a cell surveillance sensor to maintain autophagy homeostasis; in the case where autophagy is intact, FOXO3a is sequestered and degraded in the cytoplasm, but upon autophagy perturbation FOXO3a
is relieved from degradation and translocates to the nucleus to correct the distress or mediate apoptosis commitment (Figure 7.1).

There are unanswered questions from this work. My studies showed that the transcriptional kinetics of various apoptotic and non-apoptotic FOXO3a targets differ following pharmacological and genetic autophagy perturbation (Figure 3.5-3.6); how is FOXO3a selectively directed toward a specific set of gene targets, at what kinetics, and how do these targets influence cell fate? Above, we discussed the need for mass spectrometry experiments to understand the landscape of FOXO3a post-translational modifications that may direct FOXO3a to favor the activation of individual targets. Understanding FOXO3a post-translational modifications may also help us understand how FOXO3a is selectively recruited to phagophores, and what signals allow it to avoid autophagic turnover and become activated upon autophagy inhibition. Additionally, data from Chapter V show that FOXO3a is degraded by both the proteasome and autophagy; however, inhibiting autophagy but not the proteasome causes an increase in PUMA mRNA (Figure 5.1) suggesting that there may be different pools of FOXO3a destined for different modes of degradation and cellular signaling, which may also be explained by different post-translational modifications. Understanding the underlying biology of FOXO3a signaling may uncover the molecular consequences of blocking autophagy. In the next section, I will discuss how the mechanisms described above can be leveraged to improve anti-cancer drugs.

**Autophagy inhibition improves anti-cancer drugs via FOXO3a**

Chapter IV of this Thesis shows that I can take advantage of the molecular connection between autophagy and apoptosis that I revealed in Chapter III to make
Figure 7.1
FOXO3a may act as a cell surveillance mechanism to maintain autophagy homeostasis.
(left) Basal autophagy flux is upheld and homeostasis is maintained. FOXO3 is targeted for degradation by basal autophagy to confer low apoptosis sensitivity. (right) FOXO3 avoids autophagic turnover upon autophagy inhibition (genetic deletion of essential autophagy genes or pharmacological inhibition). FOXO3 transactivates autophagy-related genes and attempts to compensate for the perturbation in autophagy flux; if this correction is not achieved, FOXO3 leads to BBC3/PUMA transactivation and apoptosis sensitization.
anti-cancer drugs work better. I show that this molecular mechanism—autophagy’s ability to control PUMA levels via FOXO3a—explains why cancer cells are sensitized to undergo apoptosis upon autophagy inhibition (Figure 7.2). How can this mechanism allow us to improve cancer therapy? I showed that lysosomal disruption by chloroquine (a drug that is used in people to inhibit autophagy, but also affect other lysosomal-related processes and has lysosome independent effects as well) sensitizes cells to apoptosis upon treatment with the DNA damaging agents such as doxorubicin and etoposide, but this combinatorial effect was dependent on the ability of FOXO3a to bind to the PUMA locus at the FHRE (Figures 4.1-4.2). I took the mechanism a step further to change the mode of action of an anti-cancer drug called Nutlin from cancer cell growth arrest to causing apoptosis commitment upon autophagy inhibition (Figure 4.4). These in vitro data indicate that Nutlin and lysosomal inhibition cooperate to cause cancer cells to commit to apoptosis, but this cooperation is dependent on the ability of FOXO3a to bind to the PUMA locus (Figure 7.3). Similar results were obtained for genetic autophagy inhibition and Nutlin combination, indicating that these effects involve macroautophagy’s ability to regulate apoptosis and not autophagy-independent effects of chloroquine (Figure 4.4). Consistent with these data, mice with xenograft tumors grown from parental HCT116 cells and treated with the combination of Nutlin and chloroquine had significantly better survival based on tumor burden compared to the two drugs alone (Figure 4.5). However, mice with tumors grown from cells lacking the endogenous FHRE in the PUMA locus failed to respond to this combination treatment and displayed no better survival than parental tumors treated with Nutlin alone (Figure 4.5). These data indicate that the combination of chloroquine and Nutlin can reduce
Figure 7.2
Autophagy determines sensitivity to chemotherapy.
Autophagic turnover of FOXO3a confers low sensitization to chemotherapy-induced apoptosis. However, upon autophagy inhibition, FOXO3a transactivation of PUMA confers high sensitization to chemotherapy-induced apoptosis.
Figure 7.3
Autophagy inhibition mediates apoptosis sensitization in cancer therapy by relieving FOXO3a turnover
Schematic of the proposed mechanistic link between autophagy and apoptosis, explaining how autophagy inhibitors can improve anti-cancer drugs by increasing sensitivity to apoptosis. The transcription factor FOXO3a, an autophagy regulator, is itself degraded by basal autophagy. Disruption of autophagy allows FOXO3a to upregulate BBC3/PUMA expression and thus cause apoptosis sensitization.
tumor burden in vivo, and that this combinatorial benefit of autophagy inhibition depends on the ability of FOXO3a to bind to the PUMA locus in these cancer cells.

