CELL DEATH RESPONSES TO NUTRIENT DEPRIVATION

By

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To my family, friends, and colleagues for their continued support.

ABSTRACT

Programmed cell death has traditionally been viewed as the induction of cell-autonomous suicide, but recent data suggest that cell death programs can also be activated non-cell autonomously. Among these, entosis, the process by which live neighboring cells are taken up by host cells and subsequently killed, occurs in human cancers and enables nutrient recovery from degraded neighbors. While the ability of entosis to provide nutrients to host cells has been established, whether nutrient withdrawal itself can induce this process is unknown. Here we identify glucose starvation as a potent inducer of entosis in both normal and tumor cell populations. We find that during glucose withdrawal, entotic cell uptake by winner cells is controlled by the energy-sensing kinase AMPK that is activated specifically within loser cells. We show that glucose starvation results in the appearance of two distinct cell populations based on their deformability, a characteristic that is predicted to result in high levels of entosis. Consistent with the model of AMPK controlling loser cell behavior, the appearance of less deformable, stiffer cells within the population is inhibited by blocking AMPK activity. Within heterogenous cell populations, engaging in this type of cell death promotes a type of cell competition that allows a subset of cells (winners) to outcompete neighboring loser cells. This competitive elimination supports the proliferation of winner cells during prolonged nutrient stress, suggesting that entosis can act as a cellular response to metabolic changes through extracellular nutrient scavenging.

BIOGRAPHICAL SKETCH

Jens Christian Hamann was born on August 27, 1990, to Barbara Susanne Hamann and Dr. Ingo Hamann in Gelnhausen, Germany. He lived in Bad Orb, Germany, with his parents and brother, Henning Hamann, until moving to Sant Just Desvern, Spain, in the spring of 1995. There, he attended the Deutsche Schule Barcelona from kindergarten through sixth grade, when, in 2002, he moved to Janesville, Wisconsin, where he finished seventh and eighth grade at Marshall Middle School, and ninth and tenth grade at Joseph A. Craig High School. In 2006, he moved to Chesterfield, Virginia, and in 2008 graduated with a high school diploma from Cosby High School in nearby Midlothian, Virginia. To pursue his interest in the biological sciences, Jens attended Virginia Polytechnic Institute and State University (Virginia Tech), where he performed research in the lab of Dr. Carla V. Finkielstein studying the regulation of cellular stress responses by circadian rhythms. After graduating magna cum laude with a Bachelor of Science degree in biochemistry in the spring of 2012, he enrolled in the Louis V. Gerstner, Jr. Graduate School of Biomedical Sciences at Memorial Sloan Kettering to further develop his interests in biomedical research. At Memorial Sloan Kettering, he worked in the lab of Dr. Michael Overholtzer examining the role and regulation of cell death pathways and their effects on cell populations.

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4E-BP1: eukaryotic translation initiation factor 4E-binding protein 1

ACC: acetyl-CoA carboxylase

AICAR: 5-aminoimidazole-4-carboxamide ribonucleotide

AKT: RAC-alpha serine/threonine-protein kinase

AMPK: 5’ AMP-activated protein kinase

AMPKAR: AMPK activity reporter

APAF1: apoptotic protease activating factor 1

Arf: ADP ribosylation factor

ARHGEF2: Rho/Rac guanine nucleotide exchange factor 2

ASC: Apoptosis-associated speck-like protein containing a caspase activation and recruitment domain (CARD)

ASCT: alanine/serine/cysteine/threonine transporter

ATF6: activating transcription factor 6

ATG: autophagy related

ATL: adipose triglyceride lipase

BAIAP2/BAIAP2L1: BAI1 associated protein 2/ BAI1 associated protein 1 like 1

Bak: Bcl-2 antagonist/killer

Bax: Bcl-2 associated X

Bcl-2: B-cell lymphoma 2

Beclin1: coiled-coil, moesin-like BCL-2 interacting protein 1

Bid: BH3-interacting domain death agonist

Bim: Bcl-2-like protein 11

BiP/Grp78: Binding immunoglobulin protein/glucose-regulated protein 78

CAD: caspase-activated DNase

CAMKK2: calcium/calmodulin-dependent protein kinase kinase 2

Cas9: CRISPR associated protein 9

CASTOR: cellular arginine sensor for mTORC1

CBS: cystathionine-β-synthase

CPT1: carnitine palmitoyltransferase 1

CRISPR: clustered regularly interspaced short palindromic repeats

CTNND1: catenin delta 1

DAMPs: damage associated molecular patterns

DFNA5: deafness, autosomal dominant 5

eF2K: elongation factor 2 kinase

EGF: epidermal growth factor

eIF4E: eukaryotic translation initiation factor 4E

ER: endoplasmic reticulum

FADD: Fas associated via death domain

FasR: Fas cell surface death receptor

FOXO: forkhead box protein O

FRET: fluorescence resonance energy transfer

GAP: GTPase activating protein

Gas6: growth arrest specific 6

GATOR1/2: GTPase activating protein activity towards Rags

GEF: guanine nucleotide exchange factor

GFAT1: glucosamine–fructose-6-phosphate aminotransferase isomerizing 1

GLUT: glucose transporter

GPT: GlcNAc phosphotransferase

GPX: glutathione peroxidase

HOPS: homotypic fusion and vacuole protein sorting

IL: interleukin

IRE1α: inositol-requiring enzyme 1α

JNK: c-Jun N-terminal kinase

K-RAS: Kirsten rat sarcoma viral oncogene homolog

KICSTOR: KPTN, ITFG2, C12orf66 and SZT2-containing regulator of mTORC1

LAMP1: lysosomal associated membrane protein 1

LAP: LC3-associated phagocytosis

LC3: microtubule-associated proteins 1A/1B light chain 3B

Lgl: lethal giant larvae

LKB1: liver kinase B1

mDia1: diaphanous related formin 1

MerTK: MER proto-oncogene tyrosine kinase

MHC: major histocompatibility complex

mLST8: mammalian lethal with Sec13 protein 8

MOMP: mitochondrial outer membrane potential

MPA: micropipette aspiration

MTHFD2: methylenetetrahydrofolate dehydrogenase (NADP+ dependent) 2

mTOR: mechanistic target of rapamycin

mTORC1/2: mTOR complex 1/2

NDP52: nuclear dot protein 52

NET1A: neuroepithelial cell transforming 1A

OPTN: optineurin

Parkin: Parkinson protein 2, E3 ubiquitin protein ligase

PDAC: pancreatic ductal adenocarcinoma

PDZ-RhoGEF: post-synaptic density protein (PSD95), Drosophila disc large tumor suppressor (Dlg1), zonula occludens-1 (ZO-1)-RhoGEF

PERK: protein kinase R-like endoplasmic reticulum kinase

PFK1: phosphofructosekinase-1

PFKFB3: 6-phosphofructo-2-kinase/fructose-2,6- bisphosphatase 3

PI3-K: phosphatidylinositol-4,5-bisphosphate 3-kinase

PIKfyve: phosphoinositide kinase, FYVE-type zinc finger containing

PINK1: PTEN induced putative kinase 1

PtdSer: phosphatidylserine

Rac1: Ras-related C3 botulinum toxin substrate 1

Rag: Ras-related GTP-binding protein

Raptor: regulatory protein associated with mTOR

Rheb: Ras homolog enriched in brain

RhoA: Ras homolog family member A

Rictor: rapamycin insensitive companion of mTOR

RIP1/3: receptor-interacting serine/threonine-protein kinase 1/3

ROCK1: Rho-associated, coiled-coil-containing protein kinase 1

RUBICON: RUN domain Beclin 1-interacting and cysteine-rich containing protein

S6K1: ribosomal protein S6 kinase beta-1

SLC38A9: solute carrier family 38 member 9

Smac: second mitochondria-derived activator of caspase

SREBP1: sterol regulatory element-binding protein 1

TFEB: transcription factor EB

TLR: toll-like receptor

TNFR1: tumor necrosis factor receptor 1

TRADD: TNFRSF1A associated via death domain

TRAIL-R: tumor necrosis factor-related apoptosis-inducing ligand receptor

TSC: tuberous sclerosis complex

TUNEL: Terminal deoxynucleotidyl transferase dUTP nick end labeling

UBA: ubiquitin associated

UDP-GlcNAc: uridine diphosphate *N*-acetylglucosamine

ULK1: unc-51 like autophagy activating kinase 1

UPR: unfolded protein response

UVRAG: UV-radiation resistance associated gene product

VPS34: vacuolar protein sorting 34

WNT: wingless-related integration site

α-KG: α-ketoglutarate

CHAPTER 1: Introduction

1.1 CELL DEATH PATHWAYS

The induction of cell death contributes to sculpting and maintaining tissues during metazoan development and adulthood and can inhibit the development of diseases such as cancer. While some cell death events are thought to occur accidentally due to irreparable cellular damage, the majority of deaths are executed actively by the initiation of specific signaling events in response to cellular insults (Green and Llambi, 2015). Early studies that described cell death events identified different types based largely on morphological changes of cells, resulting in the classification of deaths into three major types based on their appearance: type I, II, and III, which correspond to apoptotic, autophagic, and necrotic types of cell death, respectively (Figure 1.1) (Galluzzi et al., 2007).

While apoptosis, or Type I cell death, was the first-described mechanism of regulated cell death (the term apoptosis was coined in 1972 (Kerr et al., 1972)), it later became clear that the other morphological forms of cell death can also be regulated and do not simply occur accidentally (Galluzzi et al., 2018). Further, although many of the known deaths are controlled cell-autonomously and are therefore termed cellular “suicides,” non-cell autonomous mechanisms of death have recently been described, suggesting that a cell’s demise can also be controlled by neighboring cells in a form of cellular “murder” (Overholtzer and Brugge, 2008). Together, these findings have contributed to a rapidly expanding research field of programmed cell death and they raise an important question: why are there so many ways to die? In this thesis, we examined cell death

responses occurring in response to nutrient starvation, and uncovered a coordinated response involving multiple death programs, including entosis, which provides a unique population-scale effect as compared to other forms of cell death. In the following sections the different forms of known cell death mechanisms will be introduced and their regulation discussed.

1.1.1 Apoptosis

Apoptosis is characterized by distinct morphological changes, including plasma membrane blebbing and chromatin condensation that occur early during execution, and the fragmentation of nuclei and cells into subcellular pieces called “apoptotic bodies” upon cell death (Figure 1.1) (Kerr et al., 1972). As the intracellular contents of apoptotic cells are contained within intact pieces of the plasma membrane, this form of cell death is thought to shield surrounding cells from toxic intracellular contents (Poon et al., 2014). This also shields apoptotic cells from detection by the immune system and as a result, apoptosis is widely considered to be immunologically silent (Poon et al., 2014).

The genetic machinery that controls the execution of the apoptotic cell death program primarily involves the activity of caspases, cysteine proteases that cleave target proteins and thereby disrupt their normal function, leading to cellular damage and death (Crawford and Wells, 2011). The two major types of signaling pathways that impinge on caspase activation and apoptosis execution, termed the extrinsic and intrinsic pathways, are controlled by binding of ligands to their cognate death receptors, such as TNFR1, TRAIL-R, or FasR, and by

intracellular damage signals, such as DNA damage or growth factor deprivation, respectively (Taylor et al., 2008).

Upon binding of extracellular death ligands, initiator caspases, such as caspase-2, -8, and -9, are activated by dimerization that promotes their ability to cleave executioner caspases, such as caspase-3, -6, and -7 (Salvesen and Riedl, 2008). Executioner caspases have a wide variety of intracellular substrates, which upon cleavage can either become activated or inactivated. For example, caspase-mediated cleavage of the RhoA effector ROCK1 drives constitutive activation independent of upstream GTPase regulation that re-organizes the actin network and promotes blebbing of the plasma membrane, a morphological characteristic of apoptotic cell death (Coleman et al., 2001). Caspase-mediated activation of another effector called CAD, through cleavage of its inhibitor protein ICAD, leads to chromatin condensation and cleavage, another hallmark of apoptotic cell death (Sakahira et al., 1998). High levels of effector caspase activity therefore result in the disruption of multiple cellular components, which collectively dooms a cell's ability to survive.

The intrinsic apoptotic pathway can be activated by a variety of different intracellular damage signals, for example DNA damage, ER stress, and growth factor and nutrient deprivation (Okada and Mak, 2004). In response to these stresses, pro-apoptotic Bcl-2 family proteins, such as Bax and Bak, oligomerize within the outer mitochondrial membrane, resulting in MOMP that allows mitochondrial contents, such as cytochrome *c* and Smac, to enter the cytosol (Tait and Green, 2010). Formation of the apoptosome complex is dependent on

Apaf-1 oligomerization, induced by cytochrome *c* binding, and the subsequent recruitment and activation of caspase-9 (Yu et al., 2005). The release of Smac from the mitochondria also increases apoptosome activity by binding to, and inhibiting XIAP, a protein capable of binding and inhibiting caspases (Jost et al., 2009).

In addition to death receptor ligand binding or high levels of irreparable cellular damage, the loss of specific pro-growth signals can also induce apoptosis. Starvation for key nutrients and the loss of attachment to extracellular matrix have both been shown to lead to the induction of apoptosis. Cells that receive pro-survival signals from integrin receptors undergo apoptosis (called anoikis in this context) as a result of upregulation of pro-apoptotic BH3-only proteins (e.g. Bim and Bid) that cause the oligomerization of Bax and Bak and engagement of the intrinsic pathway (Reginato et al., 2003). Deprivation for key nutrients can also induce apoptosis through BH3-only protein upregulation; in case of starvation for glucose this has been attributed to a different BH3-only protein called Noxa (Alves et al., 2006). Thus, a large set of stress conditions and signals are known to regulate cell death occurring through apoptosis. How these signals also impinge upon other alternative, non-apoptotic forms of cell death will be discussed in the following sections.

1.1.2 Autophagic cell death

Autophagy is a cellular process of self-eating that is often activated in response to metabolic stress in an attempt to restore energetic balance (discussed in more detail in Chapter 1.3.1 on page 31 below) (Mizushima et al., 2008). It is therefore

usually thought of as a survival mechanism activated by cells and, as such, inhibition of this process can lead to cell death, often through apoptosis (Kroemer and Levine, 2008; Shen et al., 2012). Historically, the idea that autophagy could instead promote cell death was based largely on the morphological appearance of dying cells that accumulated the intracellular vesicle mediators of this pathway, called autophagosomes, while genetic evidence for a causative role for autophagy in cell death was lacking (Figure 1.1). More recently, though, using genetic inactivation of autophagy genes in the *Drosophila* midgut and salivary gland, it was shown that autophagy can directly contribute to positively regulating cell death, as autophagy inhibition delayed the clearance of these organ structures that is known to occur through programmed cell death (Berry and Baehrecke, 2007). Autophagy induction in this context is thought to occur primarily in response to transcriptional upregulation of ATG1 (ULK1 in humans), a key mediator of autophagy, occurring downstream of hormone signaling that initiates gland removal in development, rather than as a result of starvation.

Recently a potentially related form of autophagy-regulated cell death was reported in cultured cells, termed autosis, which can be triggered by hyperactivation of autophagy, either by starvation or by using a Tat-Beclin 1 peptide, implying that excessive autophagy can indeed trigger cell death (Liu et al., 2013). Beclin 1 is a key regulator of autophagy that functions as a binding partner for the Vps34 phosphoinositide kinase to induce autophagy (Klionsky, 2005). Autosis was argued to be distinct form other forms of cell death, as it does not share major morphological or genetic features with any known cell death

mechanisms (Liu et al., 2013). For example, autosis cannot be rescued by pharmacological inhibition of caspases that are key controllers of apoptosis, nor by inhibition of RIP1 kinase that regulates programmed necrosis (discussed in more detail on the next page), nor by deletion of pro-apoptotic proteins, such as Bax and Bak. Autosis is instead dependent on Na^+,K^+ ATPase, a cation anti-porter that maintains ion homeostasis and can regulate a variety of signaling pathways (Liu et al., 2013; Newman et al., 2008).

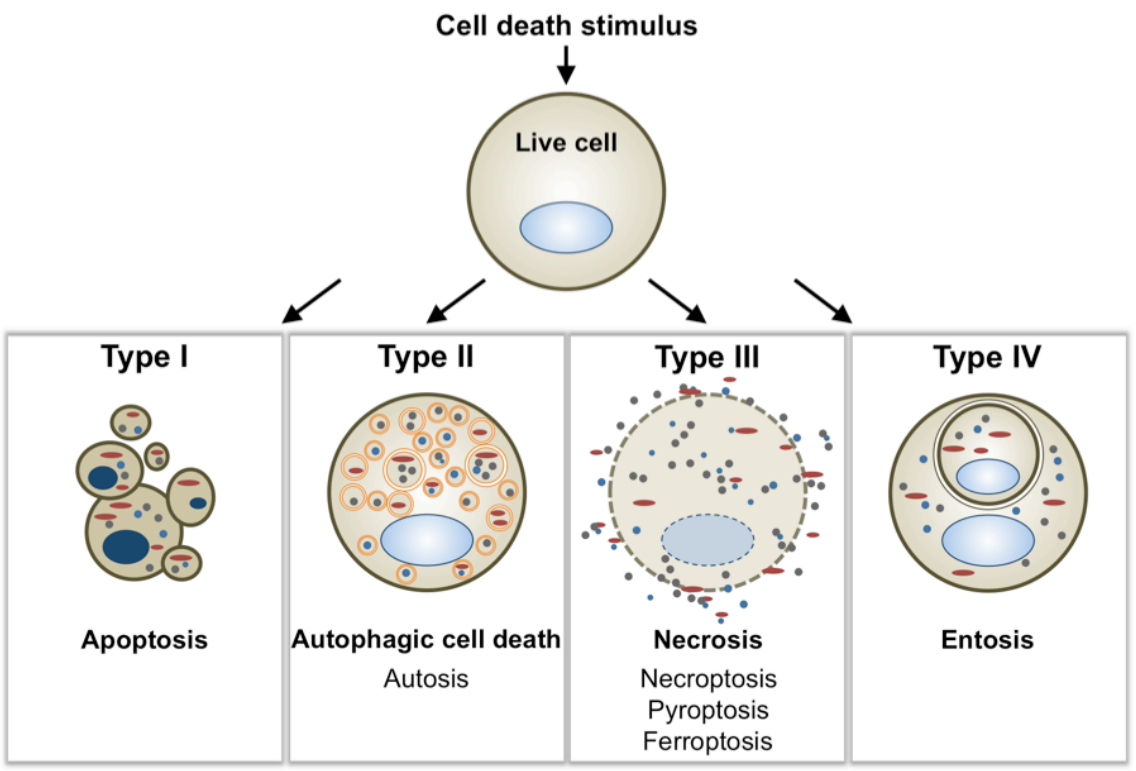


Figure 1.1. Types of cell death
Cell death can occur through multiple different mechanisms. The historical classification of cell death mechanisms (types I, II, and III) is shown, in addition to a proposed type IV cell death, entosis. Apoptotic cell death results in the fragmentation of the cell while maintaining plasma membrane integrity, which retains intracellular components (blue, grey, and red shapes). Autophagic cell death is characterized by the apperance of double-membrane autophagosomes that contain intracellular cargo. Autosis is a proposed mechanism of autophagic death induced by hyperactivation of autophagy. Type III necrotic deaths allow intracellular contents to leak out of the cell, as a result of either plasma membrane rupture or pore formation. Uptake of live cells and their "murder" by host cells by entosis has been proposed as type IV cell death, as it does not share any genetic or morphological features of the other types listed.

While the physiologic functions of autophagy-dependent cell death in contexts beyond *Drosophila* development remain uncertain, under stress conditions this form of regulated cell death could contribute to disease pathology. For example, non-apoptotic cell death occurring in response to brain ischemia has been shown to be blocked by pharmacological inhibition of autophagy or by genetic inactivation of core autophagy genes (Koike et al., 2008; Wen et al., 2008). Moreover, cardiac glycosides, steroidal compounds that bind and inhibit Na^+,K^+ ATPase, and thereby block autotic cell death, have also been shown to inhibit brain injury in murine models, suggesting this type of death might indeed be relevant in this context (Dunn et al., 2011; Liu et al., 2013). The molecular mechanism underlying how autophagy genes, and presumably the formation of autophagosomes, contribute to promote the execution of cell death remains an important, open question in the field.

1.1.3 Necrosis

Unlike apoptotic or autophagic cell death, necrotic cell death is defined by a rapid loss of plasma membrane integrity that results in the release of cytoplasmic contents into the extracellular space (Figure 1.1) (Yuan and Kroemer, 2010). In contrast to apoptosis, this type of death is considered highly immunogenic, as the lysis of cells releases DAMPs and other inflammatory cytokines that have the potential to activate cells of the immune system (Yuan and Kroemer, 2010). Historically, necrosis was thought to occur accidentally and, in contrast to apoptosis, not regulated by specific signaling pathways. Secondary necrosis was originally used to describe morphological changes similar to necrosis that

occurred independently after apoptotic or autophagic cell death was executed (Silva, 2010). Recently, however, types of necrosis have been described that are regulated and provoked by specific signaling events (Brennan and Cookson, 2000; Degterev et al., 2005; Dixon et al., 2012). These different forms of necrotic cell death will be discussed in the following sections.

1.1.3.1 Necroptosis

Necroptosis is a form of regulated necrosis mediated by the kinase RIP3 that is activated downstream of TNFR. Upon ligand binding, RIP1 is recruited to TNFR and ubiquitylated by cIAP1/2, which results in the formation of a pro-survival complex (Feoktistova et al., 2011). Upon inhibition of cIAP1/2 or deubiquitylation of RIP1, RIP1 and TRADD are released and form a cytosolic complex that is competent to activate both apoptotic and necrotic cell death programs. In addition, expression of FLIP can form heterodimers with caspase-8, which maintains inhibitory catalytic activity towards RIP3 but is unable to induce apoptosis, resulting in cell survival (Krueger et al., 2001). TRADD and RIP1 can recruit FADD and caspase-8, which results in caspase activation and cell death by apoptosis (Yuan et al., 2016). However, in the absence of FADD or caspase-8, or when caspases are blocked pharmacologically, RIP1 can bind and phosphorylate RIP3, forming the RIPoptosome, and trigger necroptosis (Tenev et al., 2011). The phosphorylation of RIP3 by RIP1 is essential for necroptosis induction and can be blocked by the small molecule necrostatin (Degterev et al., 2005). Active RIP1 also phosphorylates the pseudokinase MLKL, inducing its oligomerization and insertion into the plasma membrane, where it is thought to

generate a pore that results in the disruption of membrane asymmetry and integrity that causes necroptotic death (Cai et al., 2014). This complex regulatory mechanism allows for context-specific cell survival or induction of apoptotic or necrotic cell death. Necroptosis can be induced by viral infection upon detection of double-stranded viral DNA by the DNA sensor DAI that can bind and activate RIP3 through its RHIM domain, suggesting this type of programmed death may occur in vivo (Upton et al., 2012).

1.1.3.2 Pyroptosis

Pyroptotic cell death was initially described as a death mechanism in immune cells, including macrophages and dendritic cells, that were infected by certain pathogens, such as the enteric bacteria *Shigella flexneri* and *Salmonella typhimurium* (Shi et al., 2017). Induction of pyroptosis is dependent on caspases that are similar in function to initiator caspases required for apoptosis (caspase-2, -4, and -5 in humans). Binding of pathogen proteins, such as flagellin, to caspase-1, either directly or through the adaptor protein ASC, results in the formation of a “canonical inflammasome” (Hornung et al., 2009). The downstream effector of caspase-1 that is responsible for pyroptotic execution is Gasdermin D, a protein that, upon caspase-mediated cleavage, can oligomerize within the plasma membrane, resulting in pore formation and death (Kayagaki et al., 2015; Shi et al., 2015). CD4+ T cells infected with HIV have been shown to die through this mechanism, leading to increased T cell depletion while at the same time propagating this effect by increasing recruitment of immune cells that are then also destined to die this way (Doitsh et al., 2014). Intriguingly, these

data suggest a therapeutic approach for HIV-infected patients that involves targeting of the pyroptotic machinery in host cells, rather than eliminating the virus directly (Doitsh et al., 2014). Several caspase-1 inhibitors are currently in clinical trials and have reported to be well tolerated in humans (Vezzani et al., 2010). Preliminary studies using one of these, VX-765, in human ex vivo samples of HIV-infected tissues blocked T cell pyroptosis, suggesting inhibition of this mode of cell death may be a viable treatment strategy in this context (Doitsh et al., 2014).

1.1.3.3 Ferroptosis

The induction of the iron-dependent cell death program ferroptosis is thought to occur due to lethal accumulation of lipid peroxides that disrupt plasma membrane integrity (Dixon et al., 2012; Stockwell et al., 2017). Ferroptosis was initially described as a cellular response to the small molecule erastin, an inhibitor of system X_c^- that normally imports cystine into cells (Dixon et al., 2012). As cysteine is a key component of the tri-peptide antioxidant molecule glutathione, erastin treatment results in glutathione depletion and subsequent accumulation of reactive oxygen species that are toxic to cells. Loss of cell viability in this context appears to occur due to specific inactivation of a key lipid peroxide detoxifying enzyme, GPX4, that uses glutathione as a cofactor (Stockwell et al., 2017). Indeed, genetic deletion or pharmacological inactivation of GPX4 leads to ferroptosis induction similar to erastin treatment (Yang et al., 2014).

Interestingly, ferroptosis was recently shown to spread in a wave-like manner, where the death of one cell seemed to induce the death of neighboring

cells, suggesting an intriguing non-cell autonomous effect that could lead to the elimination of an entire cell population (Kim et al., 2016). Induction of ferroptosis in a xenograft tumor model by treatment with ultrasmall nanoparticles was observed to significantly reduce tumor burden, suggesting that ferroptosis may indeed have strong population-scale effects that could be used to target cancer, although whether this wave-like spreading occurs in vivo remains to be examined (Kim et al., 2016). Ferroptosis has also been shown to be the mode of cell death occurring in models of acute kidney injury, and this death appears to spread in a similar wave-like manner in ex vivo renal tubules, suggesting this spreading mechanism may be a common feature of ferroptosis (Linkermann et al., 2014).

Like other forms of cell death, including apoptosis, ferroptosis is regulated by the abundance of key cellular nutrients. Iron plays an important role in regulating a wide variety of cellular processes, for example by acting as a cofactor for proteins such as hemoglobin and iron-sulfur cluster proteins, and therefore maintaining iron homeostasis is essential for cell viability (Pantopoulos et al., 2012). Within the body, iron is most frequently delivered to cells via transferrin through binding to plasma membrane transferrin receptors and is subsequently delivered to the iron storage complex ferritin (Pantopoulos et al., 2012). To properly balance the need for intracellular iron, cells have evolved a form of autophagy, termed ferritinophagy, that allows cells to selectively degrade iron when necessary (Mancias et al., 2014). It was recently shown that disruption of iron homeostasis, by genetic inactivation of iron-sulfur cluster biosynthesis, triggered ferroptosis, and in a mouse model of lung adenocarcinoma, which

experiences high oxygen tension and oxidative damage, suppression of this pathway blocked tumor growth (Alvarez et al., 2017). These data suggest that, in a context-specific manner, levels of iron need to be carefully regulated to promote cell viability, and disruption of this balance may induce ferroptotic cell death.

As system X_c^- is a cystine-glutamate antiporter, high levels of extracellular glutamate, as is observed in neurological damage (Morrison et al., 2002), can induce ferroptosis by inhibiting cystine uptake (Dixon et al., 2012). Glutamine metabolism was also recently shown to regulate ferroptosis, as glutaminolysis produces α-KG, a metabolite required for ferroptosis induction (Gao et al., 2015). While the mechanism underlying how α-KG contributes to ferroptosis induction is unknown, starvation for both glutamine and cysteine inhibits ferroptosis induction, demonstrating a stringent requirement for one key nutrient to promote cell death by depletion of another (Gao et al., 2015). Given these data, it will be important to understand the regulation and implications of ferroptosis induction in a variety of different contexts, for example in cancers where nutrient availability and cellular metabolism are frequently altered.

1.1.4 Entosis

In addition to cell-autonomous death, cells can also engage in the killing of neighboring cells (Figure 1.1). While the engulfment of cells is mostly considered in the context of uptake and clearance of dead cells, many studies have shown that live cells may also be taken up by engulfing cells (Overholtzer and Brugge, 2008). The resulting structures, termed cell-in-cell structures, describe live cells

that are completely internalized within a vacuolar compartment of the host cell, an observation that dates back more than a hundred years (Humble et al., 1956; Overholtzer and Brugge, 2008).

Some cell-in-cell structures that have been observed are called heterotypic because they involve different cell types, typically leukocytes internalized inside of other cells (Overholtzer and Brugge, 2008). Entosis refers instead to homotypic events, typically involving epithelial cells or carcinoma cells ingesting each other, which frequently results in non-apoptotic death (i.e. entotic death) of the internalized cells (Overholtzer et al., 2007). This process of live cell uptake and killing was originally described in matrix-detached conditions and requires adherens junctions and a differential in cell cortex tension that is regulated by actomyosin contractility (Overholtzer et al., 2007; Sun et al., 2014a; Sun et al., 2014b). The maintenance of junctions between target and host cell is required for entosis and is mediated by epithelial cadherins and can be observed by increased immunostaining at the interface between the two cells (Overholtzer et al., 2007). Cadherins are a family of cell adhesion molecules that mediate attachment of neighboring cells to each other and are involved in a wide range of cellular functions, including maintenance of epithelial integrity and collective migration of cells (Leckband and de Rooij, 2014). Cadherins contain a cytoplasmic tail that mediates binding to proteins, such as β-catenin and p120-catenin, which can regulate the attachment to the underlying cytoskeleton and the activity of myosin (Parsons et al., 2010). As epithelial-type cadherins, such as E- and P-cadherin, are required for entotic cell uptake, cells lacking these are

deficient for entosis, but can be induced to undergo entosis by exogenous expression of either E- or P-cadherin (Sun et al., 2014a).

In addition to adherens junctions, entosis requires altered actin dynamics between neighboring cells that are driven by RhoA activation that leads to downstream ROCK1-mediated phosphorylation of myosin light chain (Overholtzer et al., 2007). Recent evidence suggests PDZ-RhoGEF acts upstream of RhoA to specifically increase actin assembly at the rear cortex of invading cells that forms a uropod-like structure (Purvanov et al., 2014). This increase in actin polymerization at the invading cell cortex can be mediated by mDia1 that regulates cell blebbing necessary for entotic invasion (Purvanov et al., 2014). Blebbing is also regulated at the transcriptional level, demonstrating a role for active transcription in entosis (Hinojosa et al., 2017). Through a positive feedback mechanism, the SRF transcription factor localizes to the nucleus in response to cell blebbing, increasing expression of Ezrin, and transcriptional induction of Ezrin is required for cells to continue blebbing to drive internalization (Hinojosa et al., 2017). Also contributing to the polarization of RhoA activity and actomyosin contraction is the recruitment of p190A RhoGAP to cell junctions, resulting in a polarized distribution of RhoA activity away from the junction that serves to increase cortical actomyosin contraction to drive entotic internalization (Sun et al., 2014a). Overall, this junction-mediated mechanism of cell uptake is in contrast to other engulfment programs that are characterized by cytoskeletal rearrangements induced by Rac1 activation specifically within engulfing cells (Segawa and Nagata, 2015).

