Electrical Pulse Stimulation of MCF7 Breast Cancer Coordinates Autophagy Reprogramming and Proliferative Failure Leading to Cellular Senescence

Mitchell A. Shiell

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Abstract

The utilization of Electrical Pulse Stimulation (EPS) has been predominantly used to study the physiological, cellular, and molecular responses of excitable cells such as nerve and muscle. Based on previous work on myotubes, non-excitable myoblasts, and non-excitable cancerous rhabdomyosarcoma cells, this study looks into the effects of EPS on breast cancer cells (MCF7s). My aim is to characterize the response of MCF7 cells to EPS and add to a working model of cell cycle arrest, autophagy, and cell death mediated by calcium signaling through cell cycle signaling proteins. This is based on the body of literature detailing the effects of EPS on excitable cell types. Ultimately, by stressing cancer cells with EPS, we can learn more about potential novel modes to induce mechanisms of proliferative failure.

Acknowledgments

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ADP	Adenosine diphosphate
AIF	Apoptosis inducing factor
AMEM	Minimum Essential Medium Eagle Alpha Modification
AMP	Adenosine monophosphate
AMPK	AMP activated protein kinase
AP	Autophagosomes
APC	Anaphase promoting complex
ATP	Adenosine triphosphate
CAK	CDK activating kinase
CAMK	Calcium/calmodulin-dependent protein kinase cdc Cell division cycle
CDK	Cyclin dependent kinase
CKI	Cyclin dependent kinase inhibitor
COXIV	Cytochrome oxidase subunit IV
CTRL	Control
CQ	Chloroquine

HCQ	Hydroxychloroquine
MEM	Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
ER	Endoplasmic Reticulum
EPS	Electrical Pulse Stimulation
FACS	Fluorescence activated cell sorting
G_0	Gap 0 phase
G_1	Gap 1 phase
G_2	Gap 2 phase
GSK	Glycogen synthase kinase
HRP	Horseradish peroxidase
INK4	Inhibitor of Kinase 4
KIP	Kinase Inhibiting Proteins
M	Mitosis
MAPK	Mitogen activated protein kinase
mTOR	Mammalian target of rapamycin

mtPTP	Mitochondrial Permeability transition pore
PE	Phosphatidylethanolamine
PI	Propidium iodide
PI3K	Phosphatidylinositol-4,5, - bisphosphate 3-kinase
PM	Plasma Membrane
PVDF	Polyvinylidene difluoride
R point	Restriction point
Rb	Retinoblastoma
RMS	Rhabdomyosarcoma
RNA	Ribonucleic acid
ROS	Reactive oxygen species
S	Synthesis phase
SASP	Senescence associated secretory profile
SAC	Spindle assembly checkpoint
SDS	Sodium dodecyl sulphate
SEM	Standard error of the mean
SR	Sarcoplasmic Reticulum

Review of Literature

1.0 Cancer

The biology and physiology of living systems displays an incredible degree of order. Any alteration that throws off this order is definitionally a diseased state. Cancer is a vast collection of diseased states with multiple characteristics at every scale. On the scale of DNA, cancer is a disease of genetic instability[1]. On the cellular scale, cancer is an imbalance of cell growth and cell death. On the physiological scale cancer takes advantage of normal biological processes in order to perpetuate its own survival. The multilayered characteristics of cancer are commonly conceptualized into what is now known as the hallmarks of cancer. The hallmarks are not definitive but are used to help organize and characterize the vast territory that the disease occupies. The six hallmarks are: sustained proliferative signaling, invasion and metastasis, evasion of growth suppressors, resisting cell death, unregulated replication, and angiogenesis[2]. There also exists many emerging hallmarks of cancer including metabolic adaptations[3] such as the Warburg effect[4], evasion of immune destruction[5], and the tumor microenvironment[6]. A cancerous cell can contain any number of these characteristics and from a harmless, undetectable cell, it can proliferate into a vast merciless disease that obstructs the order necessary for the physiological processes that make life possible.