MDM2-target therapies, like Nutlin, are currently being tested in patients with both solid and non-solid tumors in combination with other anti-cancer therapies (Burgess et al., 2016; Tisato et al., 2017). MDM2 inhibitors act as regulators of p53, a regulator of cell cycle, cell proliferation, and apoptosis. The mechanism I discovered that links autophagy and apoptosis paves the way for future studies aimed at improving the therapeutic efficacy of a cancer drug, such as Nutlin. My studies in vitro and in vivo suggest that autophagy inhibition can change the mode of action of Nutlin from growth arrest to apoptosis in cancer cells. Further, my data suggests that using autophagy inhibitors in combination with MDM2 inhibitors may be therapeutically beneficial. It is worth noting that I observed increased toxicity in mice treated with the combination of Nutlin and chloroquine (Figure 4.5). This toxicity is consistent with previous studies aiming to inhibit autophagy in combination with anti-cancer drugs (Levy et al., 2017b). One explanation for this toxicity may be that inhibiting autophagy has altered the apoptotic threshold in non-cancerous tissues. Future studies could focus on dose management in order to reduce toxicity to improve patient outcomes, and determine the effect of autophagy inhibition on the therapeutic window of anti-cancer drugs. Future studies should also determine if this mechanism applies in a range of cancer types, and in the context of other anti-cancer drugs. Additionally, are different cancer sub-types dependent on other BH3-only proteins for apoptosis sensitization upon autophagy inhibition?
Autophagy is believed to prevent tumor initiation by promoting DNA stability, mitochondrial turnover, and tissue homeostasis. However, once a tumor has been established, autophagy allows cancer cells to overcome nutrient and environmental stresses associated with tumor cell proliferation and microenvironment (Galluzzi et al., 2015). Inhibiting autophagy in cancer may be a therapeutic intervention to promote cell death (Levy et al., 2017b; Towers and Thorburn, 2016), and thus understanding the molecular mechanisms linking autophagy and apoptosis is essential (Fitzwalter and Thorburn, 2015b). Indeed, there are many ongoing clinical trials (Towers and Thorburn, 2016) using autophagy inhibitors to enhance the efficacy of anti-cancer drugs, but there is limited mechanistic insight as to why they cooperate to induce more tumor cell death. The work described in Chapters III and IV begins to provide a mechanistic rationale for combining anti-cancer drugs and autophagy inhibitors. Namely, blocking autophagy leads to apoptosis sensitivity by relieving FOXO3a from autophagic turnover, allowing for the active transcription and increase in PUMA levels. My work suggests that cancer cells may use autophagy to keep FOXO3a levels (and thereby PUMA levels) low to resist apoptosis. Hundreds of published studies show that if autophagy is inhibited in cancer cells by inhibition of specific ATG genes or with drugs such as chloroquine, those cells undergo apoptosis more easily when they are treated with an additional death stimulus. I have now provided one potential mechanism in Chapter III to explain these effects in cancer cells where autophagy is blocked.