Upon internalization, cells can undergo a variety of fates, including release, division, or most frequently, non-apoptotic death (Figure 1.2) (Overholtzer et al., 2007). Entotic cell death involves a mechanism similar to LAP, a process first described in phagosome maturation, where LC3, a protein involved in autophagy, is transiently lipidated onto the single membrane entotic vacuole to drive its maturation (Figure 1.3; described in more detail in Chapter 1.3.1 on page 31 below) (Florey et al., 2011; Sanjuan et al., 2007). Canonical autophagy induction is dependent on the activation of the ULK1 initiation complex that signals to induce autophagosome formation (Klionsky, 2005). In contrast, LC3 lipidation onto single-membrane vacuoles in LAP does not require the upstream ULK1 initiation complex (Sanjuan et al., 2007). While activation of TLRs is required for LAP in the context of phagocytosis (Sanjuan et al., 2007), the exact trigger in entotic host cells is unclear. It was recently shown that osmotic changes within the vacuole are sufficient to drive LC3 recruitment,

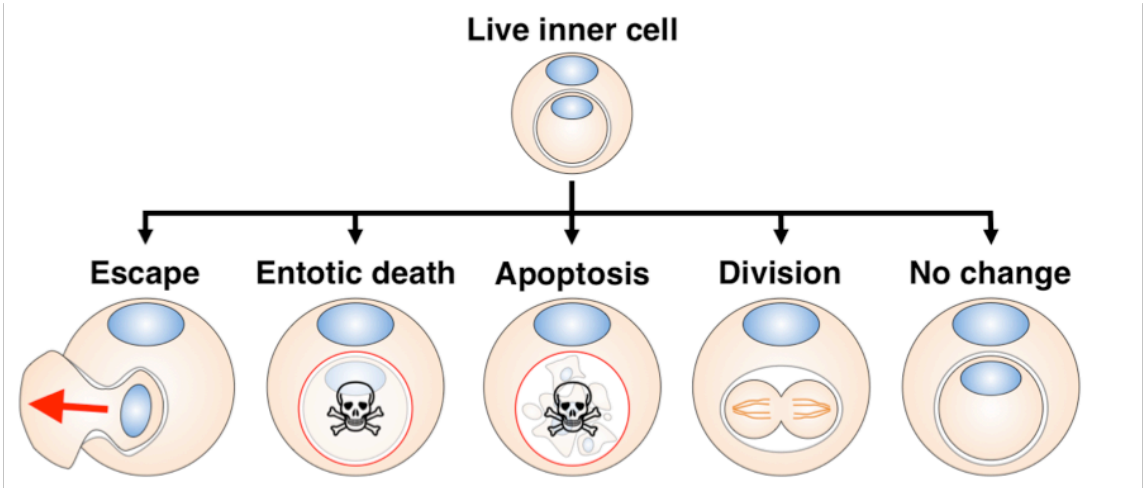


Figure 1.2. Fates of live cells internalized by entosis
Upon completion of engulfment, cells can have a variety of cell fates. Cells can escape from their hosts and remain viable, or they can undergo entotic or apoptotic death after internalization. Alternatively, inner cells may also divide, after which they can further undergo the above-mentioned fates. Depending on the length of timelapse analysis, the fate of some cells can remain unchanged.

suggesting that altered ion balance as a result of cell uptake, for example, leads to vacuole maturation (Florey et al., 2015).

The ensuing acidification of the vacuole then leads to non-apoptotic death of the inner cell (Figure 1.3) (Florey et al., 2011; Overholtzer et al., 2007). The corpse is then further degraded, allowing the vacuole to shrink in a fission process dependent on mTORC1 and PIKfyve signaling (Krajcovic et al., 2013; Krishna et al., 2016). The non-apoptotic death can be prevented by either blocking autophagy protein (e.g. ATG5) function, which inhibits LC3 recruitment, or lysosomal acidification (e.g. ConA-mediated inhibition of the lysosomal v-ATPase) (Florey et al., 2011). Frequently, this disruption in entotic cell death results in the apoptotic death of internalized cells, which can be blocked by caspase inhibition or overexpression of anti-apoptotic proteins, such as Bcl-2

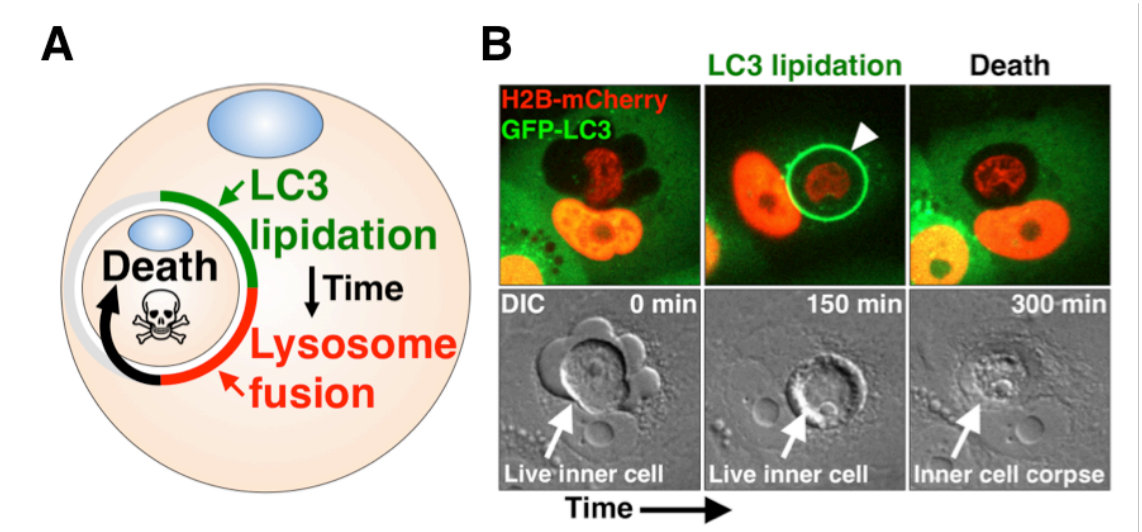


Figure 1.3. Mechanism of entotic cell death
(A) Maturation of entotic vacuole includes transient LC3 lipidation prior to death. Upon engulfment, host cells mature the single-membrane vacuole, including LC3 lipidation and subsequent lysosome fusion that results in acidification and non-apoptotic cell death. (B) Representative image sequence depicting maturation steps, beginning after completion of engulfment. Note the clear recruitment of GFP-LC3 (white arrowhead) onto the vacuole and the subsequent cell death. The inner cell undergoes non-apopototic cell death, as evidenced by the absence of blebbing and nuclear condensation. The corpse will continue to degrade and shrink after this time.

(Florey et al., 2011; Overholtzer et al., 2007). As a result, cells can remain viable for many hours in this compartment or escape (Florey et al., 2011; Overholtzer et al., 2007), but the exact mechanism that triggers cell escape from entotic vacuoles remains to be examined.

1.1.5 Perspectives

While certain cell death mechanisms engage unique molecular players, several death programs described above are regulated by proteins that function in more than one mode of execution. As the effect of cell death on surrounding cells and tissues can vary greatly depending on how the cell died, it is important to fully understand through which mechanism cells are dying and potential co-regulation of these in a context-dependent manner.

A clear example of this co-regulation is in the context of apoptosis and necroptosis, where upstream activation of death receptors is a common theme (Green and Llambi, 2015). Depending on a cell's ability to activate caspases, one of the two death modes might be induced. The inflammatory response towards cells undergoing apoptosis or necroptosis are predicted to be vastly different, as aptoptotic cells, as opposed to necrotic ones, are thought to be cleared by phagocytes before any cellular contents can be released into the extracellular space (Poon et al., 2014).

The induction of secondary necrosis after apoptotic cell death has been observed in the absence of appropriate dead cell clearance (Silva, 2010). The exact mechanism of secondary necrosis remained unknown, as the lysis of uncleared dead cells was long thought to occur accidentally due to osmotic

swelling. Recently, however, a requirement for a gasdermin-like protein, DFNA5, was shown for secondary necrosis induction (Rogers et al., 2017). DFNA5 is cleaved by the executioner caspase-3 that is activated by apoptotic stimuli, and, similar to gasdermin D, the resulting cleaved N-terminal fragment inserts into the plasma membrane to generate pores that release intracellular components (Rogers et al., 2017). As DFNA5-mediated necrosis is independent of inflammasome activation (that is, caspase-1 and -11), this mode of cell death may still allow for inflammatory cell death induced by pathogens that produce factors that inhibit inflammasome activity, such as *Yersinia* (Lamkanfi and Dixit, 2011; Rogers et al., 2017).

Autophagy can also regulate apoptosis, as autophagic degradation of active caspase-8, for example, can inhibit TRAIL-mediated apoptosis (Hou et al., 2010). Conversely, caspase activation upon apoptotic stimuli can directly cleave components of the autophagy machinery, such as Beclin 1 and ATG16L (Luo and Rubinsztein, 2010; Murthy et al., 2014). Pro-apoptotic BH3 proteins, such as Bim, can also directly bind and sequester Beclin 1, thereby suppressing its ability to activate autophagy (Luo et al., 2012). Autophagy may also play a role in inhibiting necroptotic cell death, potentially by degrading RIP1 or decreasing ROS levels, as inhibition of this pathway induced RIP1-mediated necroptosis (Bray et al., 2012). Autophagic processes are also implicated in other non-apoptotic types of death, for example by promoting the maturation of entotic vacuoles that lead to internalized cell death, and during pyroptosis in the context of *Shigella* infection, where autophagy inhibition potentiates caspase-1-

dependent cell death (Florey et al., 2011; Suzuki et al., 2007). The exact details and contributions of autophagy proteins in regulating apoptosis and other types of cell deaths, however, remain incomplete.

1.2 ENERGY HOMEOSTASIS AND SIGNALING

As discussed above, cell survival requires coordinated signaling from growth factors that license the uptake of key nutrients, allowing for energy production and macromolecular biosynthesis (Vander Heiden et al., 2009). The loss of growth factor signaling or starvation for key nutrients renders cells susceptible to cell death, which is particularly problematic for cancer cells that have constitutive growth factor pathway activation and are often deprived of key nutrients in their microenvironment (Jones and Thompson, 2009). Cells have adapted various signaling mechanisms to sense low nutrient states and to respond by upregulating survival pathways or eliminating themselves through cell death. This decision is particularly important for cancer cells that need to survive in chronically nutrient-deprived microenvironments. These nutrient sensing pathways will be discussed in the following sections.

1.2.1 AMPK

One of the energy sensors that regulates the switch between catabolism and anabolism upon nutrient stress is AMPK, a protein complex that is activated upon cellular ATP depletion by sensing the resulting increased AMP:ATP ratio (Hardie et al., 2012). The activation of AMPK results in activation of catabolic cellular processes, such as increased glucose metabolism and lipid oxidation, while simultaneously inhibiting anabolic ones, such as protein and lipid synthesis (Figure 1.4) (Hardie et al., 2012).

AMPK is a heterotrimeric protein complex composed of an α-, β-, and γ-subunit that are encoded by two (α/β) or three genes (γ), respectively (Hardie et

al., 2012). The α-subunit contains both the kinase domain that is required to signal to downstream substrates, as well as an important residue, Thr172, in its activation loop that can be phosphorylated by upstream kinases, such as LKB1 and CAMKK2 (Hardie et al., 2012). The γ-subunit contains four CBS domains that can bind ATP, ADP, and AMP, conferring upon the kinase the ability to sense the ATP:AMP ratio and therefore the cellular energy levels (Hardie et al., 2012). AMP binding to the γ-subunit activates AMPK activity by protecting the Thr172 residue from dephosphorylation, as well as by allosterically activating the kinase (Herzig and Shaw, 2018). Recent data suggests that in response to glucose starvation, AMPK can also localize to, and become activated at, lysosomes through recruitment of the WNT-related protein axin, where it might allow for co-regulation of mTOR complexes that also reside at the lysosome (Zhang et al., 2017).

AMPK activity controls a wide variety of metabolic processes that result in a switch from an anabolic to a catabolic state (Figure 1.4). Key downstream substrates include ACC1/2, whose phosphorylation leads to their inactivation, thereby inhibiting energy-consuming *de novo* lipid synthesis (Carling et al., 1987), as well as GFAT1, that results in diminished hexosamine biosynthesis and O-GlcNAc modification of proteins (Eguchi et al., 2009). AMPK also negatively regulates gene expression by inactivating, among others, SREBP1 and FOXO, proteins involved in transcriptional control of cholesterol biosynthesis and gluconeogenesis, respectively (Greer et al., 2007; Li et al., 2011).

Protein translation is inhibited by AMPK through direct modulation of the mTOR signaling pathway (see Chapter 1.2.2 on page 24 below). This can occur through direct activation of TSC2, a GAP for Rheb, which leads to inhibition of mTORC1, as well as through inhibitory phosphorylation of the mTORC1-specific protein RAPTOR (Gwinn et al., 2008; Inoki et al., 2003a; Inoki et al., 2003b). AMPK can also directly inhibit protein elongation through phosphorylation of

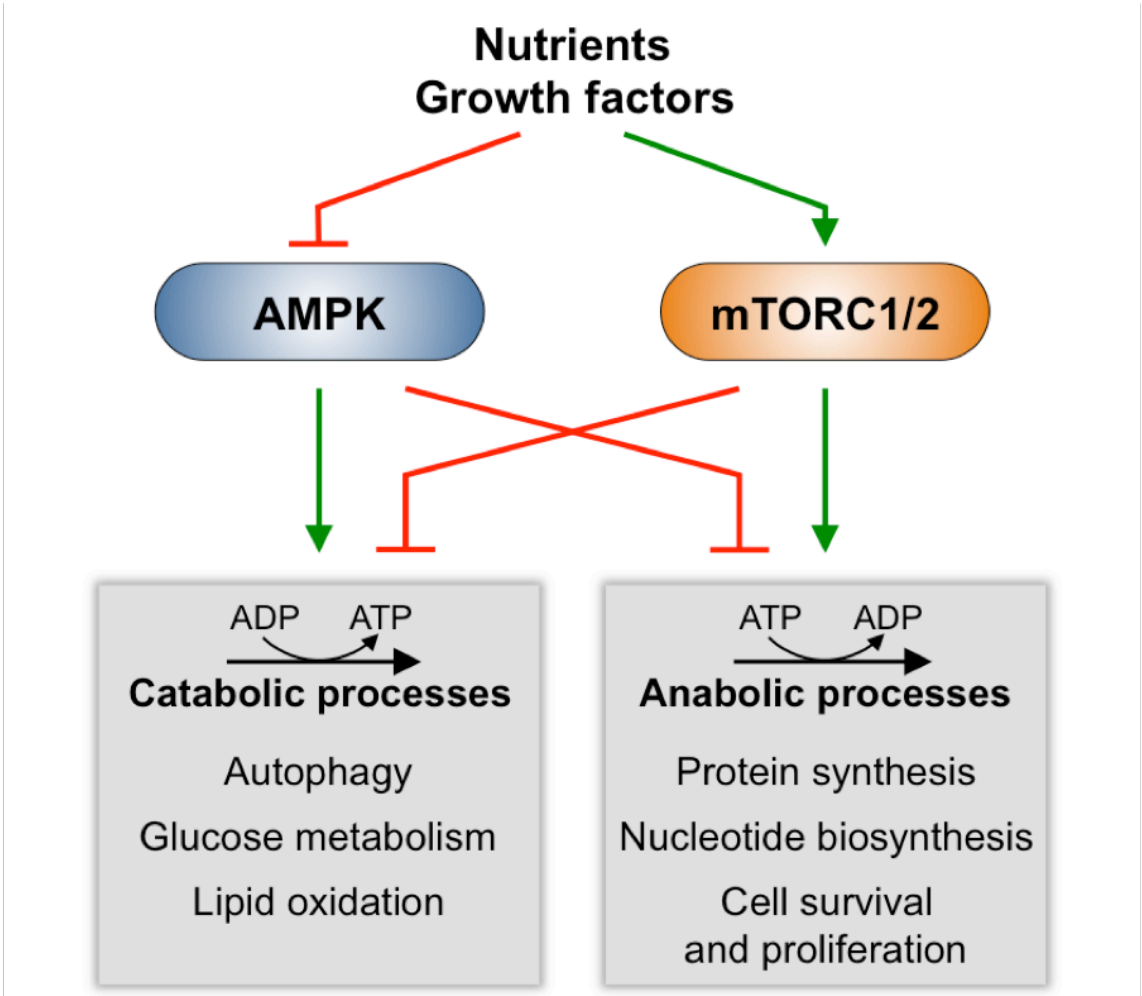


Figure 1.4. AMPK and mTOR signaling pathways
Regulation of AMPK and mTORC1/2 activity by nutrients and growth factors. AMPK is activated upon nutrient withdrawal, such as glucose starvation, that decreases intracellular ATP levels, while both mTOR complexes are activated by the presence of nutrients, such as amino acids, and growth factors. mTOR activation drives anabolic reactions, such as protein and nucleotide biosynthesis required for cell proliferation, while AMPK inhibits these and instead activates catabolic processes, such as autophagy, in an attempt to restore energetic balance.

eF2K, a negative regulator of this process (Leprivier et al., 2013).

In addition to the above-mentioned mechanisms through which AMPK functions to switch off many of the energy-consuming cellular activities in response to energetic stress, AMPK also positively regulates catabolic processes in an attempt to restore cellular energetic balance. For example, AMPK activity results in GLUT1 and GLUT4 trafficking to the plasma membrane to promote extracellular glucose uptake (Chavez et al., 2008; Wu et al., 2013), as well as increased glycolytic flux through phosphorylation of PFKFB3, a modulator of a rate-limiting glycolytic enzyme PFK1 (Bando et al., 2005), and induction of lipid store usage by activating lipases, such as ATGL (Ahmadian et al., 2011). The import of the ensuing free fatty acids into the mitochondria requires CPT1, another protein that can be modulated by AMPK through its inhibition of ACC1/2 that results in decreased levels of the CPT1 inhibitor malonyl-CoA (Saggerson, 2008).

Recycling of intracellular components through autophagy is also directly activated by AMPK. In addition to inhibiting the activity of mTORC1, a protein complex that inhibits autophagy, AMPK directly phosphorylates ULK1, a component of the autophagy initiation complex (Egan et al., 2011). Some reports suggest AMPK can also directly phosphorylate other components of the autophagy machinery, such as ATG9, a transmembrane protein involved in autophagosome formation (Weerasekara et al., 2014), as well as VPS34 (Kim et al., 2013) and Beclin1 (Zhang et al., 2016). Taken together, AMPK activation serves as a mechanism for cells to attempt to prolong survival during stress

conditions, in particular energy depletion, by shutting off macromolecular biosynthesis and inducing breakdown and turnover of metabolic substrates.

Under long-term nutrient starvation conditions where AMPK-mediated metabolic reprogramming is insufficient to promote survival, AMPK has also been shown to induce apoptosis (Okoshi et al., 2008; Song et al., 2014). Recent studies suggest that AMPK controls p53 up-regulation and transactivation and the downstream induction of apoptosis in response to glucose deprivation (Okoshi et al., 2008). AMPK is thought to directly bind p53 but how exactly this binding regulates its activity and its ability to induce apoptosis is unclear. AMPK has also been shown to phosphorylate Beclin1 (Zhang et al., 2016), which, upon cleavage, can also induce apoptosis in some contexts (Kang et al., 2011).

1.2.2 mTOR

The mTOR signaling pathway is another major sensor of energy availability and controls a wide variety of processes that regulate cell size and growth (Figure 1.4) (Saxton and Sabatini, 2017a). mTOR is a serine/threonine kinase that can assemble into two distinct protein complexes, mTORC1 and mTORC2, that are characterized, in addition to the presence of mTOR and mLST8, by association with Raptor and Rictor, respectively (Kim et al., 2002; Sarbassov et al., 2004). Active mTORC1 positively controls cell growth and anabolic metabolism, while mTORC2 is a key activator of PI3-K signaling that functions downstream of the insulin and other growth factor receptors to phosphorylate AKT at Ser473 and thereby activate substrates that lead to cell survival and proliferation (Saxton and Sabatini, 2017a).

As the induction of anabolic metabolism to produce biomass for cell growth and division must depend on the sufficient availability of required building blocks, mTORC1 activation is intimately tied to nutrient status. For example, as discussed in the previous section, growth factor pathway activation leads to the inactivation of TSC, relieving its inhibitory effect on Rheb that results in mTORC1 activation (Inoki et al., 2003a). In addition to growth factor availability, mTORC1 also integrates metabolic signals that may alter the ability of cells to proliferate, such as glucose deprivation. In addition to its negative regulation by AMPK (see Chapter 1.2.1 on page 20 above), glucose withdrawal can also be sensed directly mTORC1, as cells lacking AMPK also inhibit mTORC1 activity (Efeyan et al., 2013; Kalender et al., 2010). This is thought to occur through the Rag GTPases, but the exact mechanism of mTORC1-mediated glucose sensing remains to be elucidated (Kalender et al., 2010).

Amino acids are another necessary component for cell growth, as they are not only used for protein translation, but also contribute carbon and nitrogen to fuel other metabolic pathways. Levels of amino acids within cells are sensed by mTORC1 through the activation of Rag heterodimers (composed of RagA or RagB with RagC or RagD) that are localized to the lysosomal membrane through their interaction with the Ragulator complex (Bar-Peled et al., 2012; Sancak et al., 2010). Upon activation by GTP binding to RagA/B, this complex is competent to bind Raptor, thereby recruiting mTORC1 to the lysosomal surface (Sancak et al., 2010). Until recently, the subcellular location of the amino acid pool that mTORC1 was sensing remained unclear. Intriguingly, recent evidence suggests

that mTORC1 senses certain amino acids, such as arginine, from within the lysosomal lumen, while others, such as leucine, within the cytosol. Interaction of the lysosomal v-ATPase with the Rag-Ragulator complex enables nucleotide exchange on RagA/B, which promotes mTORC1 localization and activation (Zoncu et al., 2011). In addition, an amino acid transporter present on lysosomal membranes, SLC38A9, binds to the v-ATPase-Rag-Ragulator complex and is required for mTORC1 to be activated by arginine, suggesting that this protein may sense levels of amino acids within the lumen of the lysosome (Rebsamen et al., 2015).

The mechanism through which mTORC1 senses cytosolic amino acids, such as leucine and arginine, involves GATOR2, a protein complex that negatively regulates the GATOR1 complex that acts as a GAP towards RagA/B (Bar-Peled et al., 2013). GATOR1 recruitment to the lysosomal membrane by interaction with the KICSTOR complex is required for amino acids to activate the pathway (Wolfson et al., 2017). Sensing of cytosolic leucine requires the GATOR2-interacting protein Sestrin2, which, upon leucine binding, dissociates from, and relieves the inhibition of, the complex, thereby allowing for GATOR2-mediated negative regulation of GATOR1 to activate mTORC1 (Bar-Peled et al., 2013; Chantranupong et al., 2014). In addition to the direct sensing of leucine, Sestrin2 can also be transcriptionally induced after long-term amino acid starvation through the stress-responsive transcription factor ATF4 (Ye et al., 2015). Similar to leucine, arginine is also sensed by an amino acid-binding protein, CASTOR, which, in the absence of arginine, binds GATOR2 and inhibits

its function (Chantranupong et al., 2016; Saxton et al., 2016). Cytosolic glutamine, an important cellular source of carbon and nitrogen, has been proposed to activate mTORC1 through the Arf family of GTPases, independently of Rags (Jewell et al., 2015). Despite this complex and incomplete picture of mTORC1 regulation by nutrients, the site of activation in all cases is the lysosome, suggesting a profound role for this organelle in coordinating energetic status of the cell with signals to induce cell growth and proliferation.

Upon activation in nutrient-replete conditions, mTORC1 signaling serves to promote various anabolic pathways such as protein synthesis by phosphorylating S6K1, which activates mRNA translation initiation through direct activation of eIF4B (Holz et al., 2005), and 4E-BP1, which releases eIF4E from 4E-BP1 and allows for cap-dependent translation to proceed (Ma and Blenis, 2009). In addition to regulating overall translation, mTORC1 signaling can also promote *de novo* lipid synthesis through the SREBP family of transcription factors (Porstmann et al., 2008) and nucleotide biosynthesis, in part by increasing expression of MTHFD2, an enzyme involved in one-carbon metabolism to generate purines (Ben-Sahra et al., 2016). Glucose metabolism is also altered by mTORC1, in part by increasing flux through glycolysis by upregulating expression of enzymes such as PFK1 (Duvel et al., 2010) and by shunting glucose carbons to the pentose phosphate pathway that generates both reducing power in NADPH, as well as intermediate metabolites required for growth (Saxton and Sabatini, 2017b).

Another well-known function for mTORC1 is its inhibition of protein catabolism through autophagy (Mizushima et al., 2008; Saxton and Sabatini, 2017b). mTORC1 directly inhibits autophagosome formation by phosphorylating ULK1, preventing the ability of AMPK to activate it, as discussed above (Kim et al., 2011). The nuclear translocation, and therefore the activity, of the transcription factor TFEB is also inhibited directly by mTORC1-mediated phosphorylation, inhibiting the expression of genes encoding both core autophagy and lysosomal components (Martina et al., 2012; Roczniak-Ferguson et al., 2012).

As both mTOR complexes function at the core of multiple pathways that regulate growth and proliferation, the role of these signaling mechanisms in tumorigenesis is of great interest. Several negative regulators of mTORC1, such as LKB1 and TSC1/2, are tumor suppressors, whose loss predisposes to tumor formation (Saxton and Sabatini, 2017a). Other mutations are found in components upstream of mTORC1, including the GATOR1 complex (Bar-Peled et al., 2013) and RagC (Okosun et al., 2016), as well as in mTOR itself (Grabiner et al., 2014), suggesting that deregulation of this pathway may play a major role during tumorigenesis. The use of mTOR inhibitors in the clinic has been less successful than initially anticipated, in part due to relieving negative feedback on the pro-growth PI3-K signaling pathway (Tabernero et al., 2008), as well as through activation of autophagy and increased recycling of extracellular components that may enable cell survival in nutrient-poor conditions (topics that will be discussed in Chapter 1.3 starting on page 30 below) (Palm et al., 2015).

These findings have led to the model that in order to be clinically successful, mTOR inhibitors will likely need to be used in combination with other compounds, such as PI3-K or autophagy inhibitors (Rangwala et al., 2014).

1.3 NUTRIENT SCAVENGING

In response to nutrient starvation, which is sensed by the pathways discussed above, the ability of cells to avoid cell death and survive depends in part upon the induction of nutrient recycling and scavenging pathways that can be upregulated to restore cellular energy balance. Cells can adapt to lowered levels of extracellular nutrients by upregulating plasma membrane transporters, such as the GLUT family of transporters, for glucose, and the ASCT proteins for amino acids, such as glutamine, that enhance uptake (Jones and Thompson, 2009; van Geldermalsen et al., 2016). The expression and appropriate subcellular localization of these proteins is tightly regulated in response to nutrient availability, in large part by the mTOR and AMPK signaling pathways discussed in Chapter 1.2 above. When the import of specific substrates is unable to maintain a cell's metabolism, for example due to oncogenic mutations, or when specific nutrients are depleted from the extracellular environment, such as in poorly-vascularized regions of tumors (Mathew et al., 2007), cells can activate several additional mechanisms to cope with this stress. In addition to promoting angiogenesis in an effort to deliver additional nutrients through the formation of new vasculature, cells can catabolize intracellular proteins and organelles through autophagy, induce bulk uptake of extracellular material for lysosomal degradation, or take up whole live cells as a form of cellular cannibalism, all of which will be discussed in the following sections (Figure 1.5).

1.3.1 Autophagy

Autophagy is an evolutionarily conserved mechanism of degradation of intracellular components, such as proteins and organelles, that can be induced in response to a variety of different stresses (Mizushima et al., 2008). A major activator of autophagy is nutrient stress, when mTORC1 inactivation allows for the ULK pre-initiation complex to assemble and induce the nucleation of double membrane vesicles termed autophagosomes (Galluzzi et al., 2015). The elongation and expansion of autophagosomes is controlled by a ubiquitin-like conjugation system that catalyze the linkage between ATG5 and ATG12/ATG16L, as well as the addition of phosphatidylethanolamine to LC3 (Klionsky, 2005). Upon LC3 lipidation and closure of the autophagosome, lysosomes fuse and lead to the degradation of internalized cargo by lysosomal hydrolases (Galluzzi et al., 2015). The lipidation of LC3 onto intracellular vesicles is transient, as it can be delipidated and recycled by ATG4 (Klionsky, 2005).

Depending on the type of stress, autophagic degradation can be both specific and non-specific. For example, upon stimuli that disrupt mitochondrial function, a specialized form of autophagy termed mitophagy can respond to selectively clear depolarized mitochondria (Youle and Narendra, 2011). This process is initiated by PINK1, a mitochondrial kinase that is efficiently transported into the inner mitochondrial membrane under normal conditions, that, upon mitochondrial depolarization, accumulates in the outer mitochondrial membrane (Youle and Narendra, 2011). This allows PINK1 to phosphorylate and activate the E3 ubiquitin ligase Parkin, as well as Parkin's substrate, ubiquitin. The

depolarized mitochondria are thereby decorated with ubiquitin chains, in turn recruiting autophagy receptors, such as OPTN and NDP52, which induce autophagosome formation (Lazarou et al., 2015). Non-specific autophagy is often induced after metabolic stress and is involved in the bulk degradation of proteins. Similar to selective autophagy of damaged organelles, non-selective forms of autophagy also involve adaptor proteins, most notably p62, which can bind to nascent autophagosomes through its LC3 binding domain and to ubiquitinated proteins through its UBA domain, thereby ensuring targeted proteins are efficiently degraded (Pankiv et al., 2007).

Given autophagy's role in restoring and maintaining cellular homeostasis in response to stresses that may negatively affect healthy cells, it has been implicated in mediating a tumor-suppressive role. One piece of evidence in support of this model is the widespread deletion of a positive regulator of autophagy, Beclin1, in various cancer types and that overexpression of this protein inhibits tumorigenesis (Aita et al., 1999; Liang et al., 1999). In addition, mice carrying deletions of various autophagy genes, such as ATG5 and ATG7, develop tumors faster in both lung and pancreatic carcinoma models (Rao et al., 2014; Rosenfeldt et al., 2013). High levels of autophagy can protect cells from defects that have been shown to induce malignant growth, such as reactive oxygen species (Green et al., 2011). Indeed, autophagy-deficient cells undergo increased oxidative stress due to the inefficient clearance of damaged organelles, such as mitochondria, that may increase tumorigenesis (Scherz-Shouval and Elazar, 2007). Components of the autophagy machinery also seem

to be required for cells to combat genotoxic stress, as cells lacking ATG5 exhibit higher rates of DNA damage and aneuploidy, although the exact mechanism of this remains to be elucidated (Qiang et al., 2013).