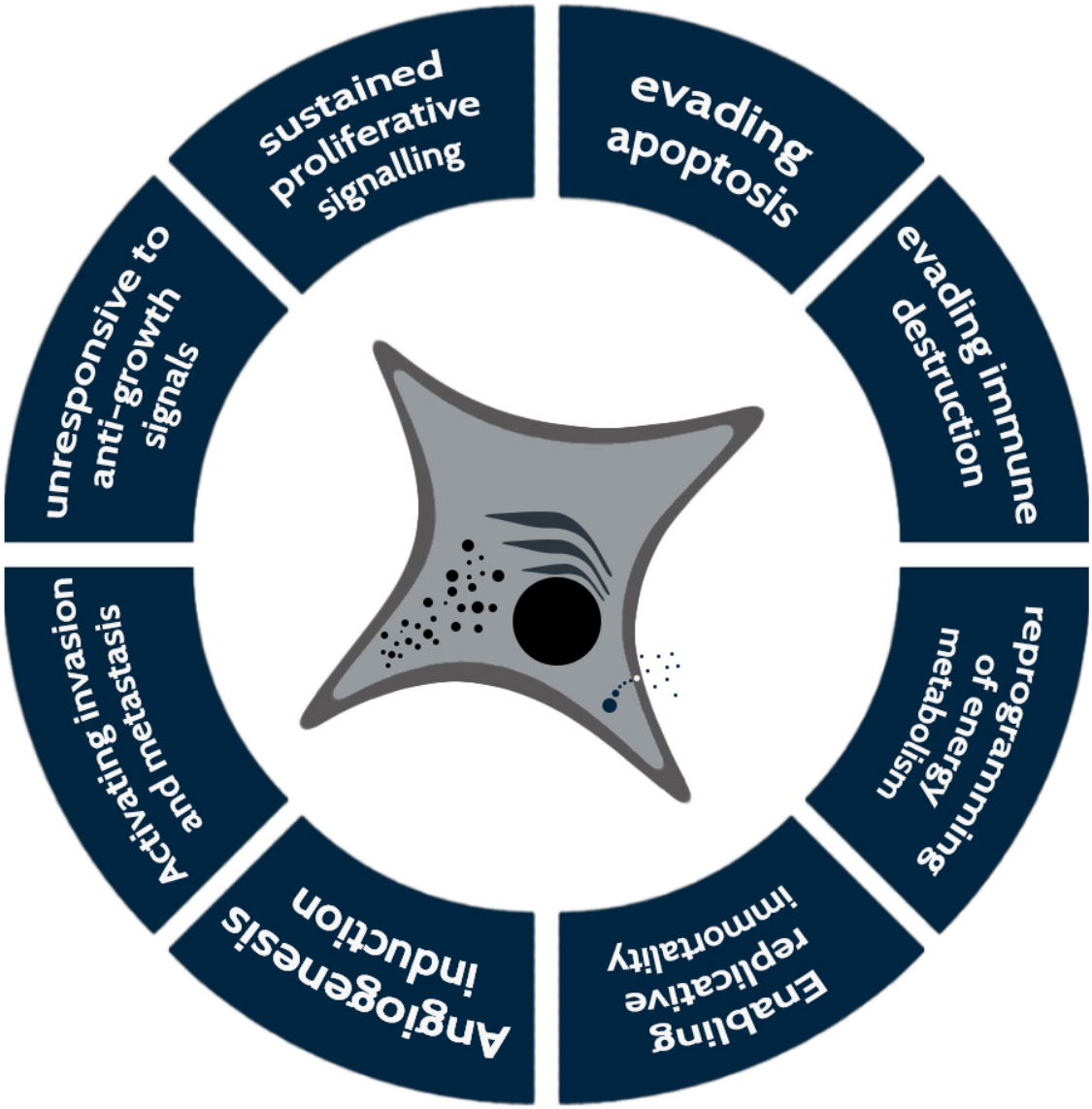

Figure 1. ***The Hallmarks of Cancer.*** The hallmarks of Cancer are common characteristics acquired in the development of human cancer. Initially six hallmarks were accepted; sustained proliferative signaling, invasion and metastasis, evasion of growth suppressors, resisting cell death, unregulated replication, and angiogenesis[1]. There also exists many emerging hallmarks of cancer including metabolic adaptations, commonly referred to as the Warburg effect, evasion of immune destruction, and the tumor microenvironment[2,3,4,5,6]. Our understanding of these common characteristics in the development of cancer contributes greatly to current and future cancer therapy research.

1.1 Cancer therapies

Cancer is by no means a new challenge, it's a disease that even predates human existence. The earliest written description of cancer was written around 3000 BC in an ancient Egyptian medical text called the Edwin Smith Papyrus. This text described a breast cancer, which the writer concluded as a grave disease with no treatment[7]. Thankfully, advances in cancer diagnosis and treatment have improved. From 2003 to 2012 the cancer mortality rate has consistently declined 1.5% annually[8]. That said, as of 2015 cancer was still the second highest cause of mortality worldwide[9] with the lifetime risk of development estimated at 48.4% and 37.3% for men and women, respectively[10]. Our modern understanding and ability to prevent, diagnose and treat cancer has improved, but it can be better.

Cancer is a difficult problem because it is not a homogenous disease. Its genetic basis means it can take on an exceedingly large amount of combinations of mutations leading to any order and magnitude of traits. Not only can each cancer be individualized to each patient but the individual cells that comprise a patient's tumor can also have a diversity of mutations and traits. Additionally, the genetic instability and increased rates of proliferation can cause mutations to continue to occur and occur more rapidly. Therefore, the heterogeneity, genetic instability and rapid replication of cancer creates a moving target for treatments.

Thus, the diversity of cancer is a direct reflection of the diversity of treatments seen today. Surgery was the first popularized and effective course of action. *De Medicina* by Aulus Celsus (25 BC-AD 50) contained a comprehensive description of cancer for the time. In his writings Celsus argued the early and aggressive removal of tumors by surgery. Sometime later Rhazes of Baghdad (860-932) in his *De Chirurgia* noted that cancer surgery should not be attempted unless the cancer can be removed completely[7]. Although the idea of cells coming from cells would only be published in 1858[11], this insight touched upon cancers replicative phenotype. It also underlined that surgery, the main treatment option at the time, would often kill the patient before the cancer would; an unfortunate ongoing theme with even modern cancer therapies. This also

addresses that surgery is only effective for certain types of cancer depending on the location, size, and aggressiveness.