**Autophagy may determine the apoptotic threshold**

For almost 20 years, one of the recognized hallmarks of cancer has been evasion of apoptosis (Hanahan and Weinberg, 2000). This has often led to the
misconception that cancer cells must be generally resistant to apoptosis compared with their normal counterparts. However, the opposite is actually true, cancer cells are generally more sensitive to apoptosis than normal cells in part because oncogenes that drive tumor cell growth also tend to ‘prime’ them to cell death (Green and Evan, 2002).

As we’ve discussed, cancer cells integrate environmental pro- and anti-apoptotic signals by using protein-protein interactions between BH3-only pro-apoptotic proteins, the apoptosis effectors BAX and BAK, and BCL-2 anti-apoptotic proteins. It is possible to directly measure the propensity of a given anti-cancer drug to release cytochrome c from mitochondria in cancer cells using a technique called BH3 profiling, which can predict therapeutic responses in patients (Ni Chonghaile et al., 2011). Cytotoxic drugs act by pushing cancer cells to commit apoptosis, and are beneficial in many cancer types and continue to be widely used (Letai, 2015). Additionally, targeted therapies capitalize on specific cancer cell vulnerabilities to promote cancer cell death. It is important to understand how both cytotoxic and targeted therapies affect apoptosis threshold to commit cancer cells to undergo apoptosis.

Studies by the Letai laboratory have determined that cancer cells reside at varying distances away from falling off of a proverbial ‘cliff’ to undergoing apoptosis—for example, some cells are highly primed to undergo apoptosis and therefore close to the cliff’s edge. Which molecular mechanisms determine a cell’s distance from the cliff? What determines the apoptotic threshold of cancer cells in a population? Could it be that autophagy—an evolutionarily conserved process connected to apoptosis—is responsible for setting the apoptotic threshold? The findings in Chapter III and IV add to our understanding of what influences a cell’s decision to live or die. From this work
(Fitzwalter et al., 2018) showing that autophagy and apoptosis are directly linked via FOXO3a transcriptionally regulating PUMA levels, and previous works from the Thorburn laboratory (Gump et al., 2014), I pose the following hypothesis: the level of autophagy preceding a death stimulus directly determines how primed that cell is to undergo apoptosis. Consistent with this hypothesis, is my data showing that I can ‘unlink’ autophagy and apoptosis by mutating the FHRE in the PUMA gene to reverse the apoptosis sensitivity conferred by autophagy inhibition (Fitzwalter et al., 2018).

Studies moving forward should utilize a method described above, BH3 profiling, to determine how primed cells are to undergo apoptosis and if the amount of autophagy flux is directly related. All cells undergo a basal amount of autophagy flux at any given moment; suppose that we could sort these cells into high and low autophagy and then subject them to BH3 profiling. If autophagy determines the apoptotic threshold, then I would expect that cells with low flux may be more primed to undergo apoptosis than high autophagy cells. Answering these questions may provide an explanation for why inhibiting autophagy in cancer patients is proving to be beneficial.

**ASAP can rapidly block autophagy**

Existing methods to inhibit autophagy are either slow (~48 hours), non-specific, or both; the experiments performed in Chapters III-IV used shRNA knockdown, CRISPR/Cas9-mediated knockout, or pharmacological blockage of autophagy. Although using these methods allowed me to uncover interesting biology linking autophagy and apoptosis, as I will discuss, some questions remain unanswerable using these existing methods. There is a need to develop genetic tools to rapidly or spatially manipulate autophagy, either inter- or intracellularly. With the advent of a new tool, future studies
could test hypotheses that are not possible to investigate with current methods of autophagy inhibition.