Autophagy can also mediate a tumor-promoting role by, for example, allowing tumor cells to survive in a nutrient-depleted microenvironment (Mathew et al., 2007). Key autophagy genes, such as ATG5, are required for viability (Tsukamoto et al., 2008), and ATG5-dependent autophagy induction provides a survival advantage under nutrient stresses in apoptosis-deficient cells (Degenhardt et al., 2006; Karantza-Wadsworth et al., 2007). Further, in a mutant K-RAS^{V12}-driven mouse model of pancreatic cancer, treatment with chloroquine, an autophagy inhibitor, retards tumor progression and prolongs survival, indicating a requirement of cancer cells for autophagy to promote tumor growth (Yang et al., 2011). Interestingly, hypoxic (i.e. nutrient-poor) regions of tumors exhibit a higher rate of autophagy induction, suggesting that cancer cells are heavily dependent on autophagic recovery of macromolecules for survival and autophagy may serve a pro-tumorigenic role in these contexts (Mathew et al., 2007). Intriguingly, recent evidence also suggests that autophagy decreased the cytotoxicity of chemotherapeutic agents, potentially through increased lysosomal degradation, as inhibition or deletion of autophagic components enhanced the ability of the topoisomerase I inhibitor camptothecin to kill tumor cells (Abedin et al., 2007). These findings have prompted clinical trials to investigate the role of autophagy inhibition in mediating susceptibility to chemotherapy (Galluzzi et al., 2015).

In addition to regulating tumorigenesis cell autonomously, autophagy induction in stromal cells can also promote tumor cell survival (Sousa et al., 2016). In a *Drosophila* malignant tumor model, induction of autophagy in surrounding tissues by tumor cells, potentially through increased ROS production, results in increased survival and growth (Katheder et al., 2017). The increased levels of autophagy in surrounding cells results in amino acid secretion into the extracellular space that tumor cells, through upregulation of specific amino acid transporters, are able to take up and utilize in support of their growth and proliferation (Katheder et al., 2017).

In the context of PDAC, a tumor type that is characterized by a high degree of stromal infiltration, high levels of autophagy in surrounding stromal cells can also regulate tumor cell growth (Sousa et al., 2016). The dense desmoplasia that surrounds and infiltrates the tumor often acts as a barrier to delivery of nutrients via the vasculature and these decreased levels of nutrients are thought to contribute to the deregulated metabolism of PDAC (Lyssiotis and Kimmelman, 2017). Recently, it was shown that pancreatic stellate cells, which make up a large part of the stromal population, play a pro-tumorigenic role through autophagic secretion of alanine (Sousa et al., 2016). This intriguing metabolic interaction among different cell types allowed for increased uptake of alanine by cancer cells to fuel the TCA cycle and support lipid biosynthesis (Sousa et al., 2016). In both cases described above, what signals the stromal cells respond to that induces autophagy is unclear. It would be interesting to determine how exactly the tumor cells activate autophagy in neighboring cells;

however, culture experiments suggest that conditioned media from stellate cells alone was sufficient to induce PDAC proliferation, suggesting this effect may not be induced by the tumor cells themselves (Sousa et al., 2016). Furthermore, how the selective export of amino acids, including alanine, is regulated remains to be elucidated. An active role for surrounding cells in this scenario might suggest co-option of cell-autonomous amino acid storage pathways that may occur on or within the lysosome.

1.3.2 Macropinocytosis

While intracellular nutrient recovery via autophagy plays a major role in mediating cell survival under nutrient-starved conditions, recent evidence suggests scavenging of extracellular substrates can also support cell growth in proliferating cells (Figure 1.5) (Commisso et al., 2013; Kamphorst et al., 2013; Palm et al., 2015; Young et al., 2013). It has long been observed that bulk uptake of extracellular fluid, or macropinocytosis, is upregulated in the context of oncogenic RAS activation (Bar-Sagi and Feramisco, 1986). It was recently shown that extracellular BSA is taken up by K-RAS^{V12}-expressing cells and subsequently degraded, fueling catabolic intermediates. These cells are specifically dependent on glutamine for survival and macropinocytic uptake and its subsequent degradation into amino acids was able to rescue the cells' dependency on glutamine (Commisso et al., 2013). In a MIA PaCa-2 xenograft model of pancreatic cancer, which carry a homozygous oncogenic *KRAS*^{G12C} allele, tumor growth could be blocked, and in some cases even regress, when mice were treated with an inhibitor of macropinocytosis (Commisso et al., 2013). These data

were among the first to suggest that in the absence of extracellular amino acids, tumor cells are able to non-selectively scavenge extracellular protein, whose degradation supported cell proliferation.

Further, both RAS-transformed and hypoxic cancer cells are able to obtain specific fatty acids from extracellular media to support their lipid metabolism, completely bypassing the need for de novo lipogenesis for cell proliferation (Kamphorst et al., 2013). Cells lacking TSC2, which display constitutive mTORC1 activity, are unable to synthesize new fatty acids in hypoxic conditions, given the requirement for O_2 in the synthesis reactions. Under these conditions,

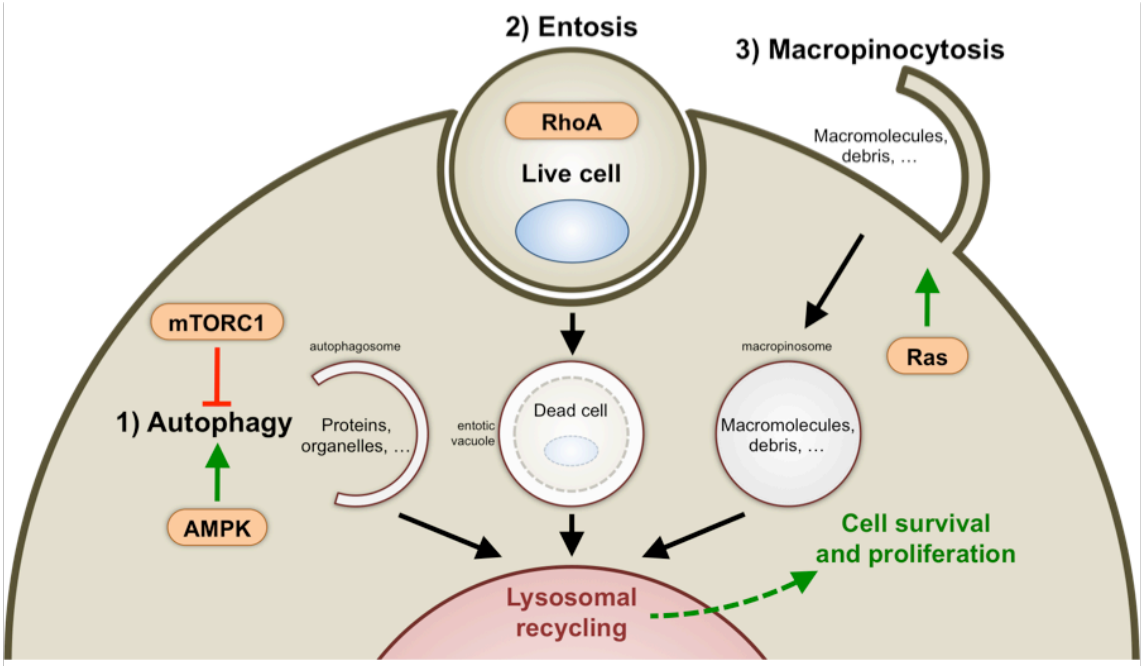


Figure 1.5. Nutrient scavenging pathways
Cells can engage in a variety of scavenging pathways to obtain nutrients under starvation conditions. 1) Cells can activate autophagy, a process inhibited by mTORC1 under nutrient-replete conditions, to catabolize intracellular components such as proteins and organelles to prolong cell survival. 2) Live neighboring cells can be ingested through entosis and subsequently killed within the host cell lysosome, which provides a survival and proliferation advantage over non-engulfing neighbors in nutrient-starved conditions. 3) Oncogenic Ras signaling can activate macropinocytosis, a process of bulk uptake of extracellular material, including macromolecules such as albumin. Degradation and recycling within the lysosome allows cells to survive and proliferate in nutrient-limiting environments.

cells are completely dependent on exogenous desaturated lipids and, as such, scavenging of serum lipids through an undefined mechanism rescues cells from death in hypoxic conditions (Young et al., 2013).

In a recent paper, mTORC1 activity was shown to inhibit the ability of RAS-transformed cells to proliferate when dependent on extracellular albumin (Palm et al., 2015). This suggested mTORC1 negatively regulates the catabolism of extracellularly-derived protein, as the overall rate of uptake of fluorescently-labeled albumin was unchanged in mTORC1-inhibited conditions (Palm et al., 2015). However, another recent study, using a ^{13}C-label tracing strategy to monitor extracellular protein catabolism and recycling, suggested that, while mTORC1 inhibition slightly increased extracellular albumin degradation, this could not explain the dramatic increase in cell proliferation observed (Nofal et al., 2017). Instead, mTORC1 inhibition in the context of exogenous protein catabolism reduces the overall translation rate, which allows cells to conserve the limited pool of amino acid that is present after BSA scavenging (Nofal et al., 2017). The arginine sensor SLC38A9 required for mTORC1 activation discussed above has recently been shown to be required for pancreatic tumor cells to export leucine from the lysosome upon its degradation from extracellular-derived protein (Wyant et al., 2017). This results in an interesting regulatory mechanism, where components required for mTORC1 activation are also necessary for the upstream scavenging of serum proteins. This is in agreement with other findings that implicated the lysosomal glutamine exporter SNAT7 in the ability of cells to scavenge extracellular protein and proliferate under these conditions (Verdon et

al., 2017). These findings suggest that inhibiting biosynthetic pathways upregulated in cancer, using mTOR inhibitors, for example, may not yield significant decreases in cell proliferation, as initially expected. Indeed, treatment of pancreatic tumors with rapamycin, an mTORC1 inhibitor, increased the proliferation rate of cells within the core of the tumor that is known to be poorly vascularized and limited for nutrients (Palm et al., 2015).

1.3.3 Cell engulfment

In addition to the bulk uptake of extracellular fluid by macropinocytosis, cells are also capable of engulfing whole cells, for example by phagocytosis, the process of engulfment of large cargo (>0.5 μm), usually dead or dying cells (Figure 1.5) (Stefater et al., 2011). While metazoans have evolved professional phagocytes, such as macrophages, dendritic cells, and other cell types within the immune system, to carry out this function, non-professional phagocytes, including epithelial cells, have also been shown to engulf dead cells (Monks et al., 2005). Phagocytosis involves the recognition of specific “eat-me” signals exposed by target cells by receptors expressed on the cell surface of phagocytes. The loss of asymmetric PtdSer distribution on the inner leaflet of the plasma membrane, due to caspase-mediated inactivation of flippase enzymes, can serve as a trigger for phagocytosis (Segawa et al., 2014). Soluble PtdSer-binding proteins, such as Protein S and Gas6, recruit phagocytes expressing cognate receptors, such as MerTK, that serve to activate Rac1, leading to actin polymerization and formation of a phagocytic cup (Segawa and Nagata, 2015). Upon completion of engulfment, internalized cargo is trafficked to the lysosome, where degradation

and recycling of the corpse occurs (Segawa and Nagata, 2015). Intriguingly, the induction of LAP was first described in the context of TLR engagement during phagocytosis that enhanced the degradation of the engulfed cargo (Sanjuan et al., 2007). The requirement for autophagy machinery in the proper maturation and clearance of engulfed cargo is intriguing, and how this process is regulated and how it contributes to the ability to efficiently clear engulfed cargo remains to be fully elucidated.

In higher organisms, a role for phagocytosis has mostly been established in the context of immunity, where it is required for the clearance of pathogenic organisms and tissue homeostasis (Poon et al., 2014). Interestingly, phagocytosis served a role in cellular feeding prior to evolving as a mechanism of immune defense (Stefater et al., 2011). In many types of amoeba, for example, bacterial phagocytosis is the major route of feeding. Engulfment of apoptotic corpses provides amino acids to engulfing macrophages and rescues cells from death in amino acid-starved conditions (Krajcovic et al., 2013). These findings suggest that this ancient function of cell engulfment remains in mammalian macrophages and can function as a feeding mechanism in low nutrient conditions.

In addition to the engulfment of dead or dying cells, live cells can also be taken up by neighbors in a form of cellular cannibalism (Overholtzer and Brugge, 2008). For example, slime molds such as *Dictyostelium caveatum*, which normally feed on surrounding bacteria, can feed on live neighboring amoeba when bacteria are absent (Waddell and Vogel, 1985). This allows them to

support their normal metabolism and provides a survival advantage. Further, during sexual development, the amoeba aggregate and a subset of winner cells begin to cannibalize on surrounding aggregated cells, resulting in the formation of a giant diploid cell that is competent to undergo recombination and meiosis to produce daughter cells (Nizak et al., 2007). These data suggest that in lower organisms, cell cannibalism serves as a mechanism to increase survival both under low nutrient conditions in the vegetative life cycle, as well as in sexual reproduction (Erdos et al., 1973).

Recent evidence has demonstrated that mammalian cells can also engage in cannibalistic behavior (see Chapter 1.1.4 on page 12 above) (Overholtzer et al., 2007). While the mechanisms of inner cell death were discussed above, the effects that live-cell engulfment has on host cells remains poorly understood. The uptake and death of cytotoxic immune cells could promote the survival of targeted tumor cells, for example, and the removal through these mechanisms of auto-reactive lymphocytes may play a role in regulating immunity within an organism (Benseler et al., 2011). However, whether upon degradation of internalized cells, hosts can recycle nutrients or other cellular components for their own consumption is an intriguing concept. This was initially hypothesized starting in the 1940's, but strong supporting evidence remained scarce (Overholtzer and Brugge, 2008). Recently, in a model of T cell uptake by melanoma cells, host cells exhibited a survival advantage over non-engulfing neighbors under low nutrient conditions (Lugini et al., 2006).

This suggested that cell cannibalism might be a nutrient scavenging mechanism, similar to *Dictyostelium*.

This model was further supported by data showing that cells engaging in entosis have not just a significant survival advantage over single neighboring cells, but were even able to proliferate when cultured in amino acid-free medium (Figure 1.5) (Krajcovic et al., 2013). mTORC1 localized to lysosomes containing dead corpses, consistent with the model that nutrients within the lysosome are sensed by this complex (Krajcovic et al., 2013; Zoncu et al., 2011). One model that might explain the consequences of this proliferation would predict the overall survival of a tumor cell population, but as one cell divides at the expense of another, the total cell number might not increase over time. However, whether host cells alter their metabolism in response to nutrient stress such that degradation of one corpse might provide sufficient nutrients for several cell divisions is unknown. Therefore, whether nutrient acquisition in this manner can support the outgrowth of a tumor cell population under continued nutrient stress is a question that will be addressed in subsequent chapters of this thesis.

1.4 THESIS AIMS

It has become increasingly clear that cells can engage in a wide variety of cell death mechanisms, each with potentially distinct consequences with regards to the larger surrounding cell population. While a lot of studies have recently uncovered the mechanisms regulating necrotic types of death, such as necroptosis and ferroptosis, still not much is known about triggers and downstream consequences of entosis. This thesis aims to therefore examine conditions under which entosis may be highly induced, as well as explore the effects such high levels of entosis may have on the cell populations in which it occurs.

In Chapter 2, we explore the role of nutrient starvation on entosis induction in cancer cells. We identify glucose withdrawal as a specific trigger for entosis induction in a variety of different cell culture systems. We then demonstrate that this high level of induction is controlled by the cellular energy sensor AMPK, as inhibition of this kinase blocks entosis under these conditions. Our data show that AMPK signaling specifically controls entosis by regulating loser cell behavior, in party by altering the deformability of cells.

In Chapter 3, we examine the outcomes of high levels of entosis on cells. We show that winner cells that remain upon glucose withdrawal proliferate at a higher rate than cells that have not engaged in entosis, suggesting that loser cells may provide essential nutrients to winners in order to survive stringent nutrient withdrawal. We also demonstrate that engaging in entosis provides a proliferation advantage not just at the single-cell level, but also on a population-

wide scale. Entosis-deficient cells are unable to proliferate under more prolonged glucose starvation, whereas entosis-competent cells were able to more than double their population size after 9 days of glucose withdrawal. We further show that this ability to engage in entosis can be selected, as long-term starved cells show competitive behavior when mixed with naïve cells, as they preferentially engulf unstarved cells.

In Chapter 4, we discuss the questions that were raised by the work in this thesis, including potential mechanisms for how AMPK may regulate entosis induction and how glucose starvation regulates the rapid entotic cell death observed. Furthermore, we explore the role that high levels of entosis induction may play in the ability of certain cancer cells to survive stringent selection conditions and how this may allow for entosis to act as a form of cell competition in this context.

CHAPTER 2: Entosis is induced by glucose starvation

2.1 INTRODUCTION

In addition to the regulated forms of cell death discussed in Chapter 1.1, other alternative forms have been reported that may represent yet additional programmed mechanisms that eliminate cells in certain contexts (Galluzzi et al., 2012). Among these, entosis is a mechanism that targets cells for death following their engulfment by neighboring cells (Overholtzer et al., 2007). Entotic cells are killed non-cell-autonomously by engulfing cells through autophagy protein-dependent lysosomal digestion (Florey et al., 2011). Entosis occurs in human cancers, and we have shown that it inhibits transformed growth by inducing cell death. However, this process also promotes the development of aneuploidy in host cells (Krajcovic et al., 2011) and facilitates nutrient recovery by engulfing cells that could function to promote tumor progression (Hamann et al., 2017; Krajcovic et al., 2013).

Previous work has shown that upon killing of internalized cells, degraded corpse cargo is rapidly distributed throughout the lysosomal network of host cells through lysosomal membrane fission (Krajcovic et al., 2013; Krishna et al., 2016). The shrinkage of this compartment and the subsequent redistribution of its cargo are regulated by the activity of the nutrient sensor mTORC1 and the lipid kinase PIKfyve, both of which localize to corpse-containing lysosomes (Krajcovic et al., 2013; Krishna et al., 2016). Earlier findings suggest that the uptake and killing of live neighboring cells through entosis provides engulfing cells with nutrients that support cell survival and proliferation in amino acid-depleted conditions

(Krajcovic et al., 2013). While recent studies have elucidated consequences of entosis on cell populations, the signals that could promote this process remain poorly characterized, with a lack of suitable matrix adhesion and aberrant mitoses due to disrupted polarity as the only clear known inducers of entosis (Durgan et al., 2017; Overholtzer et al., 2007). Given the significant survival advantage of engulfers over non-engulfing single cells, we wondered whether starvation conditions might act as a signal to induce entosis to allow cells to respond homeostatically, similar to autophagy, to these stresses. Amino acid withdrawal did not induce high levels of entosis, however, prompting us to examine if other forms of nutrient starvation could induce high rates of neighbor cell ingestion (Hamann et al., 2017).

2.2 RESULTS

2.2.1 Glucose starvation induces entosis

To identify potential entotic triggers, we cultured the human breast tumor cell line MCF-7, which undergoes high levels of entosis in matrix-detached conditions, in medium lacking individual nutrients or serum and monitored the cultures for entosis induction by imaging. After 72 hours of culture in low serum, no glucose, and no amino acids, ~30% of cells had engulfed at least one neighbor, a significant increase over cells cultured in full media conditions (Figure 2.1). As has been reported in matrix-detached conditions, we also observed more complicated structures that involved more than two cells in sequential engulfments (see Figure 2.1 i and ii). Interestingly, the withdrawal of glucose from growth medium, unlike starvation for other nutrients, was sufficient to induce a high level of cell engulfment (Figure 2.1), and re-addition of D-glucose to

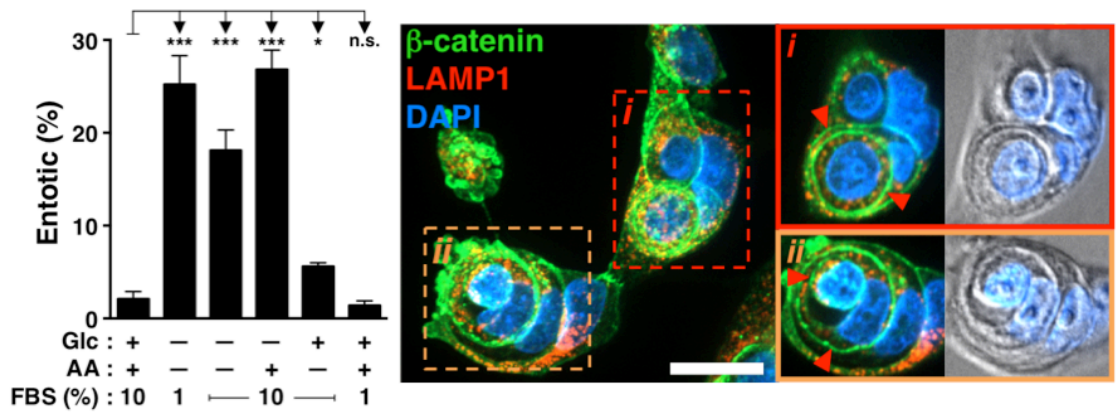


Figure 2.1. Glucose starvation induces engulfment in breast cancer cells
(A) Nutrient starvation causes engulfment. Graph shows percent of engulfments in MCF-7 cells grown in the indicated conditions for 72 hours, as determined by immunofluorescence. Glc, glucose; AA, amino acids; FBS, fetal bovine serum. Error bars depict mean ± SEM; data are from at least three independent experiments. Images show engulfed cell structures from a 72-hour glucose-starved culture. Immunostaining for β-catenin (green) and LAMP1 (red), and DAPI-stained nuclei (blue) are shown. Arrowheads show completed engulfments. Scale bar: 20 μm. This figure is adapted from Hamann et al., 2017.

glucose-free medium completely rescued this effect (Figure 2.2A). These data suggest that glucose starvation, specifically, is a trigger for live cell engulfment in adherent conditions (Hamann et al., 2017).

Entotic cell uptake is mediated by changes in cell contractility in internalizing cells, controlled by RhoA and ROCK1 signaling, as well as maintenance of cell-cell adhesions mediated by epithelial-type cadherins (e.g. E-cadherin) (Overholtzer et al., 2007). To determine whether the glucose starvation-induced live cell engulfments occurred by entosis, we examined cultures for the presence of these characteristics. First, activated myosin II, indicated by phosphorylation of myosin light chain 2 on the ROCK1-dependent site serine 19 (P-MLC-S19), was localized at the cortex of the internalizing cells, as reported (Sun et al., 2014a) (Figure 2.2B). Further, treatment with Y-27632, an inhibitor of ROCK and a potent entosis inhibitor, also completely blocked cell engulfment induced by glucose withdrawal (Figure 2.2A). Second, the cell-cell adhesion protein β-catenin localized at the interface between internalizing and engulfing cell pairs, consistent with a cell-cell adhesion-based mechanism of uptake (Figure 2.2B) (Hamann et al., 2017).

Third, disruption of E-cadherin in MCF-7 cells, by CRISPR/Cas9-mediated gene editing, significantly inhibited cell engulfment due to glucose withdrawal, which was rescued by exogenous expression of E-cadherin–GFP (Figures 2.2C and D). We also examined whether MDA-MB-231 breast cancer cells, which are E-cadherin-deficient and do not normally undergo entosis (Sun et al., 2014a), could undergo cell engulfment in response to glucose deprivation. MDA-MB-231

cells showed no evidence of engulfment in either glucose-starved or full media conditions (Figure 2.3A). However, upon expression of exogenous E-cadherin, MDA-MB-231 cells exhibited a significant rate of cell engulfment with glucose

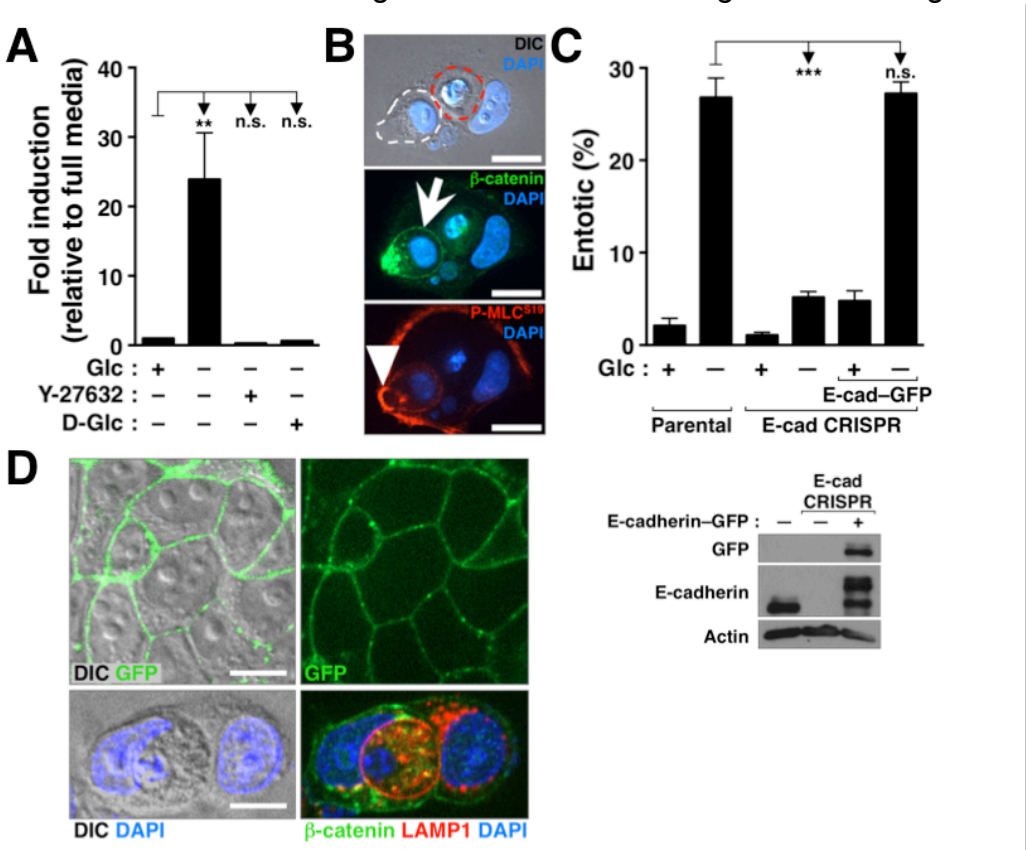


Figure 2.2. Glucose starvation induces entosis

(A) Induction of engulfment is blocked by ROCK inhibition and readdition of glucose. Graph shows quantification of engulfments throughout 72 hours, as determined by time-lapse microscopy. Error bars depict mean ± SEM; data are from at least three independent experiments. (B) Localization of cellular machinery required for entosis. Immunofluorescence image shows localization of phosphorylated myosin light chain (P-MLC^{S19}, red, arrowhead) in MCF-7 cells grown in glucose-free conditions for 72 hours. Cell-cell junctions are shown by β-catenin staining (green, arrow), DAPI-stained nuclei are shown in blue. White dashed line shows outline of partial engulfment, while red dashed line shows engulfed corpse. Scale bar: 15 μm. (C) Loss of E-cadherin blocks entosis. Graph shows percent of engulfments in parental MCF-7 cells, as well as E-cad CRISPR and E-cad CRISPR + E-cad–GFP cells, grown in the indicated media conditions for 72 hours, as determined by immunofluorescence. Error bars depict mean ± SEM; data are from at least three independent experiments. Western blot shows E-cadherin expression in parental, E-cad CRISPR, and E-cad CRISPR + E-cad-GFP MCF-7 (D) E-cadherin–GFP localizes to cell junctions. Representative images show expected plasma membrane localization of exogenous E-cadherin–GFP in MCF-7 E-cad CRISPR + E-cad–GFP cells (top row) and induction of entosis in these cells (bottom row). Immunostaining for β-catenin (green) and LAMP1 (red), and DAPI-stained nuclei (blue) are shown. Scale bar: 10 μm. This figure is adapted from Hamann et al., 2017.

starvation (Figure 2.3A) (Hamann et al., 2017). To examine whether this effect was unique to cancer cells or common among non-transformed cells as well, we cultured a non-transformed mammary epithelial cell line (MCF-10A) in glucose-free conditions for multiple days and found that levels of entosis within the culture also increased, although to a lower extent than in MCF-7 cells (Figure 2.3B). We therefore conclude that glucose starvation is an inducer of entosis in breast cancer cells and non-transformed mammary epithelial cells (Hamann et al., 2017).

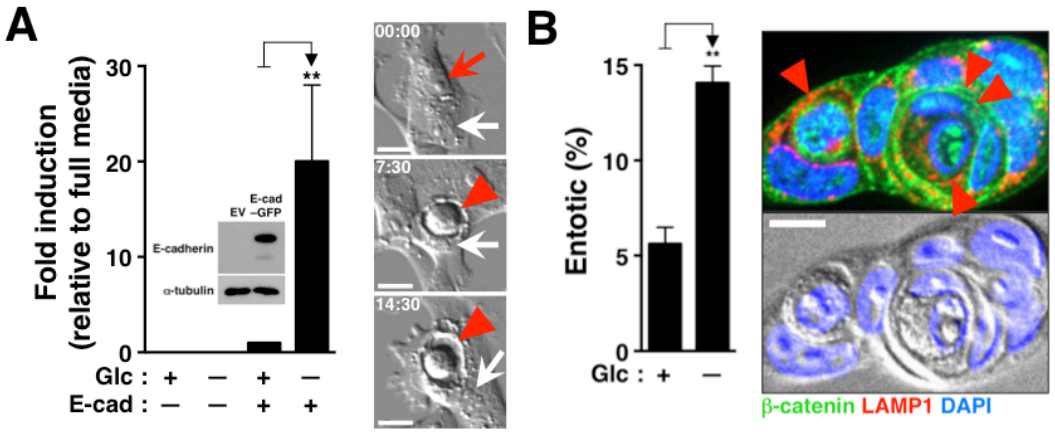


Figure 2.3. Glucose starvation triggers entosis in transformed and non-transformed cell lines

(A) Engulfment is induced during glucose starvation in MDA-MB-231 cells expressing E-cadherin. Quantification of engulfment throughout 72 hours for MDA-MB-231 cells expressing either empty vector (EV) or E-cadherin–GFP, cultured in full or glucose-free media, as determined by time-lapse microscopy. Error bars depict mean ± SEM; data are from at least three independent experiments. A representative image sequence of MDA-MB-231 cells expressing E-cadherin–GFP undergoing engulfment is shown in glucose-free conditions. Red arrow shows cell to be internalized before engulfment. White arrows show host cell. Red arrowhead shows engulfment. Time shown is in hours:minutes. Scale bar: 10 mm. Western blot shows expression of E-cadherin in parental and E-cadherin–GFP-expressing MDA-MB-231 cells. (B) Entosis is induced by glucose starvation in non-transformed MCF-10A mammary epithelial cells. Graph shows percent of engulfments in MCF-10A cells grown in in the indicated media. Error bars depict mean ± SEM; data are from at least three independent experiments. Images show immunofluorescence staining of MCF-7 cells after 72 hours of respective treatment. β-catenin stain (green) shows cell-cell junctions, while LAMP1 staining (red) marks lysosomes; DAPI-stained nuclei are shown in blue. Scale bar: 15 μm. This figure is adapted from Hamann et al., 2017.