Chemical therapy dated as far back as the Egyptians and Greeks however its introduction to the academic medical world was by the chemist and physician Paracelsus (1493-1541)[12]. He described the use of minerals such as lead, mercury, zinc and arsenic (still used today for acute promyelocytic leukemia[13]) as internal treatments for cancer. It also acknowledged that these treatments were toxic and at high enough doses deadly. This idea from 1567 is still prevalent in modern chemotherapy. The issue of maximum tolerated dose (MTD) is the main crux of chemical therapy. Being administered in the blood means it is a systemic treatment; it affects cancerous and healthy cells alike. As we age the risk of developing cancer increases drastically[14], at the same time the MTD of chemotherapy declines. Therefore, chemotherapy is the least effective on the population with the highest incidence of cancer. Additionally, the side effects of chemotherapy are undeniably terrible. Despite this chemo is the primary treatment option for cancer. It has been estimated that annually 58.4% of treatments of cancer within the US involve chemotherapy[15].

The next most popular treatment option for cancer is radiation (57.9%)[15]. Radiotherapy was discovered soon after Rontgens accidental discovery of x-rays however it would take decades for the field radiology to advance. Early attempts of radiation therapy utilized x rays to treat breast cancers and radium seeds for the treatment of cervical cancers. By the 1960s technological advances in radiotherapy allowed for the precise and deep penetration of radiation[16]. The precision of radiotherapy limits damage to surrounding tissue while deeper penetrating beams allowed it to reach a wider variety of tumors. This ability of specificity and localization are the primary advantages of radiotherapy compared to chemotherapy however, due to its mechanism of action, radio-therapy also has multiple treatment related side effects. Ionizing radiation from radiotherapy ultimately damages DNA through the formation of free radicals. Breaks in DNA initiate an apoptotic signalling cascade that causes cell death for the radiated cells[17]. Since cancer is a disease of genetic instability any intervention that damages DNA has the potential to lead to

secondary malignancies[18]. Radiation has also been implicated with other long-term side effects including cardiac disease[19], lung complications[20], osteoporosis[21] and infertility[22].

As our understanding evolves, we are forced to rethink the future of cancer therapy. In the 5000 years since the Edwin smith papyrus was written the problem of cancer has not changed. In those years we have developed a complex understanding and array of tools to prevent, diagnose and treat cancer more effectively. As the diversity of cancer demands a diversity of treatments options, modern research has seen a push towards targeted and individualized therapies[23]. Therefore, more sensitive and sophisticated diagnostic techniques combined with an expansive array of targeted treatment options is the likely future of cancer therapy.

2.0 Mammalian Cell Cycle

How cancer, from a single cell, can proliferate into a vast disease is highly dependent on alterations within the cell cycle. The importance of our cells to regulate division is fundamental to preventing tumorigenesis. Depending on the external and internal environment the cell generally has three options: to divide, quiesce or die. All three options are controlled by complex signaling events within and outside of the cell. A cell that divides uncontrollably and or refuses to die, regardless of the external or internal stimuli, has lost its ability to communicate in sync with the surrounding environment. This threatens the homeostasis of the immediate area of growth, and if left alone will destroy the homeostasis of organ systems. To prevent this the cell cycle has evolved as a complex series of checks and balances to ensure cells divide when needed. It consists of four main stages, Gap 1 (G_1), Synthesis (S), Gap 2 (G_2), Mitosis and a 5^{th} dormant stage termed G_0 or quiescence (Fig. 2). Under the appropriate conditions, the cell will exit quiescence and enter G_1 where cell growth and preparation for chromosome replication occurs. In S phase the cell duplicates its nuclear DNA and with two complete identical sets of chromosomes, the cell enters the G_2

phase where the integrity of the genetic material is inspected. If all is well, we reach the final stage, mitosis. Mitosis entails chromosomal alignment, spindle formation and the eventual separation of sister chromatids. Although not part of the cell cycle, the separation of the plasma membrane, termed cytokinesis, leaves us with two new daughter cells[24].

The main driving forces of cell cycle progression are the Cyclin/CDK complexes. CDK proteins are constitutively expressed however, the Cyclins are cyclically expressed at very specific points during the cell cycle[25]. These Cyclin/CDK complexes are mainly inhibited by two families of proteins, the inhibitors of kinase 4 (INK4) and the kinase inhibiting proteins (KIP) (Fig. 2)[26]. Protein-protein interaction, phosphorylation and localization are common themes in the regulation of cyclins, CDKs, and their inhibitors throughout the cell cycle[27].