To begin to tackle unanswered questions in the field of autophagy and cell death, I describe in Chapter VI a novel optogenetic system that can rapidly inhibit autophagy with light in cancer cells using 405 nm light. By utilizing a SNARE protein with an N-terminal truncation (STX17ΔNTD) that has been described previously (Uematsu et al., 2017), the fusion of the autophagosome and the lysosome is blocked. In the dark, STX17ΔNTD is sequestered at the mitochondrion away from sites of autophagosome-lysosome fusion, and autophagy is functional. However, upon 405nm light incidence, STX17ΔNTD is released and can act to inhibit autophagosome-lysosome fusion. An essential feature of this system is the use of an optogenetic tool called PhoCL (Zhang et al., 2017). I combined the two tools, PhoCL and STX17ΔNTD, and termed this optogenetic system ASAP for Autophagic SNARE for Abrupt Photoinhibition (Figure 6.1). Next, we used biochemistry to show that I could use ASAP to achieve autophagy inhibition in multiple different cancer cell lines, resulting in an increase in the autophagy substrates SQSTM1/p62, FOXO3a, and an increase in LC3-II levels (Figure 6.2). To provide functional evidence that ASAP is inhibiting autophagy, I used the BT549 cancer cell that is dependent on autophagy for its survival to test ASAP (Figure 6.3). We hypothesized that if I expressed ASAP in BT549 cells and expose them to 405nm light (compared to a dark control), they would undergo cell death. Indeed, I show that ASAP can dose-dependently cause rapid and transient autophagy inhibition to kill autophagy-dependent cancer cells (Figure 6.4).

Previous studies (Fitzwalter et al., 2018) showed that autophagy inhibition could
change the mode of action of the anti-cancer drug Nutlin from growth arrest to apoptosis. I wondered if I could achieve this same effect on cells using the ASAP optogenetic system to inhibit autophagy, and found that in ASAP cells the combination of 405 nm light and Nutlin treatment resulted in more growth arrest and apoptosis compared to that of the ASAP control cells (Figure 6.5). These results demonstrate that ASAP cells treated with light can effectively inhibit autophagy to improve the anti-cancer drug Nutlin.

I hypothesized that ASAP is working very rapidly (on the time scale of minutes) upon photostimulation to inhibit the autophagosome-lysosome fusion. Currently, there are no methods to measure autophagy in real time and thus test how quickly or slowly an inhibitor works. I developed another tool to determine when autophagy is inhibited by monitoring the delivery of new substrates to the lysosome for degradation in real-time by confocal microscopy. I used a fluorophore called pHRed that is strongly excited by a 561nm laser only when it resides in a low pH environment, such as a lysosome. However, at a pH-neutral environment, pHRed shows little to no fluorescence. I tagged the autophagy receptor and cargo protein p62/SQSTM1 with pHRed and observed strong co-localization of pHRed-p62 with lysosomes (Figure 6.6). I hypothesized that I could monitor the nascent delivery of p62 to lysosomes if I utilized a photobleaching workflow to observe Fluorescence Recover After Photobleaching (FRAP) (Figure 6.6). Photobleaching is an irreversible destruction of fluorescence in a region caused by short exposures to a high-intensity laser. I observed pHRed-p62 localized to lysosomes and upon photobleaching a select region of the cell, I observed rapid recovery of fluorescence. These results suggest that any fluorescence recovery is a result of new
pHRed-p62 molecules being delivered to lysosomes, and thus provides a tool for us to study autophagy substrate delivery in real time.

Using FRAP gives us the ability to monitor autophagy substrate delivery to lysosomes. This includes the ability to monitor any autophagy cargo in real time, for example the delivery of mitochondria (pHRed-Fis1) or NCOA4 (pHRed-NCOA4) to lysosomes. This powerful tool allowed us to determine that lysosomes can accommodate multiple autophagosome fusion events (Figure 6.7), and to test the hypothesis that ASAP is acting rapidly to block pHRed-p62 delivery to lysosomes via autophagic delivery (Figure 6.8). The ASAP optogenetic system and FRAP workflow of pHRed-p62 separately or in combination have capabilities to address unresolved questions in the field of autophagy and cell death. Below, I will give an overview of the potential of future uses of the ASAP system.