2.2.2 AMPK controls entosis under glucose starvation

To identify the signaling mechanisms that might control glucose starvation-induced entosis, we considered AMPK, a well-known starvation-induced kinase that allows cells to respond to starvation stress by inducing autophagy (Yuan et al., 2013). Treatment with Compound C, an inhibitor of AMPK (Zhou et al., 2001), as well as expression of dominant-negative isoforms of AMPK (AMPK-DN) (Mu et al., 2001; Young et al., 2016), inhibited entosis induction in the absence of

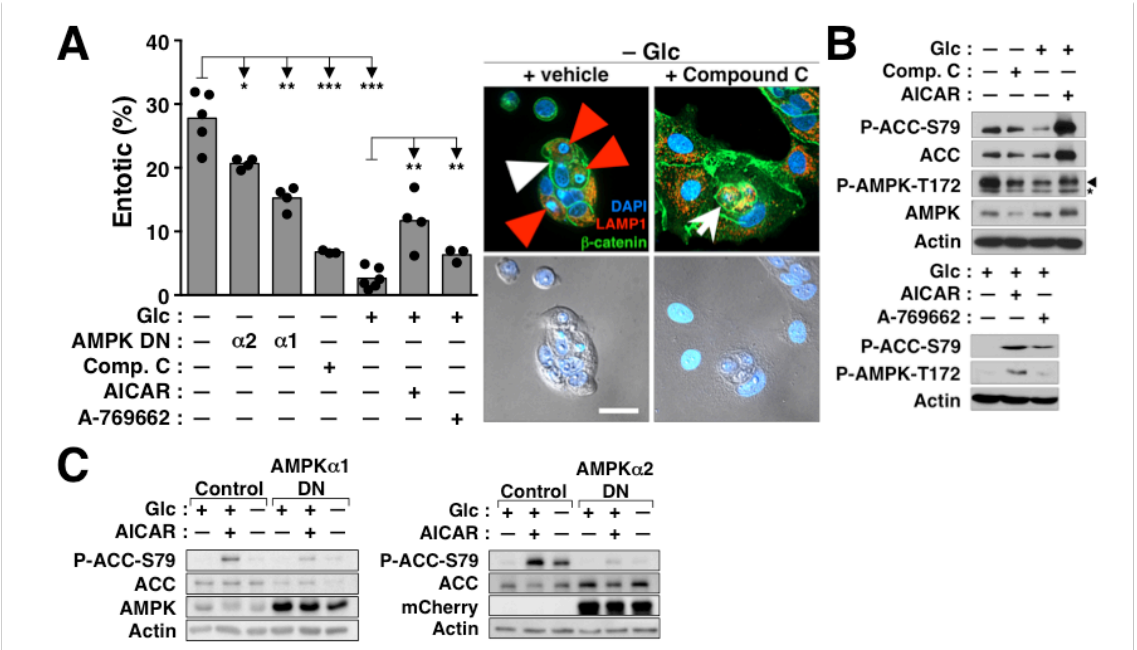


Figure 2.4. AMPK activity is required for glucose starvation-induced entosis
(A) Modulation of AMPK pathway activity alters formation of entotic structures. Graph shows number of entotic structures in MCF-7 cells grown in respective media with or without an AMPK inhibitor or two distinct activators, or MCF-7 cells expressing dominant-negative (DN) isoforms of AMPK (AMPKα1 and α2), for 72 hours, as determined by immunofluorescence. Shown are individual data points from independent experiments. Immunostaining for β-catenin (green) and LAMP1 (red), and DAPI-stained nuclei (blue) are shown. Arrow shows internalized cells, red arrowheads mark entotic corpses, and white arrowhead marks partial engulfment. Scale bar: 15 μm. (B) Western blots show activation of AMPK (by phosphorylation at T172) and downstream signaling (by phosphorylation of ACC at S79) in MCF-7 cells cultured in the indicated conditions. (C) AMPK signaling is inhibited in cells expressing either AMPKα1- or α2-DN. Western blot shows activation of AMPK and downstream signaling in control MCF-7 cells cultured in glucose-free conditions or by treatment with the AMP-analog AICAR for 6 hours. This activation is blocked in cells expressing dominant-negative isoforms of AMPK. This figure is adapted from Hamann et al., 2017.

glucose (Figure 2.4) (Hamann et al., 2017). Conversely, entosis was induced in nutrient-rich media by the induction of AMPK activity using two AMPK activators, the AMP analog AICAR (Sullivan et al., 1994) and the allosteric activator A-769662 (Goransson et al., 2007) (Figure 2.4), indicating that AMPK plays an important role in promoting this process (Hamann et al., 2017).

2.2.3 Entotic cell death is increased in glucose-starved conditions

Entosis leads to the death of internalized cells through a non-apoptotic mechanism that involves lipidation of the autophagy protein LC3 onto entotic vacuoles (Florey et al., 2011). Typically, 50-70% of internalized cells undergo death by this mechanism within a 24-hour period, while others (10-20%) manage to escape from their hosts (Florey et al., 2011; Overholtzer et al., 2007). Interestingly, under conditions of glucose withdrawal, we noted that entotic cells died more rapidly after their engulfment and rarely escaped (Figure 2.5A) (Hamann et al., 2017). More than 60% of engulfed cells died within five hours after engulfment, compared to 10% of engulfed cells cultured in full media. Ten hours after engulfment was complete, more than 90% of internalized cells cultured in glucose-free media had undergone cell death, while only ~50% of engulfed cells grown in full media had died (Figure 2.5A). Like entotic cell deaths occurring in nutrient-rich conditions, those in glucose-starved cultures involved lipidation of LC3 onto entotic vacuoles, and the frequency of cell death was reduced by knockdown of the autophagy protein Atg5 (Figures 2.5B and 2.6A) (Hamann et al., 2017). Inhibition of AMPK, either by expression of AMPK-DN or treatment with Compound C, or activation of AMPK by treatment with AICAR, did

not alter the rates of cell death once loser cells became internalized, suggesting the rapid rate of loser cell death observed in glucose-free conditions is independent of AMPK activity (Figure 2.6B) (Hamann et al., 2017).

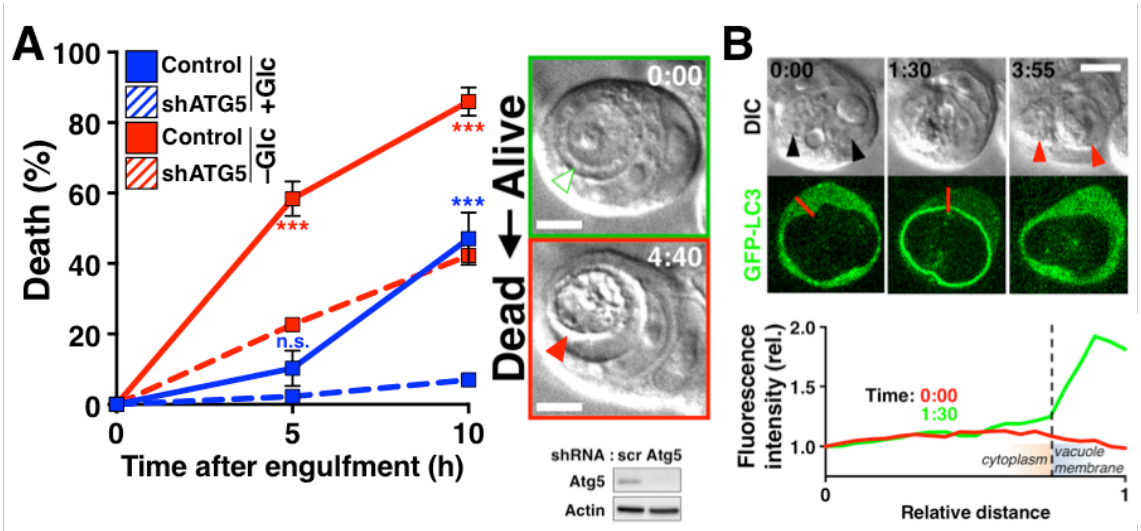


Figure 2.5. Glucose starvation increases entotic cell death
(A) Entotic death rate of internalized cells increases during glucose starvation. Graph shows death rate of internalized cells within 10 hours after completed engulfment, determined by the appearance of a vacuole within the host cell by DIC microscopy. Entotic death rates for cells cultured in full media (+Glc, blue lines) or glucose-free media (–Glc, red lines) in control MCF-7 cells (solid lines) and MCF-7 shATG5 cells (dashed lines) are shown. Error bars depict mean ± SEM; data are from at least three independent experiments, with n ≥ 40 entotic cell structures per experiment. *P*-values according to two-way ANOVA multiple comparisons test. *P*-values compare control and shATG5 cells in + or –Glc media, respectively, and are color-coded accordingly. Western blot shows expression of Atg5 relative to Actin in MCF-7 cells expressing a scrambled or Atg5-targeted shRNA. Images are representative of live and dead internalized cells as scored by DIC. Green and white arrowhead shows live internalized cell ("alive"); red arrowhead shows morphologically dead cell inside the host cell vacuole ("dead"). Time shown is in hours:minutes. Scale bar: 10 μm. (B) Internalized cell death involves transient lipidation of LC3 (light chain 3) onto entotic vacuoles. Confocal time-lapse images of MCF-7 cells expressing GFP–LC3 cultured in glucose-free media. Black arrowheads show two live engulfed cells within GFP–LC3–expressing host (one inner cell itself contains a corpse). Red arrowheads show death of both internalized cells ~4 hours after LC3 recruitment. Time shown is in hours:minutes. Scale bar: 10 μm. Graph shows relative GFP fluorescence intensity both before (0:00) and during (1:30) GFP–LC3 recruitment. Line used for fluorescence intensity profile is shown in red. This figure is adapted from Hamann et al., 2017.

2.2.4 AMPK activity controls loser cell behavior

To determine if AMPK activation occurs in winner or loser cells, we utilized a FRET-based sensor (Tsou et al., 2011) to monitor temporal AMPK dynamics. Shortly before engulfment, we observed an increase in FRET-based fluorescence (indicating increased AMPK activity) within internalizing MCF-7 cells undergoing glucose withdrawal-induced entosis (Figure 2.7A) (Hamann et al., 2017). This finding is consistent with previous reports of this sensor that showed similar fold increases in FRET signal upon glucose starvation (Banko et al., 2011; Tsou et al., 2011). We then co-cultured MCF-7 cells expressing AMPK-DN with control MCF-7 cells expressing GFP in the absence of glucose. While control mCherry-expressing MCF-7 cells were winners at ~50% frequency, MCF-7 cells

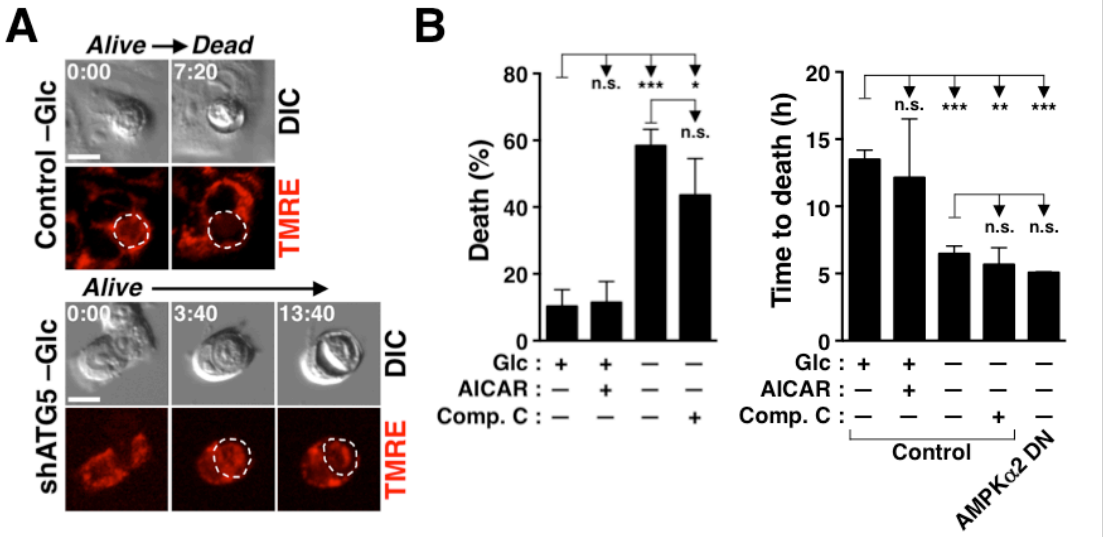


Figure 2.6. Entotic cell death is independent of AMPK
(A) Cells are engulfed alive and death can be inhibited by knockdown of Atg5. Images show both control and shATG5 cells grown in glucose-free conditions in the presence of TMRE, a dye that labels polarized mitochondria. Upon inner cell death in control cells, the fluorescent signal drops, while internalized shATG5 cells that remain alive for more than 10 hours are TMRE-positive throughout. Dashed lines mark internalized cells. Scale bar: 10 μm. (B) Entotic cell death is unchanged by AMPK inhibition. Graph on left shows quantification of entotic cell death, while graph on right shows timing of inner cell death after completion of engulfment in MCF-7 cells grown in respective conditions, as determined by time-lapse imaging. Error bars depict mean ± SEM; data are from at least three independent experiments. This figure is adapted from Hamann et al., 2017.

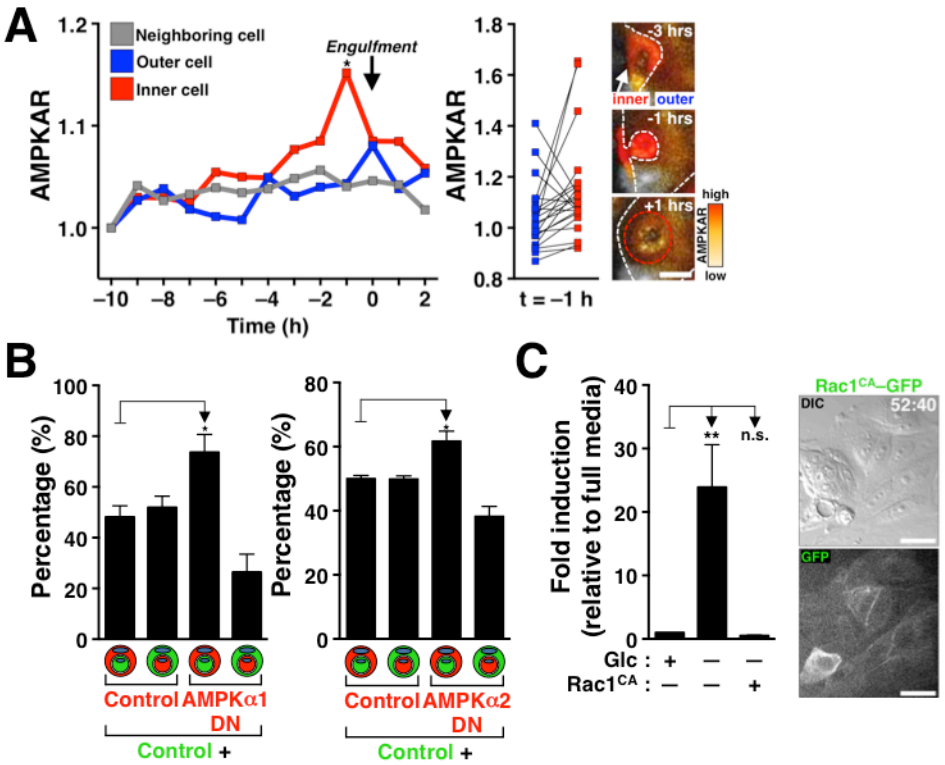


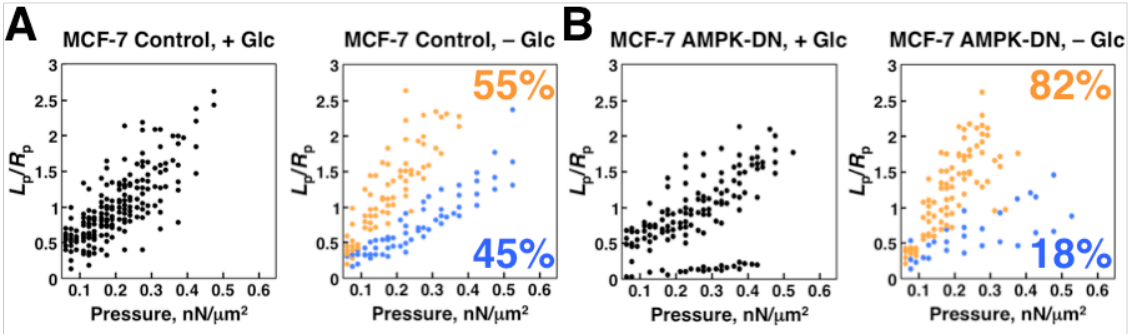
Figure 2.7. AMPK regulates loser cells during glucose starvation-induced entosis
(A) AMPK activity increases within internalizing cells during glucose starvation. Graph on the left shows normalized AMPKAR FRET ratio of internalizing cells ("inner cell") in glucose-free media throughout 12 hours (10 hours prior to completion of entosis and 2 hours after engulfment is complete, as determined by imaging), as well as AMPKAR values of entotic host cells ("outer cell") and neighboring single cells. Data is from two independent experiments (n=10 and n=12, respectively). *P*-values according to two-way ANOVA multiple comparisons test. Graph on right shows FRET values of matched outer and inner cell pairs one hour before completion of engulfment. Images show representative entotic engulfment of cells expressing AMPKAR. White dashed line marks the boundary between cells (inner cell is labeled by white arrow); red dashed line marks inner cell within outer cell after entosis. Scale bar: 10 μm. (B) Overexpression of dominant-negative AMPK blocks loser cell behavior in MCF-7 cells cultured in glucose-free media. Graph shows quantification of heterotypic entotic structures of mCherry-expressing AMPK-DN cells cultured 1:1 with parental cells (expressing GFP) and cultured in glucose-free media for 72 hours. Error bars show mean ± SEM; data are from at least three independent experiments. (C) Overexpression of constitutively active $Rac1^{V12}$ ($Rac1^{CA}$–GFP) blocks glucose starvation-induced entosis. Graph shows quantification of entosis events throughout 72 hours of parental MCF-7 cells or cells expressing constitutively active Rac1 grown in full or glucose-free media, as determined by time-lapse microscopy. Error bars show mean ± SEM; data are from at least three independent experiments. Time-lapse images show cells expressing $Rac1^{CA}$–GFP. Time shown is in hours:minutes. Scale bar: 15 μm. This figure is adapted from Hamann et al., 2017.

expressing AMPK-DN were winner cells in ~75% of heterotypic entotic structures, consistent with the idea that AMPK acts within loser cells (Figures 2.7B).

We then tested whether glucose deprivation primarily promotes entosis by inducing loser cell status. We took advantage of our previous observation that expression of a constitutively active form of Rac1 ($Rac1^{V12}$, hereafter $Rac1^{CA}$–GFP) can inhibit cell uptake by blocking loser cell internalization while promoting winner cell behavior (Sun et al., 2014b). $Rac1^{CA}$–GFP expression completely blocked the induction of entosis in glucose-depleted conditions (Figure 2.7C), consistent with the model that glucose deprivation activates entosis by stimulating loser cell behavior through increased AMPK activity, and activation of Rac1 can override this signal (Hamann et al., 2017).

2.2.5 Glucose starvation alters cell deformability

A differential in cell deformability (inverse elasticity) appears to be a requisite for entosis (Sun et al., 2014b). We therefore sought to determine if mechanical deformability is altered in glucose-starved cell populations using MPA. Control cells display a monomodal distribution of deformability (Figures 2.8A and C). However, glucose-starved cells were distributed into a bimodal population based on their mechanical profiles, with cells exhibiting relatively more (low elastic modulus) or less (high elastic modulus) deformability (Figures 2.8A and C) (Hamann et al., 2017). The measured elastic modulus of the less deformable cell population that appeared upon glucose starvation was significantly higher compared to MCF-7 cells in nutrient-rich medium, suggesting this population



C

Cell type	Elastic Modulus (nN/μm²) mean ± SEM, *n*	*P*-value
MCF-7 Control, + Glc	0.050 ± 0.003, *39*	
MCF-7 Control, – Glc	0.039 ± 0.002, *12*	0.46
MCF-7 Control, – Glc	0.072 ± 0.007, *10*	0.038
MCF-7 AMPK-DN, + Glc (upper)	0.077 ± 0.005, *19*	
MCF-7 AMPK-DN, + Glc (lower)	0.498 ± 0.05, *4*	0.0005
MCF-7 AMPK-DN, – Glc	0.029 ± 0.001, *27*	0.0003
MCF-7 AMPK-DN, – Glc	0.151 ± 0.032, *6*	0.0004

Figure 2.8. Glucose starvation alters cell deformability in an AMPK-dependent manner

(A) Glucose starvation induces two mechanically distinct cell populations. The scatterplot on the left shows individual measurements of control MCF-7 cells cultured in full media conditions for 48 hours (+Glc). The scatterplot on the right shows control MCF-7 cells cultured in glucose-free media for 48 hours (–Glc). Note the appearance of two distinct populations upon glucose starvation. The y-axis represents the deformation of individual cells (L_p) normalized by the radius of the micropipette (R_p). Cell measurements were color-coded based on each cell's degree of deformability at high pressures. The distribution of slopes for individual cells in both the control and the –Glc conditions were tested using the dip test for multimodality (R statistical package). The control dataset was consistent with a monomodal distribution (p = 0.854) while the –Glc dataset was consistent with a bimodal population distribution (p = 0.015). (B) Expression of AMPK-DN blocks the appearance of less-deformable cell population in glucose starvation. On the left are individual measurements of MCF-7 cells expressing a dominant-negative isoform of AMPK (AMPK-DN) grown in full media for 48 hours. On the right are individual measurements of AMPK-DN cells grown in glucose-free conditions for 48 hours. The number of cells present in the blue, less deformable category in glucose starvation is significantly reduced by expression of AMPK-DN (compare to –Glc graph in (A)). (C) Summary of measured mechanical parameters. Font color corresponds to populations labeled in the same color in (A) and (B), respectively. *P*-values listed for MCF-7 control, –Glc set were compared to MCF-7 control, +Glc cells; *P*-values for MCF-7 AMPK-DN, –Glc were compared to MCF-7 AMPK-DN, +Glc (upper) cells. *P*-values were obtained by ANOVA with Fisher's least significant difference test. This figure is adapted from Hamann et al., 2017.

could represent loser cells within the population, similar to what we observed previously (Sun et al., 2014b) (Figure 2.8C). To examine if AMPK plays a role in controlling the appearance of this population, we performed MPA with control and glucose-starved MCF-7 cells expressing AMPK-DN. After glucose starvation, the number of cells clustering into the high elasticity population was significantly reduced, suggesting that AMPK activity impacts loser cell behavior, in part, by altering cell deformability (Figures 2.8B and C) (Hamann et al., 2017).

Altogether, our data demonstrate that glucose starvation induces a high level of entosis in matrix-adherent breast cancer cell populations, in a manner controlled by AMPK activity in loser cells. Glucose starvation results in the emergence of a bimodal mechanical distribution of cells, where AMPK is required for the appearance of the less deformable subpopulation that we have shown previously has loser cell activity.

2.3 DISCUSSION

In this chapter, we show that nutrient deprivation in the form of glucose withdrawal is an inducer of the cell engulfment and death mechanism entosis. Thus, in addition to matrix detachment, metabolic stress resulting from insufficient glucose availability, which is known to occur during tumorigenesis (Denko, 2008), may induce entosis in human cancers (Hamann et al., 2017). We find that glucose starvation induces entosis by activating the energy-sensing kinase AMPK within loser cells. Consistent with this, the activation of Rac1, which blocks loser cell behavior, inhibits starvation-induced entosis. Mechanical measurements of cells undergoing glucose starvation revealed the appearance of a bimodal population of cells consisting of one group that is much more deformable and a second group that is less deformable in a manner dependent on AMPK activity. These data support a model where glucose starvation activates entosis by upregulating loser cell behavior, which is known to be controlled by RhoA and ROCK (Figure 2.9). Long-term glucose withdrawal can lead to AMPK-dependent cell death by apoptosis (El-Masry et al., 2012; Okoshi et al., 2008). Our data demonstrate that AMPK can also promote cell death through entosis, an effect potentially linked to the known AMPK-dependent control over myosin contractility (Bultot et al., 2009; Thaiparambil et al., 2012) (Hamann et al., 2017).

In addition to AMPK-dependent regulation of loser cell mechanics, about half of the cells in the population show increased deformability compared to controls (Figure 2.8A). This highly deformable population becomes the dominant

population upon AMPK inhibition (Figure 2.8B). Thus, glucose starvation triggers two events: high levels of AMPK activation in one population, leading to low deformability, and increased deformability in the other population, promoting winner status. In cells cultured in nutrient-replete medium, we have found that activators of AMPK (AICAR and A-769662) are sufficient to induce entosis, but to a lower extent than by glucose starvation, suggesting that winner cell mechanics induced in starved cultures could contribute significantly to entosis induction (Hamann et al., 2017). How glucose starvation promotes winner cell mechanics, as well as entotic cell death, and whether these activities could be coupled, are important questions for further study.

The mechanism of how AMPK signaling and autophagy pathway proteins coordinately regulate cell-autonomous (autophagy) versus non-cell-autonomous (entosis) processes will also be interesting to explore in future studies. Autophagy proteins in this context control the digestion of extracellular nutrients through a pathway resembling LC3-Associated Phagocytosis, or LAP, first identified in macrophages (Sanjuan et al., 2007). We find that the autophagy

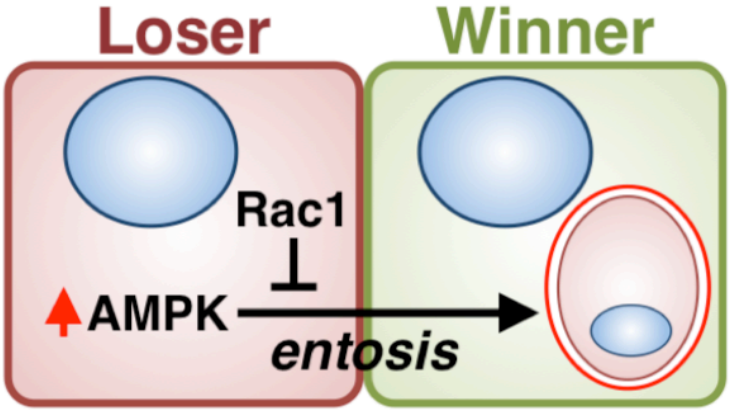


Figure 2.9. Model for entosis induction by glucose starvation
Glucose starvation-induced entosis between neighboring cells by upregulation of AMPK activity in loser cells. Activation of Rac1 in the loser cell can block its engulfment through entosis. This figure is adapted from Hamann et al., 2017.

protein-regulated death of ingested entotic cells is also controlled by glucose signaling, but in an AMPK-independent manner. How starvation impacts the death and degradation of ingested cells remains to be identified.

The recent expansion of conditions that induce entosis (Durgan et al., 2017; Hamann et al., 2017) suggest that entosis may represent a broad cellular response to stress conditions, similar to the large number of triggers for apoptotic cell death. Among all the nutrients examined, none besides glucose appear to induce entosis, suggesting a unique characteristic to glucose withdrawal and AMPK signaling (Hamann et al., 2017). However, as cell death triggers span beyond nutrient withdrawal, whether any other stress conditions induce entosis remains to be examined and will be discussed further in Chapter 4.

2.4 MATERIALS AND METHODS

2.4.1 Cell Culture and Reagents

MCF-7 cells (Lombardi Cancer Center, Georgetown University, Washington, DC) were cultured in DMEM (11965-092; Life Technologies, Grand Island, NY) supplemented with 10% heat-inactivated FBS (F2442; Sigma-Aldrich, St. Louis, MO) and penicillin/streptomycin (30-002-CI; Mediatech, Inc., Manassas, VA). MDA-MB-231 cells and its derivative were grown in RPMI (11875-093; Life Technologies, Grand Island, NY) supplemented with 10% heat-inactivated FBS with penicillin/streptomycin, as described (Sun et al., 2014a). MCF-10A cells were cultured in DMEM/F-12 (11320-033; Life Technologies, Grand Island, NY) supplemented with 5% HS (S12150; Atlanta Biologicals, Inc., Flowery Branch, GA), 20 ng/ml EGF (AF-100-15; Peprotech, Rocky Hill, NJ), 10 μg/ml insulin (I-1882; Sigma-Aldrich, St. Louis, MO), 0.5 μg/ml hydrocortisone (H-0888; Sigma-Aldrich, St. Louis, MO), 100 ng/ml cholera toxin (C-8052; Sigma-Aldrich, St. Louis, MO), and penicillin/streptomycin (30-002-CI; Mediatech, Inc., Manassas, VA), as previously described (Debnath et al., 2003). Glucose-free, amino acid-free, and glucose/amino acid-free medium was prepared by dialyzing heat-inactivated FBS for four hours at 4°C in PBS (P3813; Sigma-Aldrich, St. Louis, MO) in MWCO 3500 dialysis tubing (21-152-9; Fisherbrand, Pittsburgh, PA), followed by overnight at 4°C in fresh PBS and subsequent addition to base media prepared without respective component (glucose, amino acids, or glucose and amino acids, respectively) to a 10% final concentration. Cells expressing the H2B–mCherry nuclear marker were prepared by transducing cells with

retroviruses made with the pBabe–H2B–mCherry construct, as described (Florey et al., 2011). The mCherry–AMPK-DN construct was generated by inserting the AMPKα2 K45R gene (from Plasmid #15992; Addgene, Cambridge, MA) into the pQCXIP–mCherry retroviral vector. pEGFP–Rac1^{CA} was a gift from Dr. Alan Hall (Memorial Sloan Kettering Cancer Center, New York, NY). For disruption of E-cadherin in MCF-7 cells by CRISPR-Cas9, guide RNAs (gRNAs) were designed using the online CRISPR design tool from Feng Zhang's laboratory (http://crispr.mit.edu) and the gRNA with one of the highest theoretical mutagenic efficiency was used (5'-CGCCGAGAGCTACACGTTCACGG-3'). The vector encoding for Cas9 (pCDNA3.3–TOPO–hCas9, Plasmid #41815; Addgene, Cambridge, MA), as well as vector encoding the gRNA (pCR–Blunt II–TOPO, Plasmid #41824; Addgene, Cambridge, MA), were introduced into control MCF-7 cells by nucleofection (Cell Line Nucleofector Kit V, VCA-1003; Lonza, Basel, Switzerland). Single cell clones were selected and examined for disruption of E-cadherin by sequencing and western blotting. Cells were treated with Y-27632 (#1254; Tocris Bioscience, Bristol, UK) at 10 μM, AICAR (#9944; Cell Signaling, Danvers, MA) at 2 mM, A-769662 at 500 nM (#3336; Tocris Bioscience, Bristol, UK) and Compound C (P5499; Sigma-Aldrich, St. Louis, MO) at 10 μM. Inhibitors (or vehicle) were added to cultures ~30 minutes before the start of biological assays unless indicated otherwise.