Understanding how these proteins facilitate the checks and balances of cell growth and proliferation are central to understanding tumorigenesis, as mutations that directly affect the regulation of the cell cycle are the primary catalysts to the uncontrolled cell division seen in cancer[1]. Mutations to regulatory proteins that cause a continuous proliferative state, cancer, are termed oncogenes, while mutations that abolish a proteins function in preventing aberrant division are termed tumor suppressors. Oncogenic and tumor suppressor proteins involved in the regulation of cell cycle control are critical targets and areas of study in order to understand, prevent, and treat cancer.

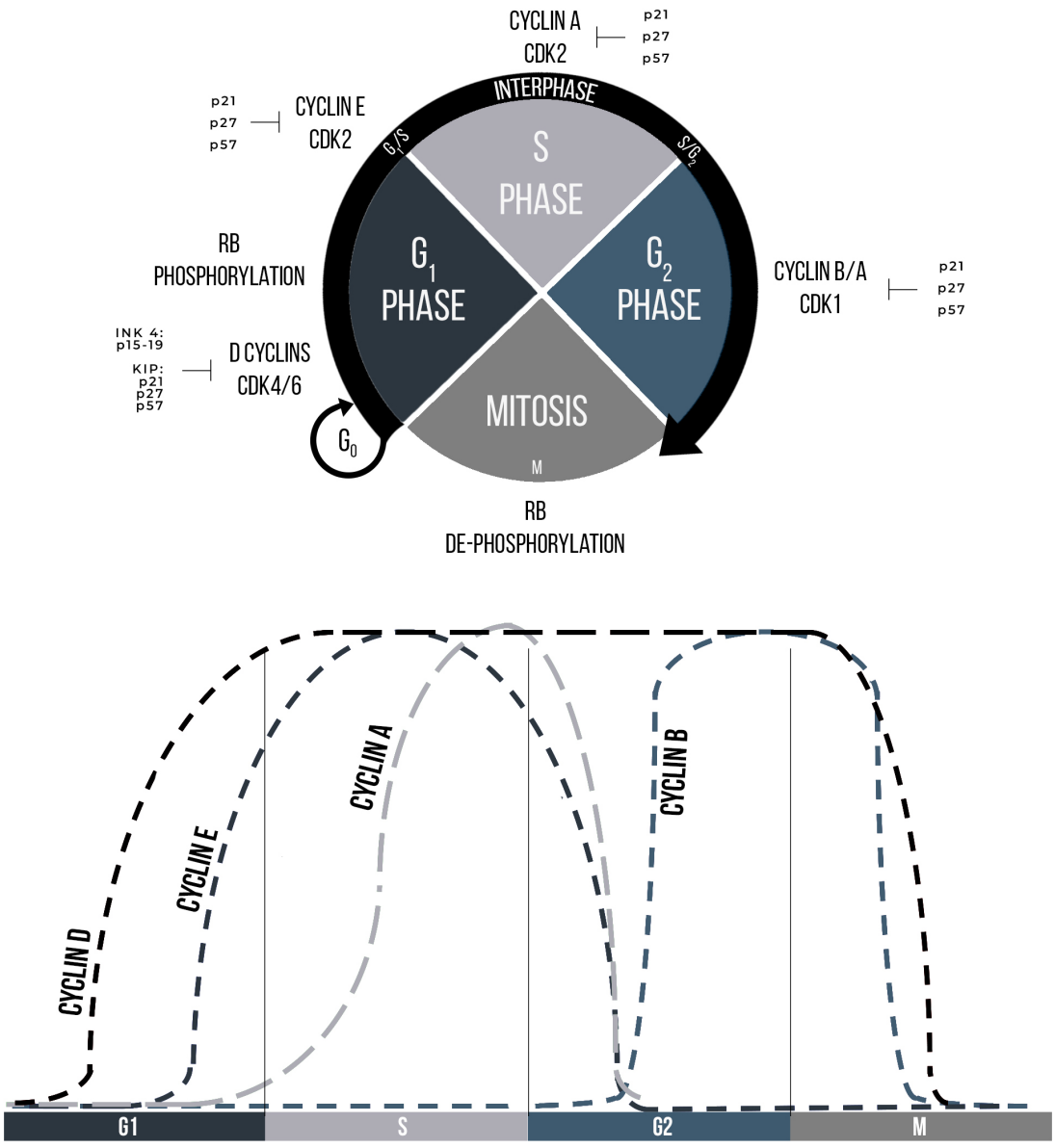

Figure 2. ***Stages of the mammalian cell cycle***. In G_0 the cell remains quiescent. During G_1 the cell begins to grow, monitor its environment and prepare for chromosomal replication. In S phase the chromosomes duplicate. With two sets of DNA the cell enters the G_2 phase. In G_2 the cell works to ensure the integrity of all DNA before entering Mitosis. In Mitosis we see chromosomal alignment and the separation of sister chromatids. Following cytokinesis, we are left with two new daughter cells. The main drivers of the cell cycle are the Cyclin CDK complexes. CDKs are expressed constitutively while cyclins are cyclically expressed depending on the phase of the cycle. The main inhibitors of the cell cycle are the INK and KIP families of proteins[1].