It is known that cancer cells induce autophagy quickly in response to chemotherapy drugs and radiation, but the effect of this chemotherapy-induced autophagy on cell death is not known (Bristol et al., 2012; Gewirtz, 2014; Wilson et al., 2011). Some studies suggest that induced autophagy during apoptosis may be protective (Lindqvist et al., 2018; Lindqvist et al., 2014). Is this drug-induced autophagy promoting cell death, preventing it, or simply an inconsequential side effect? Is the drug-induced autophagy molecularly distinct from other forms of autophagy, like starvation-induced autophagy?

A rapid genetic system is necessary to address these questions, and is not possible with current methods. Using ASAP, I can determine the effect of specifically blocking drug-induced autophagy on apoptosis; blocking drug-induced autophagy with
light may result in augmented, decreased, or unaffected apoptosis compared to cells kept in the dark. If inhibiting drug-induced autophagy using ASAP results in more apoptosis, this would suggest that the autophagy is protecting the cell from the impending death. However, if less apoptosis occurs, this suggests that autophagy may be important for mediating the cell death. Either of these scenarios may uncover the unknown significance drug-induced autophagy, and could have an influence on the way patients are treated with autophagy inhibitors in the clinic.

Future studies using ASAP could also uncover the immediate transcriptional changes, post-translational modifications, and cellular adaptation mechanisms following genetic autophagy inhibition. Additionally, ASAP could be used in vivo in transparent organisms, like zebrafish to test the contribution of autophagy to physiological processes. One possibility would be to express ASAP under a cell type-specific promoter to determine the role of autophagy in a particular tissue or during a particular time during development. Zebrafish models of neurodegeneration, cancer, and ageing are currently available to use for these studies (Martin-Jimenez et al., 2015; Mione and Trede, 2010).

Concluding remarks

In conclusion, I have described a fundamental molecular mechanism that links autophagy and apoptosis, and I leverage this mechanism to show that autophagy inhibition causes anti-cancer drugs to become more effective at inducing apoptosis. In addition to increased apoptosis sensitization, this mechanism is able to change the mode of action of an MDM2-targeted drug from growth arrest to cell death, suggesting that combining Nutlin therapies and autophagy inhibition could be especially efficacious.
All of these effects are reliant on a single FOXO3a binding site in the PUMA gene; simply mutating this site is sufficient to reverse these biological effects. Equally surprising is the finding that a transcription factor, FOXO3a, known to regulate many gene targets would mediate the connection between autophagy and apoptosis through transactivation of a single BH3 protein, PUMA. Further, the regulation of FOXO3a activity by autophagic degradation, but not proteasomal degradation, suggest that there are still interesting aspects of FOXO3a biology to be uncovered. To my knowledge, this work provides the first demonstration that autophagy can selectively degrade a transcription factor to influence its transcriptional activity.

I have discussed work from the Letai laboratory and stressed the importance of the apoptosis threshold in cancer cells, and how chemotherapies are effective by taking advantage of the difference in normal cells and cancer cells. The most important implication of this Thesis work is that autophagy directly determines the apoptotic threshold in cancer cells, and by manipulating autophagy we can push cells to commit to apoptosis. By combining autophagy inhibitors and anti-cancer drugs, we create an opportunity to improve cancer therapeutics by expanding the therapeutic window.

In this Thesis, I show the development of a powerful optogenetic system, ASAP, to rapidly inhibit autophagosome-lysosome fusion. Current methods to block autophagy are slow and non-specific, but ASAP provides both rapid and genetic autophagy inhibition with small light doses. The advent of this method will allow us to gain mechanistic insight into how autophagy regulates cell death and other cellular processes.
This thesis provides a body of work that begins to make sense of the molecular links between autophagy and cell death. Continued efforts to gain mechanistic insight will bring us closer to understanding the precise consequences of altering autophagy in patients; we have only just begun to explore the potential of targeting this evolutionarily-ancient pathway.
REFERENCES


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