2.4.2 Western blotting

Cells were lysed in ice-cold RIPA buffer and western blotting was performed as described (Florey et al., 2011). The following antibodies were used: anti-E-

cadherin (1:500; 3195, Cell Signaling, Danvers, MA), anti-tubulin (1:2000; 3873, Cell Signaling, Danvers, MA), anti-Atg5 (1:500; 2630, Cell Signaling, Danvers, MA), anti-phospho-ACC-S79 (1:500; 3661, Cell Signaling, Danvers, MA), anti-ACC (1:500; 3662, Cell Signaling, Danvers, MA), anti-phospho-AMPK-T172 (1:500; 2531, Cell Signaling, Danvers, MA), anti-mCherry (1:500; ab125096, Abcam, Cambridge, MA), anti-β-actin (1:2000; A1978, Sigma-Aldrich, St. Louis, MO), anti-rabbit IgG HRP-linked antibody (1:5000; 7074, Cell Signaling, Danvers, MA), and anti-mouse IgG HRP-linked antibody (1:5000; 7076, Cell Signaling, Danvers, MA).

2.4.3 Immunofluorescence

The following antibodies were used for immunofluorescence (IF): anti-β-catenin (1:100; C2206; Sigma-Aldrich, St. Louis, MO), anti-Lamp1 (1:100; 555798; BD Biosciences, Franklin Lakes, NJ), Alexa Fluor 568 goat anti-mouse secondary (1:500; A-11031; Life Technologies, Grand Island, NY), and Alexa Fluor 488 goat anti-rabbit secondary (1:500; A-11034; Life Technologies, Grand Island, NY). IF was performed on cells cultured on glass-bottom dishes (P35G-1.5-20-C; MatTek, Ashland, MA), as described (Overholtzer et al., 2007). Briefly, cells were fixed in 1:1 methanol:acetone for five minutes at -20°C, followed by three five-minute PBS washes and blocking in 5% BSA, 100 mM glycine in PBS for 1 hour, followed by incubation with primary antibodies at 4°C overnight. Samples were then incubated with secondary antibodies and counterstained with DAPI (1:1000; D1306; Life Technologies, Grand Island, NY). Confocal microscopy was performed with the Ultraview Vox spinning-disk confocal system (Perkin Elmer,

Waltham, MA) equipped with a Yokogawa CSU-X1 spinning-disk head and an electron-multiplying charge-coupled device camera (Hamamatsu C9100-13) coupled to a Nikon Ti-E microscope; image analysis was done using Volocity software (Perkin Elmer, Waltham, MA).

2.4.4 Time-lapse microscopy

Cells were cultured on glass-bottom dishes (P06G-1.5-20-F; MatTek, Ashland, MA), and time-lapse microscopy was performed in 37°C and 5% CO_2 live-cell incubation chambers, as described (Florey et al., 2011). Fluorescence and differential interference contrast (DIC) images were acquired every 20 minutes for 72 hours using a Nikon Ti-E inverted microscope attached to a CoolSNAP charge-coupled device camera (Photometrics, Tucson, AZ) and NIS Elements software (Nikon, Melville, NY). For TMRE imaging, TMRE (T669; Thermo Fisher Scientific, Grand Island, NY) was added to cultures to be imaged at a final concentration of 100 nM.

2.4.5 Entosis quantification

For quantification of entosis in MCF-7 cells by immunofluorescence, 250,000 cells were plated on 35 mm glass-bottom dishes and allowed to adhere overnight, washed briefly three times with PBS, cultured in the indicated conditions for 72 hours, and fixed and stained as described above. The percentage of entotic cells was determined by counting at least 300 cells in each sample and quantifying the number of single cells and cell-in-cell structures; both dead (LAMP1-positive compartments) and live cells were counted as entotic. If one host cell contained two cells in separate compartments it was scored as two

engulfment events. In sequential cell-in-cell structures (see Figure 2.1 i and ii), only the outermost cell was counted as an entotic host. Fold induction of entosis in MCF-7 and MDA-MB-231 cells by time-lapse microscopy was determined as follows: engulfment events were scored by the appearance of a vacuole within the host cell throughout 72 hours and normalized to cell number at time 0 hours; normalized engulfment numbers from all conditions were then normalized to full media conditions ("fold induction"). For fate of internalized cells, time 0 of engulfment was determined by the appearance of a host cell vacuole; cell death was scored by changes in cell morphology in DIC channel. For quantification of entosis in MCF-10A cells by immunofluorescence, 500,000 cells were plated on 60 mm tissue culture dishes (353002; Corning, Inc., Corning, NY) and allowed to adhere overnight, washed briefly three times with PBS, and cultured in the indicated media for 96 hours (media was replaced after 48 hours). After 96 hours, cells were trypsinized (25053CI; Corning, Inc., Corning, NY) to achieve a single-cell suspension and 250,000 cells re-plated on 35 mm glass-bottom dishes in fresh media (either full or glucose-free) and allowed to adhere for 12 hours (for a total of 108 hours of starvation), at which point cells were fixed and stained as described above.

2.4.6 AMPK FRET measurements

A modified version of the AMPK reporter AMPKAR (Tsou et al., 2011) with an extended "EV" linker (Komatsu et al., 2011) was stably integrated into MCF-7 cells using PiggyBac transposase. Homogenous populations of reporter-expressing cells were isolated by limited dilution cloning; three independent

clones were analyzed. Time-lapse imaging was performed as previously described (Sparta et al., 2015) using a Nikon TiE with a 20x 0.75NA Plan Apochromat objective and CFP and YFP filter cubes (Chroma 49001 and 49003, respectively). Images were recorded with an Andor Zyla scMOS camera, using Nikon Elements software. For imaging, cells were plated on #1.5 glass bottom 96-well plates (P96-1.5H-N; In Vitro Scientific, Mountain View, CA) and maintained at 37°C and 5% CO_2. Raw image files were imported to ImageJ, where tracking of cells and measurement of fluorescence values was performed manually using a modified version of the "Manual Tracking" plugin. AMPKAR signals were calculated as the average background-subtracted CFP/YFP ratio within three 3-by-3-pixel regions of the cytoplasm.

2.4.7 MPA

MPA was performed as described (Sun et al., 2014b; Zhou et al., 2010). In short, after culturing cells in either full or glucose-free media for 48 hours, cells were trypsinized, pelleted by spinning down for five minutes at 1,500×g, and resuspended in the appropriate media. Prior to being measured, the cells were incubated for 15 minutes at 37°C and 5% CO_2. Cells were aspirated with 6-8 μm pipettes at varying and increasing pressures. For data analysis, the length of the deformation of the cell cortex pulled into the micropipette (L_p) was divided by the radius of the pipette (R_p), and L_p/R_p values were plotted as a function of applied pressure. These data were converted into apparent elastic moduli (Hochmuth, 2000). The distribution of slopes for individual cells in both the control and the –Glc conditions were tested using the dip test for multimodality (R statistical

package) and the resulting subpopulations were analyzed using ANOVA with Fisher's least significant difference test.

2.4.8 Statistics

The indicated *p* values were obtained using Student's *t* test unless otherwise noted. ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$; n.s., not significant.

CHAPTER 3: Outcomes of entosis induction on cell populations

3.1 INTRODUCTION

The data presented in Chapter 2 show that upon glucose withdrawal, cells engage in high levels of entosis in an AMPK-dependent manner (Hamann et al., 2017). This, in addition to matrix detachment and aberrant mitoses (Durgan et al., 2017; Hamann et al., 2017; Overholtzer et al., 2007), suggests that there are a variety of conditions under which entosis can be induced and the consequences of this entotic activity within a cell population remain poorly understood. As many entosis studies are performed with cancer cells, and entotic structures are frequently found in tumor samples, outstanding questions remaining in the field include a more detailed role for entosis in tumor cells and its effects on tumor cell population dynamics.

3.1.1 Consequences of entosis

Inhibition of entosis in soft agar promotes colony formation, suggesting a tumor suppressive function of this process (Florey et al., 2011). As even two recently-formed siblings can engage in entosis (Sun et al., 2014a), one can envision a model where engaging in this process reduces overall cell number, especially if cells are taking up multiple neighbors, as is the case during glucose starvation (Hamann et al., 2017). Furthermore, it was recently shown that entosis can also be induced by aberrant mitoses that occur due to knockdown of the GTPase Cdc42 that disrupts cell polarity (Durgan et al., 2017). In this model, increased rates of mitosis in tumor cells might be matched with an increase in clearance,

decreasing the overall growth rate of a tumor cell population that may otherwise be expected.

However, engaging in entosis also disrupts cell division in host cells by blocking the scission of the cleavage furrow during mitosis (Krajcovic et al., 2011). This results in the multinucleation of hosts that can promote aneuploidy in subsequent cell divisions (Krajcovic et al., 2011). As genomic instability is a hallmark of cancer (Hanahan and Weinberg, 2011), disruption of cell ploidy is thought to be a potent inducer of tumorigenic potential (Pfau and Amon, 2012). In agreement with this model, the frequency of cell-in-cell structures in human tumor samples increases in more proliferative, higher-grade tumors (Krajcovic et al., 2011). The divergent roles proposed for entosis in tumorigenesis are not necessarily mutually exclusive, however, and it is therefore likely that its influence will differ depending on when during tumor formation entosis occurs. For example, high levels of entosis that are not balanced with a similar increase in cell proliferation will, in the short-term, lead to a dramatic reduction in cell number, therefore acting in a tumor-suppressive manner, as discussed above. Conversely, the host cells that remain after entosis induction are shown to experience high levels of chromosomal alterations that might result in mutations that can drive oncogenesis. It is therefore important to understand and identify potent triggers of entosis and examine their consequences in a variety of different contexts.

During amino acid withdrawal, uptake and degradation of neighboring cells conferred a survival advantage upon host cells, suggesting that certain

nutrients may be reutilized for host cell metabolism (Krajcovic et al., 2013). Indeed, in a macrophage-based system of phagocytosis, engulfment of radioactively-labeled apoptotic corpses resulted in the appearance of ^{35}S-labeled methionine in macrophage proteins (Krajcovic et al., 2013; Krishna et al., 2016).

The data presented in Chapter 2 suggest that within heterogeneous tumor cell populations, cells experiencing higher levels of metabolic stress (i.e. higher levels of AMPK activation) than their neighbors are eliminated through entosis (Hamann et al., 2017). This population-scale behavior is reminiscent of cell competition, a phenomenon whereby suboptimal cells can be cleared from a population through induction of cell death by neighboring cells (Merino et al., 2016). Whether in the context of glucose starvation entosis acts competitively and functions to improve the fitness of tumor cell populations will be explored in this chapter.

3.1.2 Cell competition

The occurrence of cell competition was first described in 1975, when cells within the *Drosophila* imaginal discs carrying heterozygous mutations in a set of genes called *Minute* ($M^{-/+}$) were found to be eliminated from tissues that also contained wild-type cells (Morata and Ripoll, 1975). Intriguingly, homozygous $M^{-/+}$ tissues were viable, suggesting their viability may be regulated by the presence of wild-type cells. The *Minute* mutations were later found to encode ribosomal proteins that resulted in lowered overall gene dosage compared to wild-type cells (Kongsuwan et al., 1985). Competition was therefore suggested to serve as a detection mechanism to eliminate cells that on their own are viable, but in the

presence of ‘fitter’ winner cells are considered detrimental to the overall viability and fitness of the organism (Figure 3.1A) (Merino et al., 2016). Conversely, cells that harbor mutations resulting in an increase in relative fitness, such as high levels of myc expression, act as ‘supercompetitors’ and eliminate wild-type cells

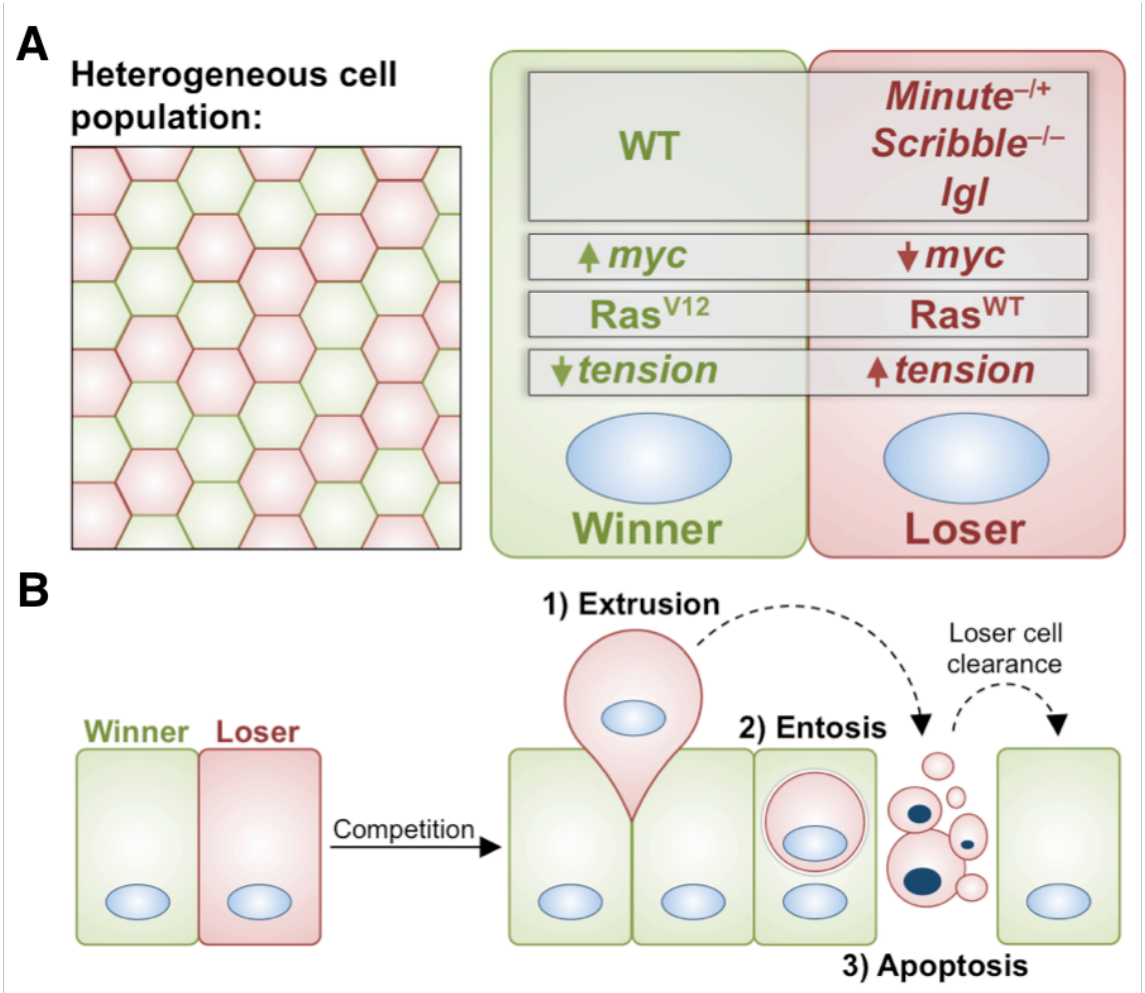


Figure 3.1. Regulation of cell competition
(A) Cell competition can occur in heterogeneous cell populations. Within mosaic tissues, winner cells (green) can engage in cell competition with loser cells (red) that is mediated by a wide variety of genetic and cellular machinery, examples of which are shown on the right. For cell competition mediated by entosis, it was recently shown that mutant Ras confers winner status, in part by regulating cell tension through actomyosin contraction (Sun et al., 2014b). (B) Elimination of loser cells during cell competition. 1) Loser cells can be extruded from the epithelium, both apically (shown) or basally, after which they may undergo apoptosis and be cleared by surrounding cells. 2) Entosis also mediates cell competition, allowing loser cells to be cleared from a population in a manner dependent on their relative cell tension. 3) Loser cells can undergo apoptosis within the epithelium and be cleared either by neighboring epithelial cells or circulating phagocytes.

in mixed heterogenous populations (Figure 3.1A) (de la Cova et al., 2004; Moreno and Basler, 2004). While the initial studies demonstrating cell competition were performed mostly in *Drosophila* model systems, a recent paper demonstrated myc-dependent competition in the mouse epiblast (Claveria et al., 2013). These studies suggested that in the developing mouse, populations of cells with varying myc levels compete in order to eliminate suboptimal cells to produce viable offspring (Claveria et al., 2013).

In addition to genetic changes that directly alter the proliferative capacity of a cell, other mutations or stress conditions can also induce competitive behavior (Menendez et al., 2010). Clones within *Drosophila* imaginal discs with disrupted apicobasal polarity, due to mutations in polarity complexes such as *lgl* and *scrib*, are eliminated from heterozygous tissues (Figure 3.1A) (Menendez et al., 2010). Homozygous *lgl* mutants form tumors in imaginal discs and this is inhibited through competitive clearance by wild-type cells in heterozygous tissues, suggesting cell competition may play a tumor-suppressive role in this context (Menendez et al., 2010). Upon whole-body UV irradiation of flies, damaged cells express a set of genes, including Azot, which encodes for a calmodulin-like protein, that have been termed ‘fitness markers’ (Merino et al., 2015). The activation of this transcriptional cascade upon environmental stress ensures that suboptimal cells are eliminated, a process that maintains viability and prolongs organismal lifespan (Merino et al., 2015).

Recently, we demonstrated that entosis acts as a form of cell competition, where the engulfment of loser cells by neighboring winners can promote clonal

selection within heterogeneous tumor cell populations (Figure 3.1B) (Sun et al., 2014b). Competition is driven by a mechanical differential between softer (reduced elasticity) cells and stiffer cells, where stiffer cells are eliminated by softer winners (Hamann et al., 2017; Sun et al., 2014b). This competitive process is regulated by key controllers of cell tension, such as Rho GTPase, Rho kinase, and myosin (Hamann and Overholtzer, 2017; Sun et al., 2014b). Oncogenic mutations, such as K-Ras^{V12}, that impinge on Rac1 activity, are sufficient to confer winner status by increasing cell deformability, likely due to inhibition of RhoA by Rac1 activity (Figure 3.1A) (Sun et al., 2014b). In this chapter, we explore in more detail the role of entosis induced by glucose starvation in altering heterogenous cell populations and its potential role in cell competition.

3.2 RESULTS

3.2.1 Glucose withdrawal induces multiple cell death pathways

We further examined the effects of entosis in glucose-starved conditions over a longer timecourse. Over the first 72 hours of glucose starvation, the population size was significantly reduced by frequent cell deaths occurring with entotic, necrotic, or apoptotic morphologies, as quantified by time-lapse microscopy (Figure 3.2). Intriguingly, we found that upon inhibiting entosis, either through deletion of E-cadherin or treatment of cells with the ROCK inhibitor Y-27632, the proportion of necrotic cell deaths that occurred under glucose starvation conditions increased (Figure 3.2) (Hamann et al., 2017). While the overall death rate of cells lacking E-cadherin was slightly reduced, the proportional increase in

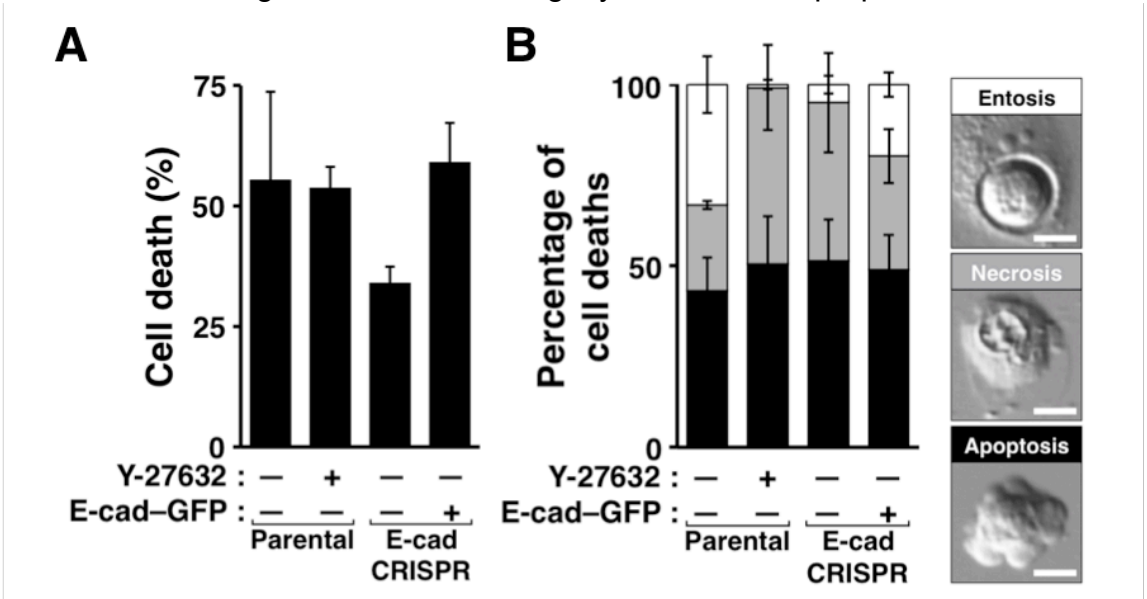


Figure 3.2. Glucose withdrawal induces multiple cell death pathways
(A) Inhibition of entosis does not increase overall rates of cell death. Graph shows quantification of all types of cell death in MCF-7 cells throughout 72 hours of glucose starvation, as determined by time-lapse microscopy. Error bars show mean ± SEM; data are from at least three independent experiments. (B) Inhibition of entosis results in increased rates of necrotic cell death. Graph shows percentage of different types of cell death observed in 72 hours of glucose starvation by time-lapse microscopy. Error bars show mean ± SEM; data are from at least three independent experiments. Representative DIC images show the three types of death morphologies used to score cell death. Scale bar: 10 μm. This figure is adapted from Hamann et al., 2017.

necrotic deaths was similar to the Y-27632-treated cells (Figure 3.2) (Hamann et al., 2017). These data raise an intriguing possibility, where engagement of necrosis or entosis may be regulated by common molecular machinery in a context-dependent manner.

3.2.2 Glucose starvation increases rates of multinucleation

Typically, entotic cell structures induce the generation of aneuploid cell lineages due to the failure of engulfing cells to divide properly (Krajcovic et al., 2011). Because most cell divisions occurring under starvation conditions involved entotic cell structures, we examined if nutrient withdrawal led to the appearance of multinucleated cells. Indeed, nutrient starvation induced a 5-fold increase in

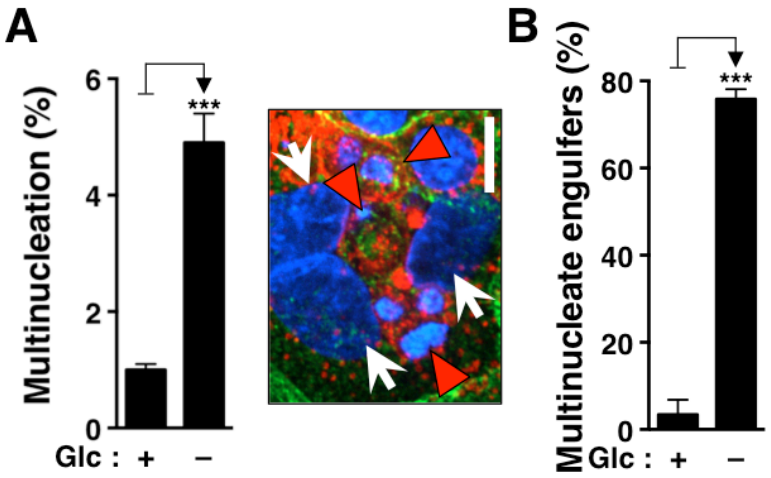


Figure 3.3. Glucose starvation induces multinucleation
(A) Graph shows quantification of multinucleated cells in cultures of MCF-7 cells grown in the presence or absence of glucose for 72 hours, as determined by immunofluorescence. Error bars show mean ± SEM; data are from at least three independent experiments. Image shows appearance of multinucleated cell after 72 hours of glucose starvation, with three nuclei (arrows) and three engulfed cells (arrowheads). Immunostaining for β-catenin (green) and LAMP1 (red), and DAPI-stained nuclei (blue) are shown. Scale bar: 10 μm. (B) Multinucleation resulting from glucose starvation is associated with the presence of entotic host cells. Graph shows quantification of percentage of multinucleated cells that are also entotic hosts in cultures of MCF-7 cells grown in the presence or absence of glucose for 72 hours, as determined by immunofluorescence. Error bars show mean ± SEM; data are from at least three independent experiments. This figure is adapted from Hamann et al., 2017.

the percentage of cells exhibiting multinucleation (Figure 3.3), suggesting that starvation can disrupt cell ploidy by inducing entosis (Hamann et al., 2017).

3.2.3 Entosis supports proliferation in nutrient-limiting conditions

We examined the consequences of glucose withdrawal-induced entosis on cell populations. We asked whether the ingestion and degradation of loser cells could provide winner cells with nutrients that support cell survival or proliferation during starvation, similar to what we have shown for amino acid starvation (Krajcovic et al., 2013). Indeed, even under stringent conditions of dual glucose and amino acid deprivation, cells that had ingested their neighbors proliferated 10-fold more frequently than control single-cell neighbors (Figures 3.4A and B) (Hamann et al., 2017).

3.2.4 Population outgrowth is sustained in cells engaging in entosis

Following 72 hours, MCF-7 cells expressing E-cadherin exhibited a population doubling after six days of continued growth in the absence of glucose (Figure 3.4C). Conversely, cells lacking E-cadherin, which are deficient for entosis induction, had an impaired ability to grow under starvation conditions, despite a slightly increased ability to proliferate under nutrient-replete conditions (Figure 3.4D). Altogether, these data are consistent with a model that entosis induced by nutrient starvation supports the proliferation of winner cells under conditions of continued nutrient withdrawal (Hamann et al., 2017).

3.2.5 Entosis enables cell competition in glucose-starved conditions

To examine the properties of cancer cell populations selected by glucose starvation, we took advantage of our observation that MCF-7 cells could be

grown in the absence of glucose for extended periods (MCF-7^{-Glc}). MCF-7^{-Glc} cells were selected in continuous culture for 36-78 days in the absence of glucose. We co-cultured MCF-7^{-Glc} cells with passage-matched parental starvation-naïve MCF-7 cells (MCF-7^{parental}) and quantified winner and loser status of each cell population in heterotypic entotic cell structures. In glucose-free conditions, MCF-7^{-Glc} displayed a marked increase in winner cell activity (Figure

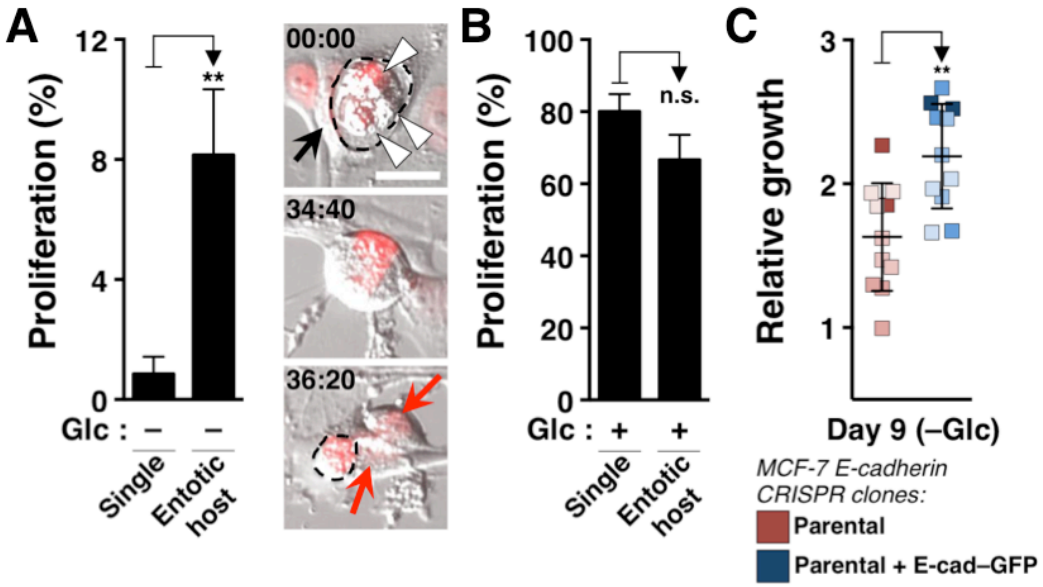


Figure 3.4. Entosis supports proliferation in nutrient-limiting conditions
(A) Entotic host cells have a proliferative advantage in starvation conditions compared to non-engulfing single cells. Graph shows quantification of proliferation events of single and entotic host cells in glucose/amino acid-free media, imaged by time-lapse microscopy over 72 hours after an initial starvation period. Error bars show mean ± SEM; data are from at least three independent experiments. Images show entotic host cell (black arrow) with three engulfed corpses (arrowheads) in glucose/amino acid-free media undergoing cell division; red arrows mark two daughter cells. Time shown is in hours:minutes. Scale bar: 15 μm. (B) Single and entotic host cells have a similar proliferation rate when cultured in full media conditions. Graph shows quantification of proliferation events of single and entotic host cells in full media, imaged by time-lapse microscopy over 72 hours after an initial starvation period (see Materials and Methods). Error bars show mean ± SEM; data are from at least three independent experiments. n.s., not significant. (C) Entosis-competent cells proliferate more than entosis-deficient cells in glucose-free conditions. Scatter plot shows number of MCF-7 E-cad CRISPR (red) and E-cad CRISPR + E-cad–GFP cells (blue) after 9 days of glucose starvation, relative to cell number after 72 hours. Shown are individual values from three independent experiments (data points from experimental replicates are shaded accordingly). Error bars show mean ± SD. This figure is adapted from Hamann et al., 2017.

3.5A). Thus, cells continually grown in glucose-depleted conditions maintain altered characteristics that confer winner status when co-cultured with parental cells under starvation conditions. Consistent with AMPK controlling loser cell activity (see Chapter 2.2.4 on page 53 above), selected cells also exhibited reduced AMPK activation, as well as lowered levels of P-MLC-S19, indicative of winner cells (Figure 3.5B) (Hamann et al., 2017).