1.2 G_1/S Transition

The first of the critical checkpoints within the cell cycle is the G_1/S transition (Fig. 3). Under normal conditions a quiescent cell is able to assess the mitogenic potential of the surrounding environment such that when the conditions permit, mitogenic signalling cascades including the well-studied mitogen activated proteins kinase pathway (MAPK) will induce the transcriptional activation of cyclin D[28,29]. MAPK has special significance as it is a commonly used marker of tumorigenesis. In cancer the ability to respond appropriately to the mitogenic potential of the micro-environment is often lost due to the mis-regulation of pathways including the MAPK cascades which in cancer leads to a state of perpetual cell cycle entry[30]. Following cyclin D translation, CDK4/6 must be phosphorylated by CAK on threonine 172 and dephosphorylated by cdc25A on tyrosine 15[31]. Cyclin D CDK4/6 must then dissociate its inhibitor INK4 which requires the binding and sequestration of p27[32] or p21[33], a process that not only activates the complex but also reduces the available pool of p27, the primary inhibitor of the Cyclin E/CDK2 complex[34]. With p27 sequestered and INK4 displaced, cyclin D CDK 4/6 phosphorylates the tumor suppressor protein Retinoblastoma (Rb)[35]. In an arrested cell the transcription factor E2F is bound to hypo-phosphorylated Rb, rendering E2F inactive[36]. Hyper-phosphorylated Rb dissociates E2F allowing it to bind to the DNA and transcribe gene targets necessary for S phase entry[32]. Gene targets include, DNA polymerase alpha, BRCA1, thymidine kinase and in a positive feedback loop more E2F and cyclin E[37]. Once formed the cyclin E CDK2 complex can phosphorylate its inhibitor, p27, on threonine 187 promoting the ubiquitination and targeting of p27 for degradation[38]. p27 also has additional phosphorylation sites with differing effects; on serine 10 phosphorylation causes nuclear export, threonine 157 results in cytosolic retention and phosphorylation of tyrosine 88 is able to disrupt the cyclin E/CDK2 binding pocket of p27[38]. p21, another member of the Kip family of proteins also has the ability to bind and inhibit cyclin CDK complexes. Similar to p27 this inhibition can be released through post translational modifications such as the phosphorylation of p21 on threonine 145 by the PI3k/AKT pathway[30]. Central to S phase entry is the cells ability to elevate

cyclin E levels above the threshold necessary to overcome the inhibition of inhibitors like p27 and p21. Once this threshold is reached the cell is able to enter into the synthesis phase. Throughout S phase cyclin E is ubiquitinated by Fbw7 targeting it for degradation while the next cyclin, cyclin A, begins to accumulate[39]. In S phase cyclin A binds to CDK2 and the genetic material is duplicated leading us out of the S phase and into G_2 where the integrity of the DNA is assessed[24].

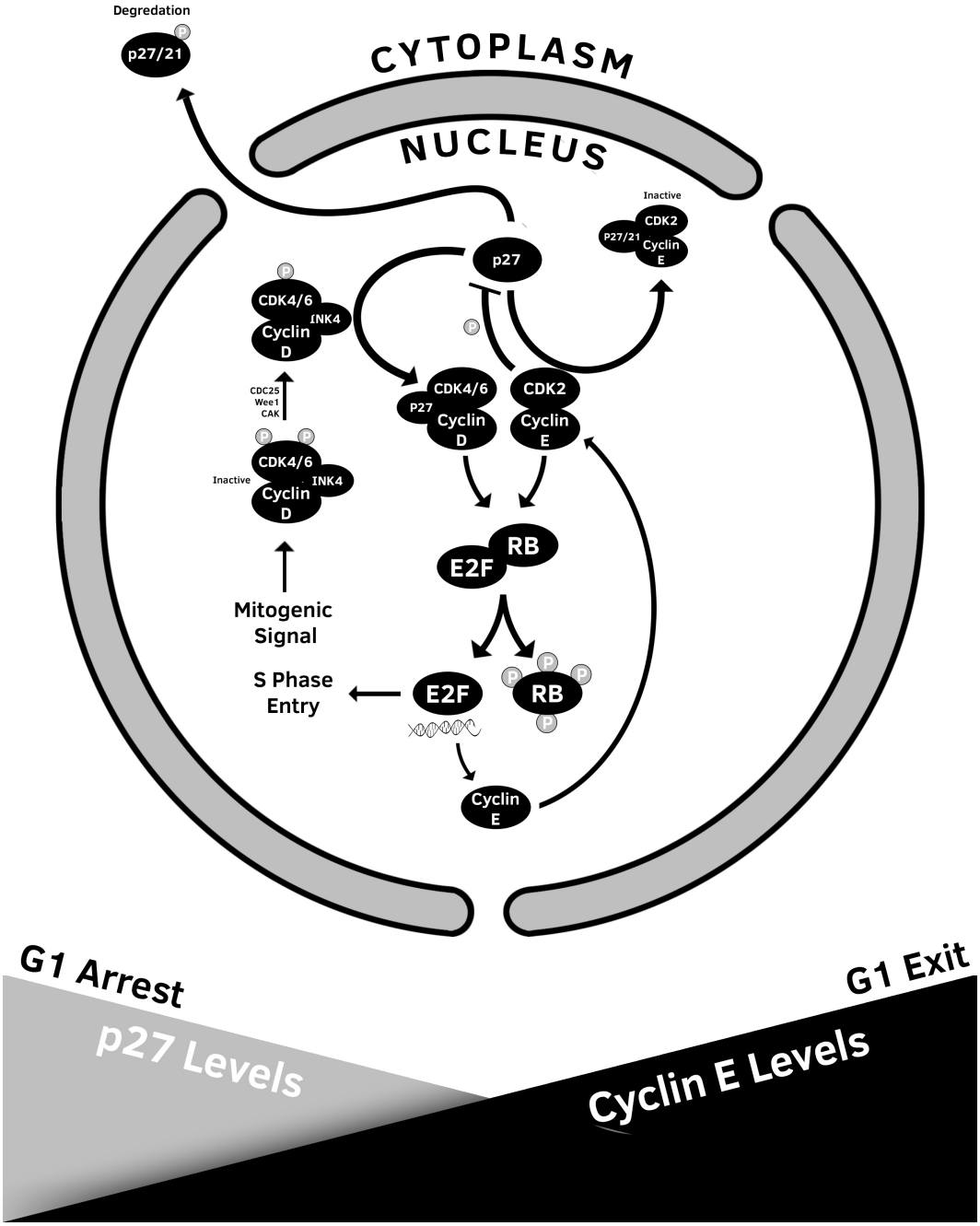

Figure 3. ***G1/S phase signalling.*** Mitogenic signals induce the G_1/S phase causing the formation of the Cyclin D cdk4/6 complex. The full activation of this complex requires p27. Once bound to the Cyclin/cdk4/6 complex p27 is no longer available to inhibit cyclin E cdk2. The Cyclin D and E CDK complex both phosphorylate Rb. This phosphorylation dissociates the E2F transcription factor. The E2F transcription factor initiates the transcription of genes critical for DNA replication and S phase entry. It also induces the transcription of Cyclin E creating a positive feedback loop. P27 can also be phosphorylated by the Cyclin E CDK complex causing its eventual degradation. Ultimately, Cyclin E levels must reach a threshold to overcome the inhibition of p27 and allowing the cell to enter the S phase[28-39].

1.3 G_2/M Transition

A long history of experimentation on a diversity of organisms has placed the protein kinase CDC2 also known as CDK1 as a central point of regulatory activity in the G_2/M transition. CDC2 is only active during the G_2/M transition and is deactivated prior to entering anaphase[40]. It has been shown to bind with cyclin A or B[41]. Within the nucleus, once fully activated, the cyclin B CDC2 complex phosphorylates substrates responsible for the transcription of critical mitotic genes[42]. In the cytoplasm, importin α and β bind to cyclin B CDC2 and subsequently transport the complex into the nucleus (Fig. 4)[43]. This import is counter-balanced by the active export of the cyclin B CDC2 complex from the nucleus by the export protein exportin 1 (CRM1)[44]. In order to deny this export cyclin B must be phosphorylated on its CRM1 binding site mediated by CDC2[45] or (polo-like kinase 1) PLK1[46]. Once Cyclin B is phosphorylated and its export from the nucleus is inhibited there are three additional posttranslational modifications required on CDC2 to fully activate the Cyclin B/CDC2 complex. The CDK activating kinase (CAK) must phosphorylate CDC2 at threonine 161[47], furthermore, two dephosphorylations on tyrosine 14 and threonine 15 by the phosphatase CDC25 (B and C) are required[48]. The Cyclin B CDC2 complex is also rapidly activated through a positive feedback mechanism involving CDC2 phosphorylation of CDC25[49]. The fulfillment of this allows the activated CDK complex to phosphorylate target proteins required to drive the cell into mitosis[40].

The G_2/M checkpoint ultimately functions to ensure the integrity of the DNA before entering mitosis. In an un-arrested cell, this transition accounts for only 15% of the complete time within a cycling cell[50]. However, in response to DNA damage the DNA damage response (DDR) is initiated, holding the cell within G_2 in order to repair the DNA or initiate apoptosis[51]. Experiments with DNA damage inducing treatments have shown that DDR arrest is associated with the rapid inactivation of CDC2[52,53]. The DDR is initiated and sensed by the ATM and ATR kinases which activate the checkpoint kinases CHK1 and CHK2[54]. CHK1/2 primarily mediate the G_2/M arrest through the phosphorylation and inactivation of the phosphatase

CDC25[55]. Once CDC25 is phosphorylated by either CHK1 or CHK2 it is able to bind to the 14-3-3 family of proteins which then functions to translocate CDC25 out of the nucleus and into the cytoplasm[55,56,57]. Without CDC25 activity in the nucleus, CDC2 has no means to overcome the inhibitory phosphorylation's on the tyrosine 15 and threonine 14 residues.