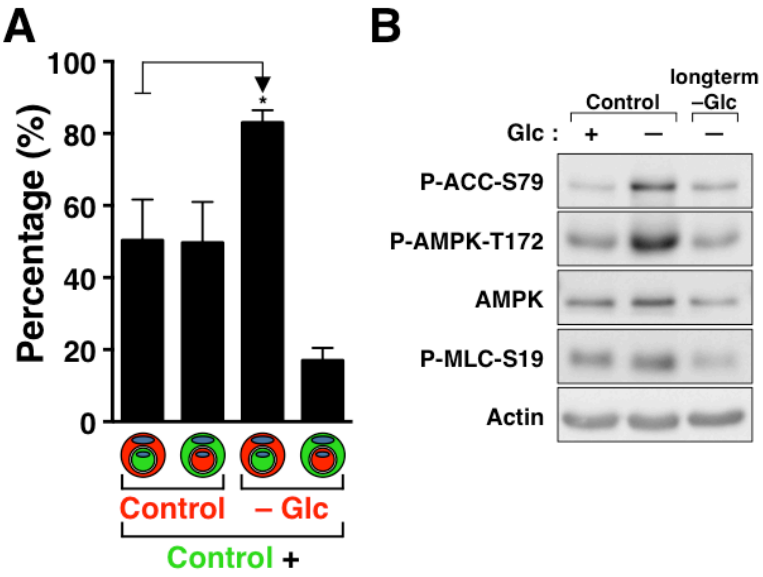


Figure 3.5. Entosis enables cell competition in glucose-starved conditions
(A) Cells cultured under prolonged glucose starvation preferentially internalize parental cells. Graph shows percentage of red-labeled cells (either parental MCF-7 or MCF-7 grown for 36-78 days in glucose-free conditions) that are hosts or internalized cells when mixed with passage-matched, green-labeled parental MCF-7 cells and cultured in glucose-free conditions for 72 hours. Error bars show mean ± SEM; data are from at least three independent experiments. (B) AMPK and myosin activity is reduced in long-term glucose-starved cells. Western blot shows activation of AMPK and downstream signaling, as well as activation of myosin light chain in MCF-7 cells cultured in the indicated conditions. This figure is adapted from Hamann et al., 2017.

3.3 DISCUSSION

In this chapter, we show that entosis induced by glucose starvation promotes the scavenging of nutrients by winner cells from losers, and its induction in this context allows cell populations to respond to starvation stress by inducing competition between cells (Hamann et al., 2017). In addition to starvation responses, such as autophagy, which promotes nutrient recycling to support cell survival (Mizushima et al., 2008), and macropinocytosis, which allows cancer cells to scavenge extracellular protein to support proliferation (Commisso et al., 2013; Nofal et al., 2017; Palm et al., 2015), entosis may be an important mechanism utilized by cancer cell populations to support metabolism under conditions of limiting nutrient availability (Hamann et al., 2017). In the long-term, some cancer cells may also activate gluconeogenesis to adapt to the continual absence of glucose, as reported (Mendez-Lucas et al., 2014).

Entosis may have the unique property of distributing nutrients to winner cells within a starved population, thereby supporting population re-growth following acute induction of cell death that initially reduces cell number. Intriguingly, we find that entosis inhibition by depletion of E-cadherin, or by treatment with Y-27632, increases rates of necrosis, while having no effect on the overall death percentage (Figure 3.2) (Hamann et al., 2017). Future studies to explore if this observed relationship results from co-regulation of these mechanisms will be informative.

We have previously found that entosis disrupts cell ploidy (Krajcovic et al., 2011), and we show here that starving cell populations exhibit multinucleation,

suggesting that an additional consequence of engaging this mechanism may be to promote tumorigenesis through the promotion of gross aneuploidy (Figure 3.3) (Hamann et al., 2017). Dying cells have been shown to provide nutrients to support the survival and proliferation of neighboring cells in single-cell yeast and bacterial populations undergoing starvation (Buttner et al., 2006; Fabrizio et al., 2004; Gourlay et al., 2006) and our data suggest that some cancer cell populations may also respond to starvation by redistributing nutrients in a manner that maintains the proliferation of selected cells (Figures 3.4 and 3.6) (Hamann et al., 2017).

These findings link a key nutrient signaling pathway and known inducer of autophagy to regulation of entosis. While autophagy promotes the recycling of intracellular nutrients to support cell survival, entosis allows winner cells in a

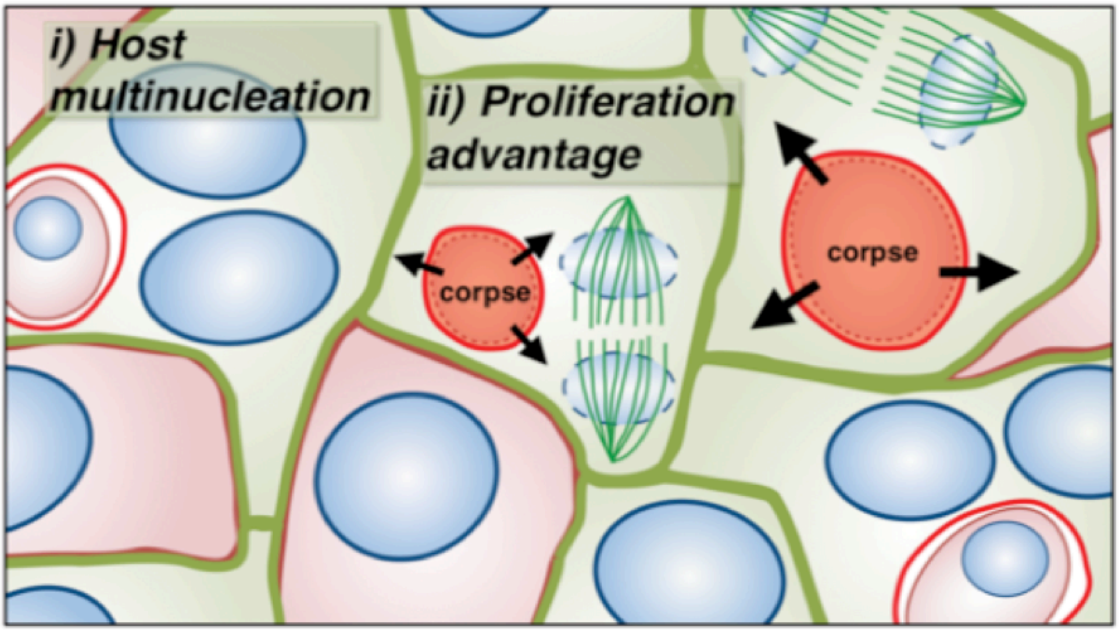


Figure 3.6. Consequences of entosis within a population
Within a cell population, entosis can (i) induce multinucleation of host cells, thereby generating aneuploid cell lineages, and (ii) confer a proliferation advantage to host cells (compared to single cells) in nutrient-restricted environments by recycling components of internalized cell corpse. This figure is adapted from Hamann et al., 2017.

population to scavenge extracellular nutrients and accumulate the biomass necessary to support proliferation (Figure 3.4) (Hamann and Overholtzer, 2017; Hamann et al., 2017). Macropinocytosis has been shown to act similarly to allow cancer cells to scavenge extracellular proteins (Krishna et al., 2016; Nofal et al., 2017; Palm et al., 2015). Entosis instead supplies bulk nutrients in the form of whole cells, where, on average, winner cells ingest two losers, providing a large nutrient supply that is well suited to support the outgrowth of selected cells in the population. Indeed, winners with ingested losers have a nearly 10-fold proliferative advantage, and entosis is required for population re-growth during long-term starvation (Hamann and Overholtzer, 2017; Hamann et al., 2017).

Our findings identify entosis as a population-scale starvation response with parallels to cell competition occurring in developing tissues (Huang et al., 2015). As mechanisms of cell competition select for the relative fitness of individual cells to promote tissue-scale fitness, entosis may similarly allow cell populations under extreme starvation stress to redistribute available nutrients to the fittest cells. Protection of the population may require sacrificing less-fit individuals, rather than rescuing those that may be under the most stress. In this model, regulation of loser cell mechanics by high levels of AMPK activity may be one signal that indicates a relative lack of fitness that could be sensed through cell junctions (Hamann and Overholtzer, 2017).

Long-term glucose starvation selects for winner cell behavior that is associated with increased proliferation, changes in cell ploidy, and a long-term ability to grow in the absence of glucose (see Figures 3.2, 3.3, 3.4, and 3.6)

(Hamann et al., 2017). Long-term glucose starvation also selects for winner cells with lowered levels of AMPK activity, and these cells are capable of ingesting naïve cells at high rates in mixed populations, demonstrating selection for the ability to scavenge nutrients through this competitive mechanism (Hamann and Overholtzer, 2017; Hamann et al., 2017). While we show that entosis is required for population re-growth, how this process links to the long-term proliferative capacity of winners is unknown, and may involve additional adaptations such as gluconeogenesis or other altered metabolic states that await discovery (Hamann and Overholtzer, 2017).

In summary, we have found that entosis is one of numerous non-apoptotic cell death mechanisms that has a unique property to promote competition between cells in a population. Recently, a regulated form of necrosis called ferroptosis was shown to have a different non-cell-autonomous effect, to spread from cell-to-cell (Kim et al., 2016). How cell population dynamics in diseased or stressed tissues are controlled may reflect in part the percentages of different types of cell death that occur and their distinct cell properties. We find that glucose starvation induces apoptosis, necrosis, and entosis and quantifying the percentages of mixed forms of cell death may be important for deciphering how different treatments influence cell selection (Hamann and Overholtzer, 2017).

3.4 MATERIALS AND METHODS

3.4.1 Proliferation advantage assay

250,000 cells per 35 mm well were plated on glass and allowed to adhere overnight. Cells were washed in PBS three times and grown in glucose/amino acid-free media for 72 hours to induce entosis. After, cells were washed in PBS and either full or glucose/amino acid-free media containing 10 μM Y-27632 was added to cells to inhibit further cell engulfment; cells were then imaged for 72 hours at 20 minute intervals. Cell fates of entotic hosts or single cells in each field of view were determined throughout this time.

3.4.2 Population growth assay

100,000 cells were seeded in triplicate in 12-well culture dishes (#3512; Corning, Corning, NY) and allowed to adhere overnight. Cells were washed in PBS three times and grown in glucose-free media for nine days, with three PBS washes and media replacement every 72 hours. For crystal violet staining, cells were washed once in PBS, fixed in 4% PFA in PBS for 15 minutes, washed once with H_2O, and subsequently stained with 0.1% crystal violet solution (in 10% ethanol) for 20 minutes. Crystal violet solution was aspirated and cells were washed three times with H_2O and allowed to air-dry overnight. The following day, crystal violet was extracted by incubating the cells with 1 ml 10% acetic acid for 20 minutes with gentle shaking, followed by absorbance measurements at 570 nm. Values were normalized to respective absorbance at day 3 of glucose starvation.

3.4.3 Quantification of winner/loser cell identity

Passage-matched cells (either control MCF-7 or –Glc MCF-7 (starved for glucose for 36-78 days with change of media every three days)) were labeled with 10 μM CellTracker dyes (green or red, C7025 and C34552, respectively; Life Technologies, Grand Island, NY) for 20 minutes at 37°C, then plated at a 1:1 ratio at a total cell density of 250,000 cells in 35 mm glass-bottom dishes overnight in media containing 10 μM Y-27632 to block entosis. The next day, cells were washed three times with PBS and glucose-free media was added for 72 hours, at which point cells were analyzed by confocal microscopy. Heterotypic cell-in-cell structures were counted and the number of structures of green-inside-red and red-inside-green determined. For competition assays with AMPKα1 DN cells, stable mCherry-expressing MCF-7 cells were transfected (using Amaxa Nucleofector (VCA-1003; Lonza, Basel, Switzerland) according to manufacturer's protocol) with either a vector expressing AMPK α1 DN (K47R) (Plasmid #79011; Addgene, Cambridge, MA) or empty vector and allowed to recover overnight. Cells were then mixed at a 1:1 ratio with stable GFP-expressing MCF-7 cells and plated and analyzed for cell-in-cell structures according to the same protocol described above. For competition assays with AMPKα2 DN cells, MCF-7 cells stably expressing either mCherry (control) or mCherry–AMPKα2 K45R DN were mixed at a 1:1 ratio with stable GFP-expressing MCF-7 cells and plated and analyzed as described above.

3.4.4 Statistics

The indicated *p* values were obtained using Student's *t* test unless otherwise noted. ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$; n.s., not significant.

CHAPTER 4: Conclusion and Future Perspectives

4.1 SUMMARY

The continuing expansion of cell death mechanisms suggests that many aspects of non-apoptotic cell death regulation remain to be discovered. Among these is entosis, a type of cell death characterized by the uptake of live neighboring cells, that was shown to be triggered by culturing cells in matrix-detached conditions (Overholtzer et al., 2007). In this thesis, we demonstrate an additional regulatory mechanism of this death, namely by glucose starvation (Figure 2.9) (Hamann et al., 2017). Entosis induced by glucose withdrawal is regulated by the energy sensor AMPK, a kinase complex that is activated when intracellular ATP concentrations decrease (Hardie et al., 2012). We further show that AMPK activity is required specifically within loser cells prior to their uptake into winners by altering the deformability of losers (Figure 4.1) (Hamann et al., 2017). In agreement with previous models, these less deformable cells are preferential loser cells when mixed with more deformable unstarved cells (Figure 3.5A) (Hamann et al., 2017; Sun et al., 2014b).

We have also found a role for entosis in the ability of cell populations to survive prolonged nutrient stress. Cells that engaged in entosis induced by glucose withdrawal exhibited a ten-fold increase in proliferation over non-participating neighbors, suggesting the ability to take up and degrade neighboring cells may provide a nutrient advantage (Figure 4.1) (Hamann et al., 2017; Krajcovic et al., 2013; Krishna et al., 2016). Further, the ability of cell populations to increase in number during long periods of nutrient withdrawal

depends on their ability to engage in entosis, as when entosis is blocked genetically or pharmacologically, cells population doublings increase at a much slower rate compared to wild-type cells (Figure 3.4C) (Hamann et al., 2017). This is again consistent with the model that entotic loser cells may act as a nutrient source in the absence of other extracellular nutrients (Figure 4.1).

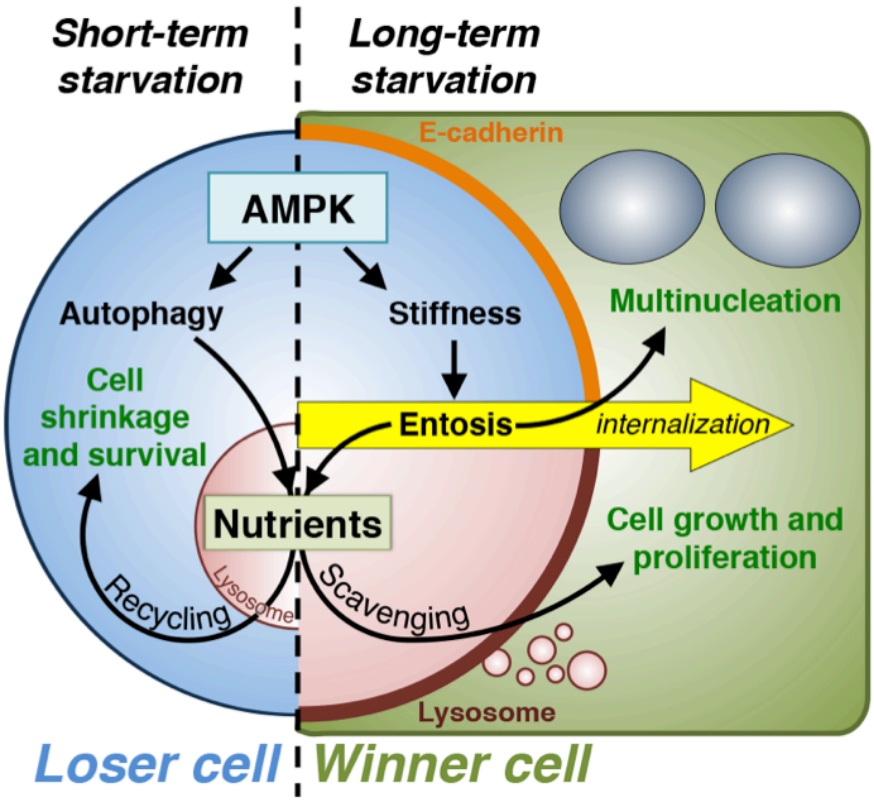


Figure 4.1. Nutrient scavenging through entosis
Entosis can occur as a result of long-term starvation. Under short-term starvation stress (left side of figure), AMPK activates autophagy, resulting in intracellular nutrient recycling from the lysosome (red half-circle) that allows cells to survive. In contrast, during long-term starvation stress (right side), AMPK controls an increase in cell tension that, in cells maintaining adherens junctions through E-cadherin (orange), leads to entotic cell internalization (yellow arrow). Uptake of live loser cells (light blue) can result in cytokinesis failure during subsequent mitoses of winner cells (green), which generates multinucleated and aneuploid cell lineages (top right). Upon killing of internalized loser cells by winner cells, the resulting corpses are degraded within winner cell lysosomes, which supports the growth and proliferation of winners that scavenge nutrients (bottom right). This figure is adapted from Hamann and Overholtzer, 2017.

4.2 FUTURE DIRECTIONS

4.2.1 Molecular mechanism of AMPK-dependent entosis

In Chapter 2, we showed that the activity of AMPK is required for entosis induced by glucose starvation specifically within loser cells (Hamann et al., 2017). This suggests AMPK may play a role in regulating loser cell behavior, for example by controlling the population of less deformable cells that appear upon glucose starvation (Hamann et al., 2017). Consistent with this model, genetic inhibition of AMPK activity blocks the appearance of this population, indicating that AMPK may regulate cell tension in some way (Hamann et al., 2017). Previous reports suggest that, in *Drosophila*, myosin light chain is a direct downstream phosphorylation target of AMPK (Lee et al., 2007). However, recent studies have cast doubt on this, and whether is also the case in human cells remains to be examined (Bultot et al., 2009). To test this hypothesis, we monitored the phosphorylation status of myosin light chain in either wild-type or AMPK-DN MCF-7 cells. Under glucose starvation conditions, there was no change in MLC phosphorylation between the two cell lines, inconsistent with a model of AMPK acting directly upstream of MLC.

Intriguingly, recent evidence suggests AMPK may have other functions that are distinct from its role in directly regulating metabolism (Banko et al., 2011; Schaffer et al., 2015). Since most cellular activities consume ATP, it is reasonable to imagine that AMPK may regulate proteins that are not necessarily involved in cellular metabolism itself but whose inhibition could nonetheless maintain energetic homeostasis. Recent efforts to identify novel AMPK

substrates have revealed proteins that are involved in aspects of cell adhesion, motility, and invasion (Schaffer et al., 2015). For example, NET1A, a RhoA GEF, is directly phosphorylated by AMPK and increases the ability of cells to invade into extracellular substrates (Schaffer et al., 2015). The exact mechanism of how AMPK-mediated phosphorylation changes NET1A activity and its downstream effects on RhoA activity remain to be elucidated. Other newly identified substrates include additional RhoA GEFs, such as ARHGEF2, as well as proteins involved in cell-cell adhesion complex stability, actin cytoskeleton rearrangements, and membrane deformability (CTNND1, BAIAP2L1, and BAIAP2, respectively) (Schaffer et al., 2015). The discovery of these new AMPK substrates is particularly interesting when considered in the context of cell deaths that involve cell-cell adhesion and changes in the actin cytoskeleton, as we have shown for entosis in this thesis.

AMPK has also been shown to regulate transcription, in part by controlling the activity of the FOXO family of transcription factors that can mediate a wide range of cellular effects, including cell proliferation and death (Hardie et al., 2012). Interestingly, the entotic engulfments observed in glucose-starved conditions occur throughout a period of 72 hours, with the highest activity beginning 36-48 hours after glucose withdrawal. This suggests a longer-term process, such as gene transcription, may be at play. It would therefore be of interest to examine the transcriptional changes that occur upon glucose starvation, both dependent and independent of AMPK. This may reveal processes that alter cellular shape and deformability that ultimately induce

entosis in a subset of cells. How AMPK activation under these conditions regulates cell death will be an important question for future studies.

4.2.2 AMPK-independent control of entosis

We have demonstrated that activation of AMPK, even in full media conditions, is sufficient to induce entosis in adherent cell populations (Hamann et al., 2017). However, the extent of cell uptake under these conditions was lower than in glucose-free media, suggesting that there may be AMPK-independent processes that also control entosis in this context. Similarly, inhibition of AMPK significantly blocked entosis, but some engulfment still occurred. Whether glucose starvation has an effect on winner cell behavior in this context will be important to examine in future studies. Our biophysical measurements suggest that in addition to the less deformable population, glucose starvation also results in the appearance of a second more deformable population (Hamann et al., 2017). Consistent with the model that AMPK-independent processes may control winner behavior, the appearance of this more deformable population is unchanged in the context of AMPK-DN cells.

In preliminary studies, we have uncovered a role for the energy sensor mTOR in controlling the induction of entosis upon glucose withdrawal. Pharmacological inhibition of mTOR during glucose starvation prevents the high levels of entosis induction observed under control conditions (Figure 4.2). Whether mTOR activity is required within loser cells, like AMPK, or is acting within winner cells remains to be examined. In previous studies, inhibition of mTORC1 blocked the shrinkage of entotic vacuoles, but had no effect on inner

cell death and degradation upstream of this process, suggesting a downstream role for mTORC1 activity (Krajcovic et al., 2013). These new results reveal that mTOR may also be playing a role upstream of engulfment, perhaps by regulating synthesis of certain substrates, as has been shown to be required for entotic invasion (Hinojosa et al., 2017). Our findings that inhibition of mTOR in glucose-free conditions is sufficient to block entosis raise the interesting possibility that mTOR may play a role in controlling winner cell behavior and it is therefore important to further examine the biophysical properties of cells under these additional conditions.

4.2.3 Regulation of entotic cell death during glucose starvation

Under control conditions, ~50% of internalized loser cells die by entotic cell death within 24 hours. In this thesis, we show that under glucose-starved conditions, within 10 hours ~90% of internalized cells die, suggesting that glucose

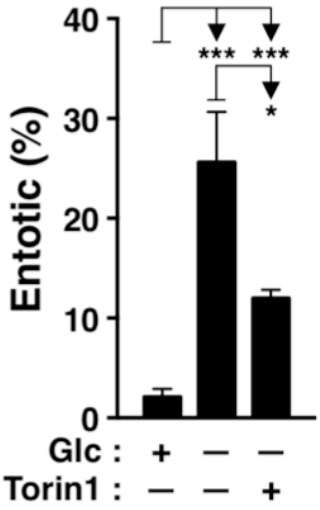


Figure 4.2. mTOR activity is required for entosis in glucose-free conditions
Inhibition of mTOR activity blocks entosis induced by glucose starvation. Graph shows quantification of entosis in adherent MCF-7 cells after 72 hours of culture in the indicated conditions, as determined by immunofluorescence. The high levels of entosis observed upon glucose starvation are partially blocked by treatment of cells with the mTOR-specific inhibitor Torin1.

withdrawal may trigger an upstream death signal (Hamann et al., 2017). The exact trigger for entotic death, even under normal conditions, remains to be elucidated, although recent insights into this LAP-like process have shed some mechanistic insight (Florey et al., 2015; Jacquin et al., 2017). It was recently shown that osmotic changes within the endolysosomal compartment are sufficient to lipidate LC3 onto this compartment, an event required upstream of entotic cell death. Whether the increased rates of death observed in glucose-free conditions could somehow be due to osmotic changes would be of interest for future studies. Previous work has shown that alteration of extracellular carbohydrate levels may change osmotic pressure, in line with the model described above (Joyner et al., 2016; Kawashima et al., 2017). Nevertheless, the signaling mechanisms that lead to LC3 lipidation in this context remain unknown, so a clear model to explain how glucose starvation could interface with LAP remains, at this point, elusive.

4.2.4 Role for entosis as a nutrient scavenging mechanism

A unique aspect of entotic cell death is the effects it has on the surviving cells within the population. As a form of cell competition, entosis results in the selection of certain cells within a heterogeneous population. These winners are likely multinucleated, as entotic host cells frequently fail to divide properly (Krajcovic et al., 2011). In the context of nutrient deprivation, entotic host cells also gain a significant survival and growth advantage over their non-engulfing neighbrors (Hamann et al., 2017; Krajcovic et al., 2013; Krishna et al., 2016). In

future studies, it will be important to identify how nutrients are exported from host cell lysosomes and which specific nutrients are recycled by host cells.

In amino acid- or glucose-depleted conditions, degradation and recycling of loser cells can support host cell metabolism. In the context of apoptotic corpse phagocytosis, we have also shown the incorporation of radioactively-labeled amino acids from apoptotic cells into host cell proteins, providing for the first time evidence for a specific metabolite that is recovered (Krajcovic et al., 2013; Krishna et al., 2016). Whether the metabolic deficiencies generated by amino acid and glucose starvation are the same in terms of their rescue through entosis remains to be examined. For example, nucleotide biosynthesis may be downregulated by both types of starvation, as amino acids provide the required nitrogen and glucose carbons are shunted through the pentose phosphate shunt. To test this model, future studies may test the ability of entotic host cells to proliferate in conditions where nucleotide synthesis is inhibited, for example by culturing cells in methotrexate. Conversely, it might be possible to rescue the low proliferation effect of single cells compared to entotic hosts in starvation conditions by supplementing the starvation media with nucleotides or their precursors. Metabolomic and metabolic flux studies may also be informative to identify certain substrates that loser cells within populations may be providing.

4.2.5 An emerging entosis regulatory network

In this thesis, we have uncovered a new activator of entosis, glucose starvation, thereby expanding what is known about the regulatory network that controls this process (Hamann et al., 2017). In addition to another recent report (Durgan et al.,

2017), it is now clear that entosis can also be induced in adherent cell populations, suggesting this mode of cell death may be more common than previously thought. In future studies, it will therefore be interesting to examine additional conditions that are known to cause cell death and determine whether entosis is induced as well. In preliminary studies, we have explored additional triggers, including ER stress and UV irradiation, and have found that entosis can also be induced under these conditions.

Unresolved chronic ER stress has long been though to induce apoptotic cell death, in part through transcriptional regulation of pro-apoptotic genes (Hetz and Papa, 2017). The activation of the ER stress signaling pathway, the ER UPR, can be provoked by a variety of insults, including glucose starvation, hypoxia, and changes in calcium signaling (Walter and Ron, 2011). The proper flux of glucose into the hexosamine biosynthetic pathway ensures that newly translated polypeptides are modified through glycosylation that can promote their correct folding, cellular localization, and function (Stowell et al., 2015). Glucose availability, therefore, directly impinges on this ER-resident process that, when deregulated, can induce ER stress. The accumulation of misfolded proteins within the ER lumen is sensed by the ER-resident chaperone BiP/Grp78 (Hetz and Papa, 2017). Under normal conditions, BiP binds and inhibits three proteins, IRE1α, PERK, and ATF6, capable of transducing downstream ER stress signals that include activation of the JNK signaling pathway, among others (Hetz and Papa, 2017). Increases in misfolded proteins causes increased BiP binding to these polypeptides, in turn releasing its inhibition on the ER stress sensors,

resulting in their activation (Walter and Ron, 2011). ATF6 is a transcription factor that becomes liberated from the ER and traffics to the nucleus and activates transcription of a variety of target genes to restore ER homeostasis and prevent cell death (Hetz and Papa, 2017). This set of genes includes BiP, which is upregulated in an attempt to increase chaperone activity within the ER (Walter and Ron, 2011).

As glucose withdrawal can induce ER stress, we wondered whether ER stress induction was sufficient to increase entosis levels. Intriguingly, when cells were treated with inhibitors of hexosamine biosynthesis, which cause ER stress, entosis was induced in MCF-7 cells to similar levels as in glucose withdrawal conditions (Figure 4.3). The compound azaserine is an inhibitor of GFAT, an aminotransferase that catalyzes the conversion of fructose-6-phosphate to glucosamine-6-phosphate, and flux of glucosamine into this pathway can completely bypass the need for this enzyme for hexosamine biosynthesis (Zhang et al., 2013). We therefore asked whether glucosamine supplementation could prevent induction of entosis observed in the presence of azaserine. This was indeed the case, suggesting the effect observed by azaserine treatment was specific to its inhibition of GFAT (Figure 4.3). Consistent with this, the effect could not be rescued by glucosamine addition in the presence of tunicamycin, which blocks UDP-GlcNAc modification downstream of glucosamine-6-phosphate production by inhibiting the enzyme GPT (Figure 4.3) (Oslowski and Urano, 2011). These data suggest that another cellular stress initially thought to induce apoptotic cell death may in fact engage other cell death programs as well. How

exactly ER stress controls the cellular changes necessary for entosis to occur remains to be elucidated. In addition, whether loser cell behavior is altered by ER stress, as with AMPK in the context of glucose starvation, or whether ER stress regulates winner cell status will be important questions to be addressed in future studies. Entosis induced by ER stress is not inhibited by AMPK inhibition, suggesting that it is likely regulated differently than glucose starvation-induced entosis, even though that condition also induces ER stress.

In unpublished studies, we have also shown that irradiation of cells with UV light is sufficient to induce entosis in adherent cell populations. Similar to the

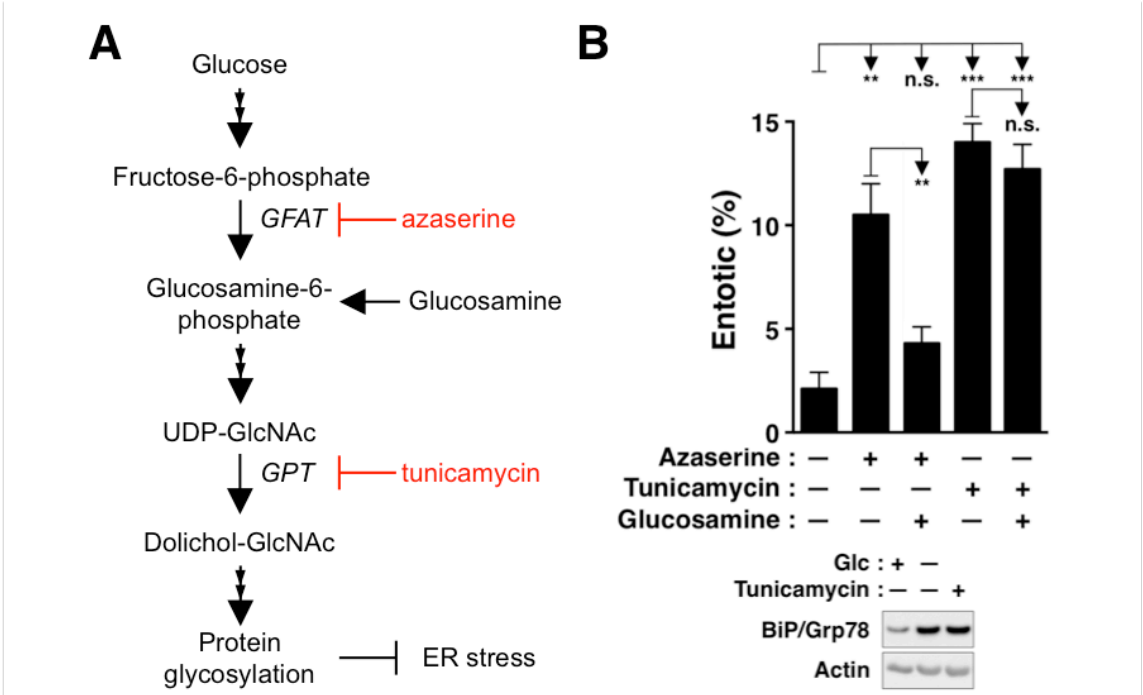


Figure 4.3. Endoplasmic reticulum stress induces entosis
(A) Role of glucose in generating protein glycosylation precursors. Shown is the relevant pathway that produces GlcNAc that can be used in protein glycosylation. Arrows with smaller arrowheads denote multiple enzymatic reactions. Enzymatic targets of pharmacological inducers of ER stress, azaserine and tunicamycin, are shown in italics. (B) Induction of ER stress results in entosis. Graph shows quantification of entotic structures in MCF-7 cells cultured in the indicated conditions, as determined by immunofluorescence. Western blot shows activation of ER stress, by glucose starvation and tunicamycin treatment, as measured by the accumulation of the ER-resident chaperone protein BiP/Grp78.

data presented in this thesis on AMPK, our data suggest that the molecular regulation of UV-induced entosis localizes to the loser cell. The activity of the stress-activated kinase JNK is required within loser cells upon UV irradiation to be taken up by neighboring cells, as cells inhibited for JNK signaling are preferential winner cells when mixed with control cells. In conclusion, in published and preliminary studies we have demonstrated that entosis is a cell death program that can be induced by a wide variety of cellular stresses and it will be important in future studies to potentially further expand this set of conditions and to gain further mechanistic insight into its regulation.