The DDR through the ATM and ATR kinases also serve to modulate the activity of the tumor suppressor protein p53 through the phosphorylation of serine 15 in response to environmental stresses such as ultra violet[58] and ionizing radiation[52]. The tumor suppressing protein and transcription factor p53 is primarily known for its roles in apoptosis, but it also has critical functions in cell cycle arrest and senescence[59]. p53 mediated cell cycle arrest can be initiated by the transcriptional increase of p21 which induces arrest through interactions with PCNA and multiple cyclin CDK complexes[60,61]. In a G_2 arrest p21 can inhibit the activity of CAK, a critical enzyme responsible for the activating phosphorylation of CDC2 on threonine 161 however, experiments have shown no interaction between CAK and Kip proteins (p21, p27, p16, p18) suggesting the inhibition of CAK phosphorylation by p21 relies on an interaction with the cyclin CDK complex that makes the phosphorylation site on the CDK inaccessible to CAK[62].

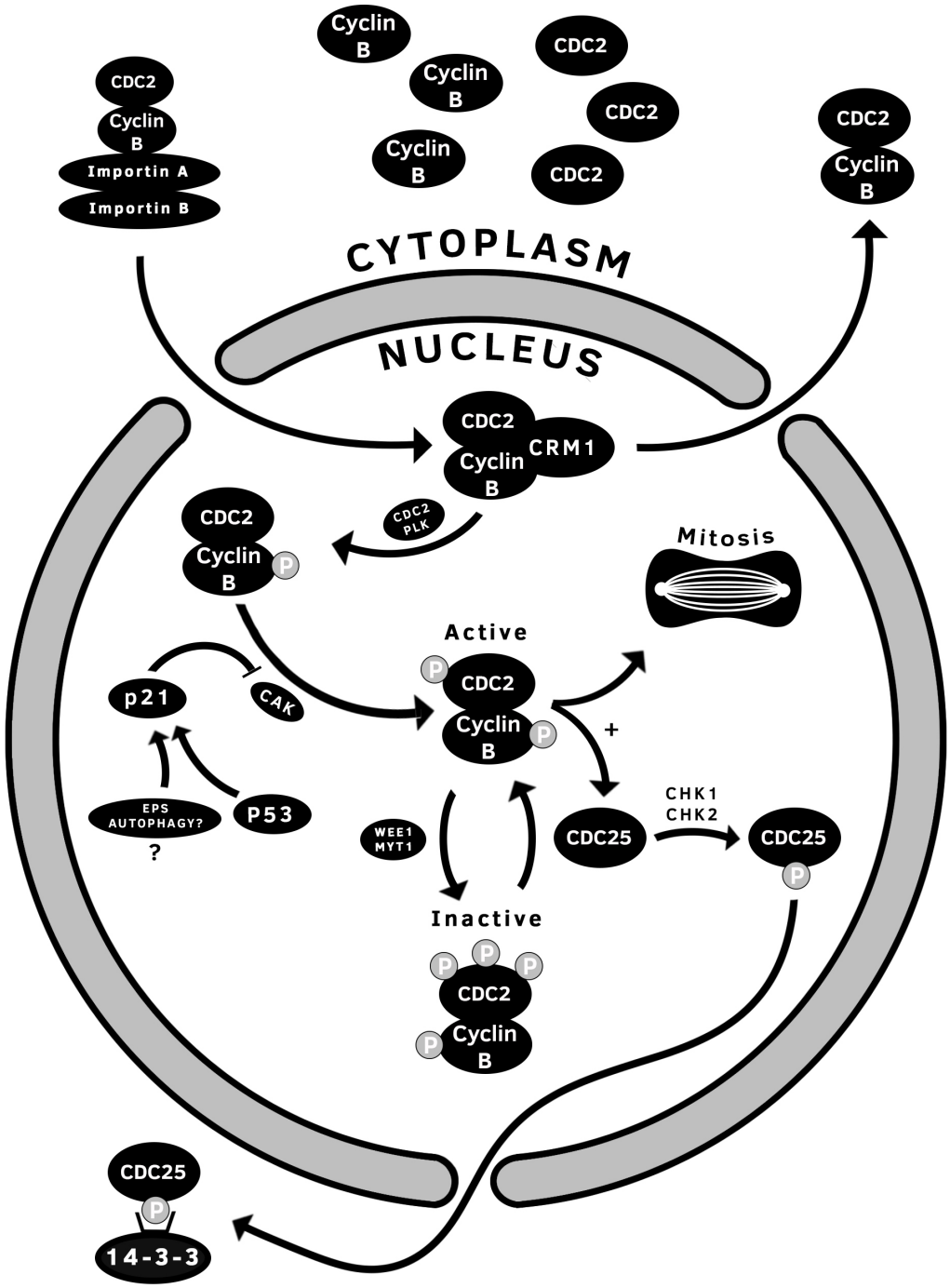

Figure 4. ***G₂/M phase signalling.*** Central to G2/M phase regulation is the Cyclin B/CDC2 complex. Once Cyclin B is stabilized into the nucleus it is able to form a complex with CDC2. The Cyclin B CDC2 complex requires a series of phosphorylations and dephosphorylations to reach its full active state. In its active state Cyclin B CDC2 phosphorylates target genes necessary for mitotic progression.[33]

1.4 Mitosis

Exiting G_2, cyclin B/CDK1 is able to phosphorylate target proteins leading the cell into the 5 stage process of mitosis[63]. The ultimate goal of mitosis is to divide two equal copies of the genome into two daughter cells. In prophase the DNA condenses. During prometaphase the nuclear envelope dissolves and mitotic spindles begin to form. Within metaphase chromosomes align preparing for their separation in anaphase. Lastly the DNA decondenses, the nuclear envelope is made again, and the cell undergoes cytokinesis.