4.2.6 Entosis and cell competition

As we have again shown in this thesis that entosis is a competitive process that results in the elimination of a subset of cells, how this type of cellular behavior fits into the larger field of cell competition will be important to understand in future studies. While cell competition was first described in *Drosophila* models, recent evidence suggests competition can also occur in mammalian systems, such as during mouse development (Claveria et al., 2013). In the mouse embryo, it was recently shown that differential levels of myc within the epiblast cell population led to competitive interactions that allowed high myc expressing cells to accumulate, a model that has direct parallels to ‘supercompetition’ originally identified as a result of myc overexpression in the fly (Claveria et al., 2013). Recently, this type of myc-mediated supercompetition was also demonstrated in a cell culture model using MCF-7 cells, where cells with lowered myc expression, achieved by small hairpin RNA-mediated knockdown, were eliminated by cells

expressing control levels of myc (Patel et al., 2017). These data suggest that myc expression levels may control cell competition in numerous different models but the molecular mechanisms that lead to loser cell elimination are still debated.

In flies, it has been shown that neighboring cells can engulf each other as a mechanism to induce loser cell death (Li and Baker, 2007). While this idea has clear parallels to entosis, the engulfment mechanism was argued instead to resemble phagocytosis, as it required phagocytic genes, such as *draper*, *WASP*, and *ELMO* (Li and Baker, 2007). Moreover, the induction of apoptosis was also implicated in contributing to loser cell clearance (Li and Baker, 2007). Indeed, subsequent studies have contributed to the current model for competition in flies involving induction of apoptosis upstream of engulfment that is thought to primarily function to clear dead losers, not to induce cell death (Lolo et al., 2012; Ohsawa et al., 2011).

Similar to the model in *Drosophila*, recent examination of cell competition occurring in the mouse epiblast revealed a clear role for apoptosis in mediating elimination of loser cells when adjacent to myc-overexpressing supercompetitors (Claveria et al., 2013). Their data also include, however, images of engulfed loser cells that do not show signs of cell death, such as TUNEL-positivity, suggesting that some cells may still be alive and engulfment could occur upstream of death (Claveria et al., 2013). Some engulfed loser cells also maintained adherens junctions with winner cells, marked by E-cadherin staining, potentially consistent with the model for entotic live cell engulfment (Claveria et al., 2013; Overholtzer et al., 2007).

Furthermore, in a culture system of MCF-7 cells, cells with differential c-myc expression were recently shown to engage in competitive behavior (Patel et al., 2017). Loser cells were shown to be engulfed by winners in the absence of any apoptotic markers, supporting a model of live cell engulfment (Patel et al., 2017). These two studies may suggest that under certain conditions of cell competition, loser cells could be cleared by winners prior to their death, perhaps through a live-cell engulfment program resembling entosis.

In order to more closely examine the role that entosis plays in other forms of cell competition, it will be interesting in future studies to perform careful imaging-based analyses using these model systems. As it is still unclear in what context cell engulfment is required for cell competition, it will also be important to examine entosis machinery (e.g. E-cadherin and Rho kinase) in other model systems, such as *Drosophila* or the mouse epiblast, for potential effects of loss of function on loser cell elimination.

4.3 CONCLUSION

The findings presented and discussed here identify entosis as a major type of cell death induced upon glucose withdrawal (Hamann et al., 2017). An intracellular energy sensor that is activated by glucose starvation, AMPK, is required for live cell engulfment by entosis. Intriguingly, the kinase activity of this complex acts within internalizing cells by regulating the deformability of cells, which confers loser status. This competitive engulfment process induced by starvation allows heterogeneous cell populations to eliminate cells that are sensed to be less fit, thereby potentially promoting overall population survival. This activity results in the selection of winner cells through long-term glucose starvation that are able to proliferate at a higher rate than parental cells, consistent with this model. Further work will help uncover more detailed mechanistic insight into how AMPK regulates loser cell behavior as well as AMPK-independent regulation of entotic cell death that is increased by glucose starvation. Induction of entosis may also play an important physiological role, especially in the context of tumorigenesis, where cell-in-cell structures are frequently observed. As metabolic deregulation is frequently observed in cancers, it will be important to further understand the consequences that high levels of entosis may have on cancer cell populations and whether these are ultimately pro- or anti-tumorigenic.

REFERENCES

Abedin, M.J., Wang, D., McDonnell, M.A., Lehmann, U., and Kelekar, A. (2007). Autophagy delays apoptotic death in breast cancer cells following DNA damage. Cell Death Differ *14*, 500-510.

Ahmadian, M., Abbott, M.J., Tang, T., Hudak, C.S., Kim, Y., Bruss, M., Hellerstein, M.K., Lee, H.Y., Samuel, V.T., Shulman, G.I.*, et al.* (2011). Desnutrin/ATGL is regulated by AMPK and is required for a brown adipose phenotype. Cell metabolism *13*, 739-748.

Aita, V.M., Liang, X.H., Murty, V.V., Pincus, D.L., Yu, W., Cayanis, E., Kalachikov, S., Gilliam, T.C., and Levine, B. (1999). Cloning and genomic organization of beclin 1, a candidate tumor suppressor gene on chromosome 17q21. Genomics *59*, 59-65.

Alvarez, S.W., Sviderskiy, V.O., Terzi, E.M., Papagiannakopoulos, T., Moreira, A.L., Adams, S., Sabatini, D.M., Birsoy, K., and Possemato, R. (2017). NFS1 undergoes positive selection in lung tumours and protects cells from ferroptosis. Nature *551*, 639-643.

Alves, N.L., Derks, I.A., Berk, E., Spijker, R., van Lier, R.A., and Eldering, E. (2006). The Noxa/Mcl-1 axis regulates susceptibility to apoptosis under glucose limitation in dividing T cells. Immunity *24*, 703-716.

Bando, H., Atsumi, T., Nishio, T., Niwa, H., Mishima, S., Shimizu, C., Yoshioka, N., Bucala, R., and Koike, T. (2005). Phosphorylation of the 6-phosphofructo-2-kinase/fructose 2,6-bisphosphatase/PFKFB3 family of glycolytic regulators in human cancer. Clin Cancer Res *11*, 5784-5792.

Banko, M.R., Allen, J.J., Schaffer, B.E., Wilker, E.W., Tsou, P., White, J.L., Villen, J., Wang, B., Kim, S.R., Sakamoto, K.*, et al.* (2011). Chemical genetic screen for AMPKalpha2 substrates uncovers a network of proteins involved in mitosis. Mol Cell *44*, 878-892.

Bar-Peled, L., Chantranupong, L., Cherniack, A.D., Chen, W.W., Ottina, K.A., Grabiner, B.C., Spear, E.D., Carter, S.L., Meyerson, M., and Sabatini, D.M. (2013). A Tumor suppressor complex with GAP activity for the Rag GTPases that signal amino acid sufficiency to mTORC1. Science *340*, 1100-1106.

Bar-Peled, L., Schweitzer, L.D., Zoncu, R., and Sabatini, D.M. (2012). Ragulator is a GEF for the rag GTPases that signal amino acid levels to mTORC1. Cell *150*, 1196-1208.

Bar-Sagi, D., and Feramisco, J.R. (1986). Induction of membrane ruffling and fluid-phase pinocytosis in quiescent fibroblasts by ras proteins. Science *233*, 1061-1068.

Ben-Sahra, I., Hoxhaj, G., Ricoult, S.J.H., Asara, J.M., and Manning, B.D. (2016). mTORC1 induces purine synthesis through control of the mitochondrial tetrahydrofolate cycle. Science *351*, 728-733.

Benseler, V., Warren, A., Vo, M., Holz, L.E., Tay, S.S., Le Couteur, D.G., Breen, E., Allison, A.C., van Rooijen, N., McGuffog, C.*, et al.* (2011). Hepatocyte entry leads to degradation of autoreactive CD8 T cells. Proc Natl Acad Sci U S A *108*, 16735-16740.

Berry, D.L., and Baehrecke, E.H. (2007). Growth arrest and autophagy are required for salivary gland cell degradation in Drosophila. Cell *131*, 1137-1148.

Bray, K., Mathew, R., Lau, A., Kamphorst, J.J., Fan, J., Chen, J., Chen, H.Y., Ghavami, A., Stein, M., DiPaola, R.S.*, et al.* (2012). Autophagy suppresses RIP kinase-dependent necrosis enabling survival to mTOR inhibition. PLoS One *7*, e41831.

Brennan, M.A., and Cookson, B.T. (2000). Salmonella induces macrophage death by caspase-1-dependent necrosis. Molecular microbiology *38*, 31-40.

Bultot, L., Horman, S., Neumann, D., Walsh, M.P., Hue, L., and Rider, M.H. (2009). Myosin light chains are not a physiological substrate of AMPK in the control of cell structure changes. FEBS Lett *583*, 25-28.

Buttner, S., Eisenberg, T., Herker, E., Carmona-Gutierrez, D., Kroemer, G., and Madeo, F. (2006). Why yeast cells can undergo apoptosis: death in times of peace, love, and war. The Journal of cell biology *175*, 521-525.

Cai, Z., Jitkaew, S., Zhao, J., Chiang, H.C., Choksi, S., Liu, J., Ward, Y., Wu, L.G., and Liu, Z.G. (2014). Plasma membrane translocation of trimerized MLKL protein is required for TNF-induced necroptosis. Nature cell biology *16*, 55-65.

Carling, D., Zammit, V.A., and Hardie, D.G. (1987). A common bicyclic protein kinase cascade inactivates the regulatory enzymes of fatty acid and cholesterol biosynthesis. FEBS Lett *223*, 217-222.

Chantranupong, L., Scaria, S.M., Saxton, R.A., Gygi, M.P., Shen, K., Wyant, G.A., Wang, T., Harper, J.W., Gygi, S.P., and Sabatini, D.M. (2016). The CASTOR Proteins Are Arginine Sensors for the mTORC1 Pathway. Cell *165*, 153-164.

Chantranupong, L., Wolfson, R.L., Orozco, J.M., Saxton, R.A., Scaria, S.M., Bar-Peled, L., Spooner, E., Isasa, M., Gygi, S.P., and Sabatini, D.M. (2014). The Sestrins interact with GATOR2 to negatively regulate the amino-acid-sensing pathway upstream of mTORC1. Cell Rep *9*, 1-8.

Chavez, J.A., Roach, W.G., Keller, S.R., Lane, W.S., and Lienhard, G.E. (2008). Inhibition of GLUT4 translocation by Tbc1d1, a Rab GTPase-activating protein

abundant in skeletal muscle, is partially relieved by AMP-activated protein kinase activation. J Biol Chem *283*, 9187-9195.

Claveria, C., Giovinazzo, G., Sierra, R., and Torres, M. (2013). Myc-driven endogenous cell competition in the early mammalian embryo. Nature *500*, 39-44.

Coleman, M.L., Sahai, E.A., Yeo, M., Bosch, M., Dewar, A., and Olson, M.F. (2001). Membrane blebbing during apoptosis results from caspase-mediated activation of ROCK I. Nature cell biology *3*, 339-345.

Commisso, C., Davidson, S.M., Soydaner-Azeloglu, R.G., Parker, S.J., Kamphorst, J.J., Hackett, S., Grabocka, E., Nofal, M., Drebin, J.A., Thompson, C.B.*, et al.* (2013). Macropinocytosis of protein is an amino acid supply route in Ras-transformed cells. Nature *497*, 633-637.

Crawford, E.D., and Wells, J.A. (2011). Caspase substrates and cellular remodeling. Annu Rev Biochem *80*, 1055-1087.

de la Cova, C., Abril, M., Bellosta, P., Gallant, P., and Johnston, L.A. (2004). Drosophila myc regulates organ size by inducing cell competition. Cell *117*, 107-116.

Debnath, J., Muthuswamy, S.K., and Brugge, J.S. (2003). Morphogenesis and oncogenesis of MCF-10A mammary epithelial acini grown in three-dimensional basement membrane cultures. Methods *30*, 256-268.

Degenhardt, K., Mathew, R., Beaudoin, B., Bray, K., Anderson, D., Chen, G., Mukherjee, C., Shi, Y., Gelinas, C., Fan, Y.*, et al.* (2006). Autophagy promotes tumor cell survival and restricts necrosis, inflammation, and tumorigenesis. Cancer Cell *10*, 51-64.

Degterev, A., Huang, Z., Boyce, M., Li, Y., Jagtap, P., Mizushima, N., Cuny, G.D., Mitchison, T.J., Moskowitz, M.A., and Yuan, J. (2005). Chemical inhibitor of nonapoptotic cell death with therapeutic potential for ischemic brain injury. Nat Chem Biol *1*, 112-119.

Denko, N.C. (2008). Hypoxia, HIF1 and glucose metabolism in the solid tumour. Nat Rev Cancer *8*, 705-713.

Dixon, S.J., Lemberg, K.M., Lamprecht, M.R., Skouta, R., Zaitsev, E.M., Gleason, C.E., Patel, D.N., Bauer, A.J., Cantley, A.M., Yang, W.S.*, et al.* (2012). Ferroptosis: an iron-dependent form of nonapoptotic cell death. Cell *149*, 1060-1072.

Doitsh, G., Galloway, N.L., Geng, X., Yang, Z., Monroe, K.M., Zepeda, O., Hunt, P.W., Hatano, H., Sowinski, S., Munoz-Arias, I.*, et al.* (2014). Cell death by pyroptosis drives CD4 T-cell depletion in HIV-1 infection. Nature *505*, 509-514.

Dunn, D.E., He, D.N., Yang, P., Johansen, M., Newman, R.A., and Lo, D.C. (2011). In vitro and in vivo neuroprotective activity of the cardiac glycoside oleandrin from Nerium oleander in brain slice-based stroke models. J Neurochem *119*, 805-814.

Durgan, J., Tseng, Y.Y., Hamann, J.C., Domart, M.C., Collinson, L., Hall, A., Overholtzer, M., and Florey, O. (2017). Mitosis can drive cell cannibalism through entosis. Elife *6*.

Duvel, K., Yecies, J.L., Menon, S., Raman, P., Lipovsky, A.I., Souza, A.L., Triantafellow, E., Ma, Q., Gorski, R., Cleaver, S.*, et al.* (2010). Activation of a metabolic gene regulatory network downstream of mTOR complex 1. Mol Cell *39*, 171-183.

Efeyan, A., Zoncu, R., Chang, S., Gumper, I., Snitkin, H., Wolfson, R.L., Kirak, O., Sabatini, D.D., and Sabatini, D.M. (2013). Regulation of mTORC1 by the Rag GTPases is necessary for neonatal autophagy and survival. Nature *493*, 679-683.

Egan, D.F., Shackelford, D.B., Mihaylova, M.M., Gelino, S., Kohnz, R.A., Mair, W., Vasquez, D.S., Joshi, A., Gwinn, D.M., Taylor, R.*, et al.* (2011). Phosphorylation of ULK1 (hATG1) by AMP-activated protein kinase connects energy sensing to mitophagy. Science *331*, 456-461.

Eguchi, S., Oshiro, N., Miyamoto, T., Yoshino, K., Okamoto, S., Ono, T., Kikkawa, U., and Yonezawa, K. (2009). AMP-activated protein kinase phosphorylates glutamine : fructose-6-phosphate amidotransferase 1 at Ser243 to modulate its enzymatic activity. Genes Cells *14*, 179-189.

El-Masry, O.S., Brown, B.L., and Dobson, P.R. (2012). Effects of activation of AMPK on human breast cancer cell lines with different genetic backgrounds. Oncology letters *3*, 224-228.

Erdos, G.W., Raper, K.B., and Vogen, L.K. (1973). Mating Types and Macrocyst Formation in Dictyostelium discoideum. Proc Natl Acad Sci U S A *70*, 1828-1830.

Fabrizio, P., Battistella, L., Vardavas, R., Gattazzo, C., Liou, L.L., Diaspro, A., Dossen, J.W., Gralla, E.B., and Longo, V.D. (2004). Superoxide is a mediator of an altruistic aging program in Saccharomyces cerevisiae. The Journal of cell biology *166*, 1055-1067.

Feoktistova, M., Geserick, P., Kellert, B., Dimitrova, D.P., Langlais, C., Hupe, M., Cain, K., MacFarlane, M., Hacker, G., and Leverkus, M. (2011). cIAPs block Ripoptosome formation, a RIP1/caspase-8 containing intracellular cell death complex differentially regulated by cFLIP isoforms. Mol Cell *43*, 449-463.

Florey, O., Gammoh, N., Kim, S.E., Jiang, X., and Overholtzer, M. (2015). V-ATPase and osmotic imbalances activate endolysosomal LC3 lipidation. Autophagy *11*, 88-99.

Florey, O., Kim, S.E., Sandoval, C.P., Haynes, C.M., and Overholtzer, M. (2011). Autophagy machinery mediates macroendocytic processing and entotic cell death by targeting single membranes. Nature cell biology *13*, 1335-1343.

Galluzzi, L., Maiuri, M.C., Vitale, I., Zischka, H., Castedo, M., Zitvogel, L., and Kroemer, G. (2007). Cell death modalities: classification and pathophysiological implications. Cell Death Differ *14*, 1237-1243.

Galluzzi, L., Pietrocola, F., Bravo-San Pedro, J.M., Amaravadi, R.K., Baehrecke, E.H., Cecconi, F., Codogno, P., Debnath, J., Gewirtz, D.A., Karantza, V.*, et al.* (2015). Autophagy in malignant transformation and cancer progression. EMBO J *34*, 856-880.

Galluzzi, L., Vitale, I., Aaronson, S.A., Abrams, J.M., Adam, D., Agostinis, P., Alnemri, E.S., Altucci, L., Amelio, I., Andrews, D.W.*, et al.* (2018). Molecular mechanisms of cell death: recommendations of the Nomenclature Committee on Cell Death 2018. Cell Death Differ.

Galluzzi, L., Vitale, I., Abrams, J.M., Alnemri, E.S., Baehrecke, E.H., Blagosklonny, M.V., Dawson, T.M., Dawson, V.L., El-Deiry, W.S., Fulda, S.*, et al.* (2012). Molecular definitions of cell death subroutines: recommendations of the Nomenclature Committee on Cell Death 2012. Cell death and differentiation *19*, 107-120.

Gao, M., Monian, P., Quadri, N., Ramasamy, R., and Jiang, X. (2015). Glutaminolysis and Transferrin Regulate Ferroptosis. Mol Cell *59*, 298-308.

Goransson, O., McBride, A., Hawley, S.A., Ross, F.A., Shpiro, N., Foretz, M., Viollet, B., Hardie, D.G., and Sakamoto, K. (2007). Mechanism of action of A-769662, a valuable tool for activation of AMP-activated protein kinase. J Biol Chem *282*, 32549-32560.

Gourlay, C.W., Du, W., and Ayscough, K.R. (2006). Apoptosis in yeast--mechanisms and benefits to a unicellular organism. Molecular microbiology *62*, 1515-1521.

Grabiner, B.C., Nardi, V., Birsoy, K., Possemato, R., Shen, K., Sinha, S., Jordan, A., Beck, A.H., and Sabatini, D.M. (2014). A diverse array of cancer-associated MTOR mutations are hyperactivating and can predict rapamycin sensitivity. Cancer Discov *4*, 554-563.

Green, D.R., Galluzzi, L., and Kroemer, G. (2011). Mitochondria and the autophagy-inflammation-cell death axis in organismal aging. Science *333*, 1109-1112.

Green, D.R., and Llambi, F. (2015). Cell Death Signaling. Cold Spring Harb Perspect Biol *7*.

Greer, E.L., Oskoui, P.R., Banko, M.R., Maniar, J.M., Gygi, M.P., Gygi, S.P., and Brunet, A. (2007). The energy sensor AMP-activated protein kinase directly regulates the mammalian FOXO3 transcription factor. J Biol Chem *282*, 30107-30119.

Gwinn, D.M., Shackelford, D.B., Egan, D.F., Mihaylova, M.M., Mery, A., Vasquez, D.S., Turk, B.E., and Shaw, R.J. (2008). AMPK phosphorylation of raptor mediates a metabolic checkpoint. Mol Cell *30*, 214-226.

Hamann, J.C., and Overholtzer, M. (2017). Entosis enables a population response to starvation. Oncotarget *8*, 57934-57935.

Hamann, J.C., Surcel, A., Chen, R., Teragawa, C., Albeck, J.G., Robinson, D.N., and Overholtzer, M. (2017). Entosis Is Induced by Glucose Starvation. Cell Rep *20*, 201-210.

Hanahan, D., and Weinberg, R.A. (2011). Hallmarks of cancer: the next generation. Cell *144*, 646-674.

Hardie, D.G., Ross, F.A., and Hawley, S.A. (2012). AMPK: a nutrient and energy sensor that maintains energy homeostasis. Nat Rev Mol Cell Biol *13*, 251-262.

Herzig, S., and Shaw, R.J. (2018). AMPK: guardian of metabolism and mitochondrial homeostasis. Nat Rev Mol Cell Biol *19*, 121-135.

Hetz, C., and Papa, F.R. (2017). The Unfolded Protein Response and Cell Fate Control. Mol Cell.

Hinojosa, L.S., Holst, M., Baarlink, C., and Grosse, R. (2017). MRTF transcription and Ezrin-dependent plasma membrane blebbing are required for entotic invasion. The Journal of cell biology *216*, 3087-3095.

Hochmuth, R.M. (2000). Micropipette aspiration of living cells. J Biomech *33*, 15-22.

Holz, M.K., Ballif, B.A., Gygi, S.P., and Blenis, J. (2005). mTOR and S6K1 mediate assembly of the translation preinitiation complex through dynamic protein interchange and ordered phosphorylation events. Cell *123*, 569-580.

Hornung, V., Ablasser, A., Charrel-Dennis, M., Bauernfeind, F., Horvath, G., Caffrey, D.R., Latz, E., and Fitzgerald, K.A. (2009). AIM2 recognizes cytosolic dsDNA and forms a caspase-1-activating inflammasome with ASC. Nature *458*, 514-518.

Hou, W., Han, J., Lu, C., Goldstein, L.A., and Rabinowich, H. (2010). Autophagic degradation of active caspase-8: a crosstalk mechanism between autophagy and apoptosis. Autophagy *6*, 891-900.

Huang, H., Chen, Z., and Sun, Q. (2015). Mammalian Cell Competitions, Cell-in-Cell Phenomena and Their Biomedical Implications. Current molecular medicine *15*, 852-860.

Humble, J.G., Jayne, W.H., and Pulvertaft, R.J. (1956). Biological interaction between lymphocytes and other cells. Br J Haematol *2*, 283-294.

Inoki, K., Li, Y., Xu, T., and Guan, K.L. (2003a). Rheb GTPase is a direct target of TSC2 GAP activity and regulates mTOR signaling. Genes & development *17*, 1829-1834.

Inoki, K., Zhu, T., and Guan, K.L. (2003b). TSC2 mediates cellular energy response to control cell growth and survival. Cell *115*, 577-590.

Jacquin, E., Leclerc-Mercier, S., Judon, C., Blanchard, E., Fraitag, S., and Florey, O. (2017). Pharmacological modulators of autophagy activate a parallel noncanonical pathway driving unconventional LC3 lipidation. Autophagy *13*, 854-867.

Jewell, J.L., Kim, Y.C., Russell, R.C., Yu, F.X., Park, H.W., Plouffe, S.W., Tagliabracci, V.S., and Guan, K.L. (2015). Metabolism. Differential regulation of mTORC1 by leucine and glutamine. Science *347*, 194-198.

Jones, R.G., and Thompson, C.B. (2009). Tumor suppressors and cell metabolism: a recipe for cancer growth. Genes & development *23*, 537-548.

Jost, P.J., Grabow, S., Gray, D., McKenzie, M.D., Nachbur, U., Huang, D.C., Bouillet, P., Thomas, H.E., Borner, C., Silke, J.*, et al.* (2009). XIAP discriminates between type I and type II FAS-induced apoptosis. Nature *460*, 1035-1039.

Joyner, R.P., Tang, J.H., Helenius, J., Dultz, E., Brune, C., Holt, L.J., Huet, S., Muller, D.J., and Weis, K. (2016). A glucose-starvation response regulates the diffusion of macromolecules. Elife *5*.

Kalender, A., Selvaraj, A., Kim, S.Y., Gulati, P., Brule, S., Viollet, B., Kemp, B.E., Bardeesy, N., Dennis, P., Schlager, J.J.*, et al.* (2010). Metformin, independent of AMPK, inhibits mTORC1 in a rag GTPase-dependent manner. Cell metabolism *11*, 390-401.

Kamphorst, J.J., Cross, J.R., Fan, J., de Stanchina, E., Mathew, R., White, E.P., Thompson, C.B., and Rabinowitz, J.D. (2013). Hypoxic and Ras-transformed cells support growth by scavenging unsaturated fatty acids from lysophospholipids. Proc Natl Acad Sci U S A *110*, 8882-8887.

Kang, R., Zeh, H.J., Lotze, M.T., and Tang, D. (2011). The Beclin 1 network regulates autophagy and apoptosis. Cell Death Differ *18*, 571-580.

Karantza-Wadsworth, V., Patel, S., Kravchuk, O., Chen, G., Mathew, R., Jin, S., and White, E. (2007). Autophagy mitigates metabolic stress and genome damage in mammary tumorigenesis. Genes & development *21*, 1621-1635.

Katheder, N.S., Khezri, R., O'Farrell, F., Schultz, S.W., Jain, A., Rahman, M.M., Schink, K.O., Theodossiou, T.A., Johansen, T., Juhasz, G.*, et al.* (2017). Microenvironmental autophagy promotes tumour growth. Nature *541*, 417-420.

Kawashima, K.I., Ishiuchi, Y., Konnai, M., Komatsu, S., Sato, H., Kawaguchi, H., Miyanishi, N., Lamartine, J., Nishihara, M., and Nedachi, T. (2017). Glucose deprivation regulates the progranulin-sortilin axis in PC12 cells. FEBS Open Bio *7*, 149-159.

Kayagaki, N., Stowe, I.B., Lee, B.L., O'Rourke, K., Anderson, K., Warming, S., Cuellar, T., Haley, B., Roose-Girma, M., Phung, Q.T.*, et al.* (2015). Caspase-11 cleaves gasdermin D for non-canonical inflammasome signalling. Nature *526*, 666-671.

Kerr, J.F., Wyllie, A.H., and Currie, A.R. (1972). Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. Br J Cancer *26*, 239-257.

Kim, D.H., Sarbassov, D.D., Ali, S.M., King, J.E., Latek, R.R., Erdjument-Bromage, H., Tempst, P., and Sabatini, D.M. (2002). mTOR interacts with raptor to form a nutrient-sensitive complex that signals to the cell growth machinery. Cell *110*, 163-175.

Kim, J., Kim, Y.C., Fang, C., Russell, R.C., Kim, J.H., Fan, W., Liu, R., Zhong, Q., and Guan, K.L. (2013). Differential regulation of distinct Vps34 complexes by AMPK in nutrient stress and autophagy. Cell *152*, 290-303.

Kim, J., Kundu, M., Viollet, B., and Guan, K.L. (2011). AMPK and mTOR regulate autophagy through direct phosphorylation of Ulk1. Nature cell biology *13*, 132-141.

Kim, S.E., Zhang, L., Ma, K., Riegman, M., Chen, F., Ingold, I., Conrad, M., Turker, M.Z., Gao, M., Jiang, X.*, et al.* (2016). Ultrasmall nanoparticles induce ferroptosis in nutrient-deprived cancer cells and suppress tumour growth. Nature nanotechnology *11*, 977-985.

Klionsky, D.J. (2005). The molecular machinery of autophagy: unanswered questions. J Cell Sci *118*, 7-18.

Koike, M., Shibata, M., Tadakoshi, M., Gotoh, K., Komatsu, M., Waguri, S., Kawahara, N., Kuida, K., Nagata, S., Kominami, E.*, et al.* (2008). Inhibition of

autophagy prevents hippocampal pyramidal neuron death after hypoxic-ischemic injury. Am J Pathol *172*, 454-469.

Komatsu, N., Aoki, K., Yamada, M., Yukinaga, H., Fujita, Y., Kamioka, Y., and Matsuda, M. (2011). Development of an optimized backbone of FRET biosensors for kinases and GTPases. Mol Biol Cell *22*, 4647-4656.

Kongsuwan, K., Yu, Q., Vincent, A., Frisardi, M.C., Rosbash, M., Lengyel, J.A., and Merriam, J. (1985). A Drosophila Minute gene encodes a ribosomal protein. Nature *317*, 555-558.

Krajcovic, M., Johnson, N.B., Sun, Q., Normand, G., Hoover, N., Yao, E., Richardson, A.L., King, R.W., Cibas, E.S., Schnitt, S.J.*, et al.* (2011). A non-genetic route to aneuploidy in human cancers. Nature cell biology *13*, 324-330.

Krajcovic, M., Krishna, S., Akkari, L., Joyce, J.A., and Overholtzer, M. (2013). mTOR regulates phagosome and entotic vacuole fission. Molecular biology of the cell *24*, 3736-3745.

Krishna, S., Palm, W., Lee, Y., Yang, W., Bandyopadhyay, U., Xu, H., Florey, O., Thompson, C.B., and Overholtzer, M. (2016). PIKfyve Regulates Vacuole Maturation and Nutrient Recovery following Engulfment. Dev Cell *38*, 536-547.

Kroemer, G., and Levine, B. (2008). Autophagic cell death: the story of a misnomer. Nat Rev Mol Cell Biol *9*, 1004-1010.

Krueger, A., Schmitz, I., Baumann, S., Krammer, P.H., and Kirchhoff, S. (2001). Cellular FLICE-inhibitory protein splice variants inhibit different steps of caspase-8 activation at the CD95 death-inducing signaling complex. J Biol Chem *276*, 20633-20640.

Lamkanfi, M., and Dixit, V.M. (2011). Modulation of inflammasome pathways by bacterial and viral pathogens. J Immunol *187*, 597-602.

Lazarou, M., Sliter, D.A., Kane, L.A., Sarraf, S.A., Wang, C., Burman, J.L., Sideris, D.P., Fogel, A.I., and Youle, R.J. (2015). The ubiquitin kinase PINK1 recruits autophagy receptors to induce mitophagy. Nature *524*, 309-314.

Leckband, D.E., and de Rooij, J. (2014). Cadherin adhesion and mechanotransduction. Annu Rev Cell Dev Biol *30*, 291-315.

Lee, J.H., Koh, H., Kim, M., Kim, Y., Lee, S.Y., Karess, R.E., Lee, S.H., Shong, M., Kim, J.M., Kim, J.*, et al.* (2007). Energy-dependent regulation of cell structure by AMP-activated protein kinase. Nature *447*, 1017-1020.

Leprivier, G., Remke, M., Rotblat, B., Dubuc, A., Mateo, A.R., Kool, M., Agnihotri, S., El-Naggar, A., Yu, B., Somasekharan, S.P.*, et al.* (2013). The eEF2 kinase

confers resistance to nutrient deprivation by blocking translation elongation. Cell *153*, 1064-1079.

Li, W., and Baker, N.E. (2007). Engulfment is required for cell competition. Cell *129*, 1215-1225.

Li, Y., Xu, S., Mihaylova, M.M., Zheng, B., Hou, X., Jiang, B., Park, O., Luo, Z., Lefai, E., Shyy, J.Y.*, et al.* (2011). AMPK phosphorylates and inhibits SREBP activity to attenuate hepatic steatosis and atherosclerosis in diet-induced insulin-resistant mice. Cell metabolism *13*, 376-388.

Liang, X.H., Jackson, S., Seaman, M., Brown, K., Kempkes, B., Hibshoosh, H., and Levine, B. (1999). Induction of autophagy and inhibition of tumorigenesis by beclin 1. Nature *402*, 672-676.

Linkermann, A., Skouta, R., Himmerkus, N., Mulay, S.R., Dewitz, C., De Zen, F., Prokai, A., Zuchtriegel, G., Krombach, F., Welz, P.S.*, et al.* (2014). Synchronized renal tubular cell death involves ferroptosis. Proc Natl Acad Sci U S A *111*, 16836-16841.

Liu, Y., Shoji-Kawata, S., Sumpter, R.M., Jr., Wei, Y., Ginet, V., Zhang, L., Posner, B., Tran, K.A., Green, D.R., Xavier, R.J.*, et al.* (2013). Autosis is a Na+,K+-ATPase-regulated form of cell death triggered by autophagy-inducing peptides, starvation, and hypoxia-ischemia. Proc Natl Acad Sci U S A *110*, 20364-20371.

Lolo, F.N., Casas-Tinto, S., and Moreno, E. (2012). Cell competition time line: winners kill losers, which are extruded and engulfed by hemocytes. Cell Rep *2*, 526-539.

Lugini, L., Matarrese, P., Tinari, A., Lozupone, F., Federici, C., Iessi, E., Gentile, M., Luciani, F., Parmiani, G., Rivoltini, L.*, et al.* (2006). Cannibalism of live lymphocytes by human metastatic but not primary melanoma cells. Cancer Res *66*, 3629-3638.

Luo, S., Garcia-Arencibia, M., Zhao, R., Puri, C., Toh, P.P., Sadiq, O., and Rubinsztein, D.C. (2012). Bim inhibits autophagy by recruiting Beclin 1 to microtubules. Mol Cell *47*, 359-370.

Luo, S., and Rubinsztein, D.C. (2010). Apoptosis blocks Beclin 1-dependent autophagosome synthesis: an effect rescued by Bcl-xL. Cell Death Differ *17*, 268-277.

Lyssiotis, C.A., and Kimmelman, A.C. (2017). Metabolic Interactions in the Tumor Microenvironment. Trends Cell Biol *27*, 863-875.

Ma, X.M., and Blenis, J. (2009). Molecular mechanisms of mTOR-mediated translational control. Nat Rev Mol Cell Biol *10*, 307-318.

Mancias, J.D., Wang, X., Gygi, S.P., Harper, J.W., and Kimmelman, A.C. (2014). Quantitative proteomics identifies NCOA4 as the cargo receptor mediating ferritinophagy. Nature *509*, 105-109.

Martina, J.A., Chen, Y., Gucek, M., and Puertollano, R. (2012). MTORC1 functions as a transcriptional regulator of autophagy by preventing nuclear transport of TFEB. Autophagy *8*, 903-914.

Mathew, R., Karantza-Wadsworth, V., and White, E. (2007). Role of autophagy in cancer. Nat Rev Cancer *7*, 961-967.

Mendez-Lucas, A., Hyrossova, P., Novellasdemunt, L., Vinals, F., and Perales, J.C. (2014). Mitochondrial phosphoenolpyruvate carboxykinase (PEPCK-M) is a pro-survival, endoplasmic reticulum (ER) stress response gene involved in tumor cell adaptation to nutrient availability. J Biol Chem *289*, 22090-22102.

Menendez, J., Perez-Garijo, A., Calleja, M., and Morata, G. (2010). A tumor-suppressing mechanism in Drosophila involving cell competition and the Hippo pathway. Proc Natl Acad Sci U S A *107*, 14651-14656.

Merino, M.M., Levayer, R., and Moreno, E. (2016). Survival of the Fittest: Essential Roles of Cell Competition in Development, Aging, and Cancer. Trends Cell Biol *26*, 776-788.

Merino, M.M., Rhiner, C., Lopez-Gay, J.M., Buechel, D., Hauert, B., and Moreno, E. (2015). Elimination of unfit cells maintains tissue health and prolongs lifespan. Cell *160*, 461-476.

Mizushima, N., Levine, B., Cuervo, A.M., and Klionsky, D.J. (2008). Autophagy fights disease through cellular self-digestion. Nature *451*, 1069-1075.

Monks, J., Rosner, D., Geske, F.J., Lehman, L., Hanson, L., Neville, M.C., and Fadok, V.A. (2005). Epithelial cells as phagocytes: apoptotic epithelial cells are engulfed by mammary alveolar epithelial cells and repress inflammatory mediator release. Cell Death Differ *12*, 107-114.

Morata, G., and Ripoll, P. (1975). Minutes: mutants of drosophila autonomously affecting cell division rate. Dev Biol *42*, 211-221.

Moreno, E., and Basler, K. (2004). dMyc transforms cells into super-competitors. Cell *117*, 117-129.

Morrison, B., 3rd, Pringle, A.K., McManus, T., Ellard, J., Bradley, M., Signorelli, F., Iannotti, F., and Sundstrom, L.E. (2002). L-arginyl-3,4-spermidine is neuroprotective in several in vitro models of neurodegeneration and in vivo ischaemia without suppressing synaptic transmission. Br J Pharmacol *137*, 1255-1268.

Mu, J., Brozinick, J.T., Jr., Valladares, O., Bucan, M., and Birnbaum, M.J. (2001). A role for AMP-activated protein kinase in contraction- and hypoxia-regulated glucose transport in skeletal muscle. Mol Cell *7*, 1085-1094.

Murthy, A., Li, Y., Peng, I., Reichelt, M., Katakam, A.K., Noubade, R., Roose-Girma, M., DeVoss, J., Diehl, L., Graham, R.R.*, et al.* (2014). A Crohn's disease variant in Atg16l1 enhances its degradation by caspase 3. Nature *506*, 456-462.

Newman, R.A., Yang, P., Pawlus, A.D., and Block, K.I. (2008). Cardiac glycosides as novel cancer therapeutic agents. Mol Interv *8*, 36-49.

Nizak, C., Fitzhenry, R.J., and Kessin, R.H. (2007). Exploitation of other social amoebae by Dictyostelium caveatum. PLoS One *2*, e212.

Nofal, M., Zhang, K., Han, S., and Rabinowitz, J.D. (2017). mTOR Inhibition Restores Amino Acid Balance in Cells Dependent on Catabolism of Extracellular Protein. Mol Cell *67*, 936-946 e935.

Ohsawa, S., Sugimura, K., Takino, K., Xu, T., Miyawaki, A., and Igaki, T. (2011). Elimination of oncogenic neighbors by JNK-mediated engulfment in Drosophila. Dev Cell *20*, 315-328.

Okada, H., and Mak, T.W. (2004). Pathways of apoptotic and non-apoptotic death in tumour cells. Nat Rev Cancer *4*, 592-603.

Okoshi, R., Ozaki, T., Yamamoto, H., Ando, K., Koida, N., Ono, S., Koda, T., Kamijo, T., Nakagawara, A., and Kizaki, H. (2008). Activation of AMP-activated protein kinase induces p53-dependent apoptotic cell death in response to energetic stress. J Biol Chem *283*, 3979-3987.

Okosun, J., Wolfson, R.L., Wang, J., Araf, S., Wilkins, L., Castellano, B.M., Escudero-Ibarz, L., Al Seraihi, A.F., Richter, J., Bernhart, S.H.*, et al.* (2016). Recurrent mTORC1-activating RRAGC mutations in follicular lymphoma. Nat Genet *48*, 183-188.

Oslowski, C.M., and Urano, F. (2011). Measuring ER stress and the unfolded protein response using mammalian tissue culture system. Methods Enzymol *490*, 71-92.

Overholtzer, M., and Brugge, J.S. (2008). The cell biology of cell-in-cell structures. Nat Rev Mol Cell Biol *9*, 796-809.

Overholtzer, M., Mailleux, A.A., Mouneimne, G., Normand, G., Schnitt, S.J., King, R.W., Cibas, E.S., and Brugge, J.S. (2007). A nonapoptotic cell death process, entosis, that occurs by cell-in-cell invasion. Cell *131*, 966-979.

Palm, W., Park, Y., Wright, K., Pavlova, N.N., Tuveson, D.A., and Thompson, C.B. (2015). The Utilization of Extracellular Proteins as Nutrients Is Suppressed by mTORC1. Cell *162*, 259-270.

Pankiv, S., Clausen, T.H., Lamark, T., Brech, A., Bruun, J.A., Outzen, H., Overvatn, A., Bjorkoy, G., and Johansen, T. (2007). p62/SQSTM1 binds directly to Atg8/LC3 to facilitate degradation of ubiquitinated protein aggregates by autophagy. J Biol Chem *282*, 24131-24145.

Pantopoulos, K., Porwal, S.K., Tartakoff, A., and Devireddy, L. (2012). Mechanisms of mammalian iron homeostasis. Biochemistry *51*, 5705-5724.

Parsons, J.T., Horwitz, A.R., and Schwartz, M.A. (2010). Cell adhesion: integrating cytoskeletal dynamics and cellular tension. Nat Rev Mol Cell Biol *11*, 633-643.

Patel, M.S., Shah, H.S., and Shrivastava, N. (2017). c-Myc-Dependent Cell Competition in Human Cancer Cells. J Cell Biochem *118*, 1782-1791.

Pfau, S.J., and Amon, A. (2012). Chromosomal instability and aneuploidy in cancer: from yeast to man. EMBO Rep *13*, 515-527.

Poon, I.K., Lucas, C.D., Rossi, A.G., and Ravichandran, K.S. (2014). Apoptotic cell clearance: basic biology and therapeutic potential. Nat Rev Immunol *14*, 166-180.

Porstmann, T., Santos, C.R., Griffiths, B., Cully, M., Wu, M., Leevers, S., Griffiths, J.R., Chung, Y.L., and Schulze, A. (2008). SREBP activity is regulated by mTORC1 and contributes to Akt-dependent cell growth. Cell metabolism *8*, 224-236.

Purvanov, V., Holst, M., Khan, J., Baarlink, C., and Grosse, R. (2014). G-protein-coupled receptor signaling and polarized actin dynamics drive cell-in-cell invasion. Elife *3*.

Qiang, L., Wu, C., Ming, M., Viollet, B., and He, Y.Y. (2013). Autophagy controls p38 activation to promote cell survival under genotoxic stress. J Biol Chem *288*, 1603-1611.

Rangwala, R., Chang, Y.C., Hu, J., Algazy, K.M., Evans, T.L., Fecher, L.A., Schuchter, L.M., Torigian, D.A., Panosian, J.T., Troxel, A.B.*, et al.* (2014). Combined MTOR and autophagy inhibition: phase I trial of hydroxychloroquine and temsirolimus in patients with advanced solid tumors and melanoma. Autophagy *10*, 1391-1402.

Rao, S., Tortola, L., Perlot, T., Wirnsberger, G., Novatchkova, M., Nitsch, R., Sykacek, P., Frank, L., Schramek, D., Komnenovic, V.*, et al.* (2014). A dual role for autophagy in a murine model of lung cancer. Nat Commun *5*, 3056.

Rebsamen, M., Pochini, L., Stasyk, T., de Araujo, M.E., Galluccio, M., Kandasamy, R.K., Snijder, B., Fauster, A., Rudashevskaya, E.L., Bruckner, M.*, et al.* (2015). SLC38A9 is a component of the lysosomal amino acid sensing machinery that controls mTORC1. Nature *519*, 477-481.

Reginato, M.J., Mills, K.R., Paulus, J.K., Lynch, D.K., Sgroi, D.C., Debnath, J., Muthuswamy, S.K., and Brugge, J.S. (2003). Integrins and EGFR coordinately regulate the pro-apoptotic protein Bim to prevent anoikis. Nature cell biology *5*, 733-740.

Roczniak-Ferguson, A., Petit, C.S., Froehlich, F., Qian, S., Ky, J., Angarola, B., Walther, T.C., and Ferguson, S.M. (2012). The transcription factor TFEB links mTORC1 signaling to transcriptional control of lysosome homeostasis. Sci Signal *5*, ra42.

Rogers, C., Fernandes-Alnemri, T., Mayes, L., Alnemri, D., Cingolani, G., and Alnemri, E.S. (2017). Cleavage of DFNA5 by caspase-3 during apoptosis mediates progression to secondary necrotic/pyroptotic cell death. Nat Commun *8*, 14128.

Rosenfeldt, M.T., O'Prey, J., Morton, J.P., Nixon, C., MacKay, G., Mrowinska, A., Au, A., Rai, T.S., Zheng, L., Ridgway, R.*, et al.* (2013). p53 status determines the role of autophagy in pancreatic tumour development. Nature *504*, 296-300.

Saggerson, D. (2008). Malonyl-CoA, a key signaling molecule in mammalian cells. Annu Rev Nutr *28*, 253-272.

Sakahira, H., Enari, M., and Nagata, S. (1998). Cleavage of CAD inhibitor in CAD activation and DNA degradation during apoptosis. Nature *391*, 96-99.

Salvesen, G.S., and Riedl, S.J. (2008). Caspase mechanisms. Adv Exp Med Biol *615*, 13-23.

Sancak, Y., Bar-Peled, L., Zoncu, R., Markhard, A.L., Nada, S., and Sabatini, D.M. (2010). Ragulator-Rag complex targets mTORC1 to the lysosomal surface and is necessary for its activation by amino acids. Cell *141*, 290-303.

Sanjuan, M.A., Dillon, C.P., Tait, S.W., Moshiach, S., Dorsey, F., Connell, S., Komatsu, M., Tanaka, K., Cleveland, J.L., Withoff, S.*, et al.* (2007). Toll-like receptor signalling in macrophages links the autophagy pathway to phagocytosis. Nature *450*, 1253-1257.

Sarbassov, D.D., Ali, S.M., Kim, D.H., Guertin, D.A., Latek, R.R., Erdjument-Bromage, H., Tempst, P., and Sabatini, D.M. (2004). Rictor, a novel binding partner of mTOR, defines a rapamycin-insensitive and raptor-independent pathway that regulates the cytoskeleton. Curr Biol *14*, 1296-1302.

Saxton, R.A., Chantranupong, L., Knockenhauer, K.E., Schwartz, T.U., and Sabatini, D.M. (2016). Mechanism of arginine sensing by CASTOR1 upstream of mTORC1. Nature *536*, 229-233.

Saxton, R.A., and Sabatini, D.M. (2017a). mTOR Signaling in Growth, Metabolism, and Disease. Cell *169*, 361-371.

Saxton, R.A., and Sabatini, D.M. (2017b). mTOR Signaling in Growth, Metabolism, and Disease. Cell *168*, 960-976.

Schaffer, B.E., Levin, R.S., Hertz, N.T., Maures, T.J., Schoof, M.L., Hollstein, P.E., Benayoun, B.A., Banko, M.R., Shaw, R.J., Shokat, K.M.*, et al.* (2015). Identification of AMPK Phosphorylation Sites Reveals a Network of Proteins Involved in Cell Invasion and Facilitates Large-Scale Substrate Prediction. Cell metabolism *22*, 907-921.

Scherz-Shouval, R., and Elazar, Z. (2007). ROS, mitochondria and the regulation of autophagy. Trends Cell Biol *17*, 422-427.

Segawa, K., Kurata, S., Yanagihashi, Y., Brummelkamp, T.R., Matsuda, F., and Nagata, S. (2014). Caspase-mediated cleavage of phospholipid flippase for apoptotic phosphatidylserine exposure. Science *344*, 1164-1168.

Segawa, K., and Nagata, S. (2015). An Apoptotic 'Eat Me' Signal: Phosphatidylserine Exposure. Trends Cell Biol *25*, 639-650.

Shen, S., Kepp, O., and Kroemer, G. (2012). The end of autophagic cell death? Autophagy *8*, 1-3.

Shi, J., Gao, W., and Shao, F. (2017). Pyroptosis: Gasdermin-Mediated Programmed Necrotic Cell Death. Trends Biochem Sci *42*, 245-254.

Shi, J., Zhao, Y., Wang, K., Shi, X., Wang, Y., Huang, H., Zhuang, Y., Cai, T., Wang, F., and Shao, F. (2015). Cleavage of GSDMD by inflammatory caspases determines pyroptotic cell death. Nature *526*, 660-665.

Silva, M.T. (2010). Secondary necrosis: the natural outcome of the complete apoptotic program. FEBS Lett *584*, 4491-4499.

Song, X., Kim, S.Y., Zhang, L., Tang, D., Bartlett, D.L., Kwon, Y.T., and Lee, Y.J. (2014). Role of AMP-activated protein kinase in cross-talk between apoptosis and autophagy in human colon cancer. Cell Death Dis *5*, e1504.

Sousa, C.M., Biancur, D.E., Wang, X., Halbrook, C.J., Sherman, M.H., Zhang, L., Kremer, D., Hwang, R.F., Witkiewicz, A.K., Ying, H.*, et al.* (2016). Pancreatic stellate cells support tumour metabolism through autophagic alanine secretion. Nature *536*, 479-483.

Sparta, B., Pargett, M., Minguet, M., Distor, K., Bell, G., and Albeck, J.G. (2015). Receptor Level Mechanisms Are Required for Epidermal Growth Factor (EGF)-stimulated Extracellular Signal-regulated Kinase (ERK) Activity Pulses. J Biol Chem *290*, 24784-24792.

Stefater, J.A., 3rd, Ren, S., Lang, R.A., and Duffield, J.S. (2011). Metchnikoff's policemen: macrophages in development, homeostasis and regeneration. Trends Mol Med *17*, 743-752.

Stockwell, B.R., Friedmann Angeli, J.P., Bayir, H., Bush, A.I., Conrad, M., Dixon, S.J., Fulda, S., Gascon, S., Hatzios, S.K., Kagan, V.E.*, et al.* (2017). Ferroptosis: A Regulated Cell Death Nexus Linking Metabolism, Redox Biology, and Disease. Cell *171*, 273-285.

Stowell, S.R., Ju, T., and Cummings, R.D. (2015). Protein glycosylation in cancer. Annu Rev Pathol *10*, 473-510.

Sullivan, J.E., Carey, F., Carling, D., and Beri, R.K. (1994). Characterisation of 5'-AMP-activated protein kinase in human liver using specific peptide substrates and the effects of 5'-AMP analogues on enzyme activity. Biochemical and biophysical research communications *200*, 1551-1556.

Sun, Q., Cibas, E.S., Huang, H., Hodgson, L., and Overholtzer, M. (2014a). Induction of entosis by epithelial cadherin expression. Cell research *24*, 1288-1298.

Sun, Q., Luo, T., Ren, Y., Florey, O., Shirasawa, S., Sasazuki, T., Robinson, D.N., and Overholtzer, M. (2014b). Competition between human cells by entosis. Cell research *24*, 1299-1310.

Suzuki, T., Franchi, L., Toma, C., Ashida, H., Ogawa, M., Yoshikawa, Y., Mimuro, H., Inohara, N., Sasakawa, C., and Nunez, G. (2007). Differential regulation of caspase-1 activation, pyroptosis, and autophagy via Ipaf and ASC in Shigella-infected macrophages. PLoS Pathog *3*, e111.

Tabernero, J., Rojo, F., Calvo, E., Burris, H., Judson, I., Hazell, K., Martinelli, E., Ramon y Cajal, S., Jones, S., Vidal, L.*, et al.* (2008). Dose- and schedule-dependent inhibition of the mammalian target of rapamycin pathway with everolimus: a phase I tumor pharmacodynamic study in patients with advanced solid tumors. J Clin Oncol *26*, 1603-1610.

Tait, S.W., and Green, D.R. (2010). Mitochondria and cell death: outer membrane permeabilization and beyond. Nat Rev Mol Cell Biol *11*, 621-632.

Taylor, R.C., Cullen, S.P., and Martin, S.J. (2008). Apoptosis: controlled demolition at the cellular level. Nat Rev Mol Cell Biol *9*, 231-241.

Tenev, T., Bianchi, K., Darding, M., Broemer, M., Langlais, C., Wallberg, F., Zachariou, A., Lopez, J., MacFarlane, M., Cain, K.*, et al.* (2011). The Ripoptosome, a signaling platform that assembles in response to genotoxic stress and loss of IAPs. Mol Cell *43*, 432-448.

Thaiparambil, J.T., Eggers, C.M., and Marcus, A.I. (2012). AMPK regulates mitotic spindle orientation through phosphorylation of myosin regulatory light chain. Molecular and cellular biology *32*, 3203-3217.

Tsou, P., Zheng, B., Hsu, C.H., Sasaki, A.T., and Cantley, L.C. (2011). A fluorescent reporter of AMPK activity and cellular energy stress. Cell metabolism *13*, 476-486.

Tsukamoto, S., Kuma, A., Murakami, M., Kishi, C., Yamamoto, A., and Mizushima, N. (2008). Autophagy is essential for preimplantation development of mouse embryos. Science *321*, 117-120.

Upton, J.W., Kaiser, W.J., and Mocarski, E.S. (2012). DAI/ZBP1/DLM-1 complexes with RIP3 to mediate virus-induced programmed necrosis that is targeted by murine cytomegalovirus vIRA. Cell Host Microbe *11*, 290-297.

van Geldermalsen, M., Wang, Q., Nagarajah, R., Marshall, A.D., Thoeng, A., Gao, D., Ritchie, W., Feng, Y., Bailey, C.G., Deng, N.*, et al.* (2016). ASCT2/SLC1A5 controls glutamine uptake and tumour growth in triple-negative basal-like breast cancer. Oncogene *35*, 3201-3208.

Vander Heiden, M.G., Cantley, L.C., and Thompson, C.B. (2009). Understanding the Warburg effect: the metabolic requirements of cell proliferation. Science *324*, 1029-1033.

Verdon, Q., Boonen, M., Ribes, C., Jadot, M., Gasnier, B., and Sagne, C. (2017). SNAT7 is the primary lysosomal glutamine exporter required for extracellular protein-dependent growth of cancer cells. Proc Natl Acad Sci U S A *114*, E3602-E3611.

Vezzani, A., Balosso, S., Maroso, M., Zardoni, D., Noe, F., and Ravizza, T. (2010). ICE/caspase 1 inhibitors and IL-1beta receptor antagonists as potential therapeutics in epilepsy. Curr Opin Investig Drugs *11*, 43-50.

Waddell, D.R., and Vogel, G. (1985). Phagocytic behavior of the predatory slime mold, Dictyostelium caveatum. Cell nibbling. Exp Cell Res *159*, 323-334.

Walter, P., and Ron, D. (2011). The unfolded protein response: from stress pathway to homeostatic regulation. Science *334*, 1081-1086.

Weerasekara, V.K., Panek, D.J., Broadbent, D.G., Mortenson, J.B., Mathis, A.D., Logan, G.N., Prince, J.T., Thomson, D.M., Thompson, J.W., and Andersen, J.L. (2014). Metabolic-stress-induced rearrangement of the 14-3-3zeta interactome

promotes autophagy via a ULK1- and AMPK-regulated 14-3-3zeta interaction with phosphorylated Atg9. Mol Cell Biol *34*, 4379-4388.

Wen, Y.D., Sheng, R., Zhang, L.S., Han, R., Zhang, X., Zhang, X.D., Han, F., Fukunaga, K., and Qin, Z.H. (2008). Neuronal injury in rat model of permanent focal cerebral ischemia is associated with activation of autophagic and lysosomal pathways. Autophagy *4*, 762-769.

Wolfson, R.L., Chantranupong, L., Wyant, G.A., Gu, X., Orozco, J.M., Shen, K., Condon, K.J., Petri, S., Kedir, J., Scaria, S.M.*, et al.* (2017). KICSTOR recruits GATOR1 to the lysosome and is necessary for nutrients to regulate mTORC1. Nature *543*, 438-442.

Wu, N., Zheng, B., Shaywitz, A., Dagon, Y., Tower, C., Bellinger, G., Shen, C.H., Wen, J., Asara, J., McGraw, T.E.*, et al.* (2013). AMPK-dependent degradation of TXNIP upon energy stress leads to enhanced glucose uptake via GLUT1. Mol Cell *49*, 1167-1175.

Wyant, G.A., Abu-Remaileh, M., Wolfson, R.L., Chen, W.W., Freinkman, E., Danai, L.V., Vander Heiden, M.G., and Sabatini, D.M. (2017). mTORC1 Activator SLC38A9 Is Required to Efflux Essential Amino Acids from Lysosomes and Use Protein as a Nutrient. Cell *171*, 642-654 e612.

Yang, S., Wang, X., Contino, G., Liesa, M., Sahin, E., Ying, H., Bause, A., Li, Y., Stommel, J.M., Dell'antonio, G.*, et al.* (2011). Pancreatic cancers require autophagy for tumor growth. Genes & development *25*, 717-729.

Yang, W.S., SriRamaratnam, R., Welsch, M.E., Shimada, K., Skouta, R., Viswanathan, V.S., Cheah, J.H., Clemons, P.A., Shamji, A.F., Clish, C.B.*, et al.* (2014). Regulation of ferroptotic cancer cell death by GPX4. Cell *156*, 317-331.

Ye, J., Palm, W., Peng, M., King, B., Lindsten, T., Li, M.O., Koumenis, C., and Thompson, C.B. (2015). GCN2 sustains mTORC1 suppression upon amino acid deprivation by inducing Sestrin2. Genes & development *29*, 2331-2336.

Youle, R.J., and Narendra, D.P. (2011). Mechanisms of mitophagy. Nat Rev Mol Cell Biol *12*, 9-14.

Young, N.P., Kamireddy, A., Van Nostrand, J.L., Eichner, L.J., Shokhirev, M.N., Dayn, Y., and Shaw, R.J. (2016). AMPK governs lineage specification through Tfeb-dependent regulation of lysosomes. Genes & development *30*, 535-552.

Young, R.M., Ackerman, D., Quinn, Z.L., Mancuso, A., Gruber, M., Liu, L., Giannoukos, D.N., Bobrovnikova-Marjon, E., Diehl, J.A., Keith, B.*, et al.* (2013). Dysregulated mTORC1 renders cells critically dependent on desaturated lipids for survival under tumor-like stress. Genes & development *27*, 1115-1131.

Yu, X., Acehan, D., Menetret, J.F., Booth, C.R., Ludtke, S.J., Riedl, S.J., Shi, Y., Wang, X., and Akey, C.W. (2005). A structure of the human apoptosome at 12.8 A resolution provides insights into this cell death platform. Structure *13*, 1725-1735.

Yuan, H.X., Xiong, Y., and Guan, K.L. (2013). Nutrient sensing, metabolism, and cell growth control. Molecular cell *49*, 379-387.

Yuan, J., and Kroemer, G. (2010). Alternative cell death mechanisms in development and beyond. Genes & development *24*, 2592-2602.

Yuan, J., Najafov, A., and Py, B.F. (2016). Roles of Caspases in Necrotic Cell Death. Cell *167*, 1693-1704.

Zhang, C.S., Hawley, S.A., Zong, Y., Li, M., Wang, Z., Gray, A., Ma, T., Cui, J., Feng, J.W., Zhu, M.*, et al.* (2017). Fructose-1,6-bisphosphate and aldolase mediate glucose sensing by AMPK. Nature *548*, 112-116.

Zhang, D., Wang, W., Sun, X., Xu, D., Wang, C., Zhang, Q., Wang, H., Luo, W., Chen, Y., Chen, H.*, et al.* (2016). AMPK regulates autophagy by phosphorylating BECN1 at threonine 388. Autophagy *12*, 1447-1459.

Zhang, W., Liu, J., Tian, L., Liu, Q., Fu, Y., and Garvey, W.T. (2013). TRIB3 mediates glucose-induced insulin resistance via a mechanism that requires the hexosamine biosynthetic pathway. Diabetes *62*, 4192-4200.

Zhou, G., Myers, R., Li, Y., Chen, Y., Shen, X., Fenyk-Melody, J., Wu, M., Ventre, J., Doebber, T., Fujii, N.*, et al.* (2001). Role of AMP-activated protein kinase in mechanism of metformin action. J Clin Invest *108*, 1167-1174.

Zhou, Q., Kee, Y.S., Poirier, C.C., Jelinek, C., Osborne, J., Divi, S., Surcel, A., Will, M.E., Eggert, U.S., Muller-Taubenberger, A.*, et al.* (2010). 14-3-3 coordinates microtubules, Rac, and myosin II to control cell mechanics and cytokinesis. Curr Biol *20*, 1881-1889.

Zoncu, R., Bar-Peled, L., Efeyan, A., Wang, S., Sancak, Y., and Sabatini, D.M. (2011). mTORC1 senses lysosomal amino acids through an inside-out mechanism that requires the vacuolar H(+)-ATPase. Science *334*, 678-683.