During prophase, DNA condensation is mediated by posttranslational modifications of Histones[64]. The molecular signalling events of DNA condensation is still unclear however Aurora B Kinase has been implicated in the phosphorylation of Histone H3 during mitosis[65]. Cyclin B-CDK1 plays multiple roles during mitosis including the hyper-phosphorylation of lamins which disassembles the nuclear envelope[66]. Shortly after G_2 exit, newly duplicated centrosomes sperate to opposing poles. The migration of the centrosomes has been shown to be regulated by multiple kinases including cyclin B-cdk1 and Plk1 which both phosphorylate and activate the kinesin related motor protein Eg5[67]. Once the centrosomes are positioned, microtubule nucleation begins to form the mitotic spindles necessary for chromosome separation. Microtubules extend in a charge dependent process towards the kinetochores which are responsible for the attachment of chromosomes to microtubules[68]. At the kinetochore the spindle assembly checkpoint (SAC), senses and regulates the proper chromosomal attachment to microtubules. The primary function of the SAC is to inhibit progression until the microtubules are correctly attached to the kinetochores[69]. Prior to mitotic spindle attachment the SAC inhibits mitotic progression through the generation of the mitotic checkpoint complex (MMC) which actively inhibits the anaphase promoting complex (APC/C). The stable attachment of microtubules to the kinetochore inactivates the MCC releasing the inhibition on the APC/C and thus propelling the cell into anaphase[70]. APC/C is further activated by

cyclin B/CDK1 phosphorylation[71] which consequently causes the ubiquitination and degradation of both cyclin B and securin. The degradation of Cyclin B inactivates CDK1 while the degradation of securin frees the enzyme seperase which allows for the opening of the cohesin ring structures that bind together sister chromatids[72]. Combined together, this leads to mitotic exit by allowing the separation of sister chromatids in anaphase eventually leading to cytokinesis and the formation of two daughter cell.

Seeing as cancer cells go through more mitotic divisions than normal cells, many chemotherapeutic drugs aim to target mitosis. All currently approved drugs that target mitosis act to inhibit the proper formation of microtubules[73]. With improper spindle formation the SAC sustains a prolonged arrest leading the cell to apoptosis[74]. Additional mechanisms of cell death from mitotic drug targets exist, including mitotic catastrophe[75] and treatment induced senescence[76].

1.5 Senescence

Cellular Senescence is a stable and permanent cell cycle arrest. For most of history it was thought that cells were immortal and could divide indefinitely until Leonard Hayflick refuted this by demonstrating that normal cells had a finite amount of divisions before permanently arresting into a state termed senescence[77]. The ability to bypass cellular senescence is one of the first hurdles a cell has to overcome in tumorigenesis. Seeing as cancer is a disease of uncontrolled cell growth treatment induced senescence through DNA damage, oxidative stress, and or oncogene activation is a promising avenue of cancer intervention especially so for apoptotic deficient tumors[78].

One of the distinguishing factors between senescence and quiescent cells is their ability to resume proliferation. While senescent cells are metabolically active, they do not respond to mitogenic signals like their quiescent counter parts. It has also been shown that senescent cells are much more resistant to apoptosis. Bio-markers of cellular senescence include chronically elevated p16 and p21 as well as

senescence associated βeta-galactosidase activity. Morphologically, senescent cells present themselves as enlarged and flattened, additionally, senescent cells do not resume replication in response to passaging or to the exposure of growth factors. In essence, senescent cells remain viable and metabolically active yet lack any replicative ability[79]. On the molecular level the senescence programming is still very much an area of ongoing study. In response to DNA damage there is an initial rise in p53 and p21 levels which typically declines over a time course of days. p16, an inhibitor of multiple CDKs, has also been shown to steadily increase. These elevations have been linked to both the DNA damage response (DDR) and ARF pathways[80]. Another characteristic of senescence is the senescence associated secretory phenotype (SASP). SASP is a proinflammatory response of senescence. Senescent cells have been shown to release cytokines as well as matrix metalloproteinases (MMP) which is speculated to have a negative effect on malignancy and the tumor microenvironment. Current data suggests the material required for SASP is fed through the process of autophagy[81].

3.0 Autophagy

Our understanding of the complete autophagy pathway is still infantile and evolving however it has been implicated in a multitude of roles in a diversity of organisms. 2013 marked the 50th anniversary of Christian de Duve's coining of the term "autophagy"[4]. Considered the father of autophagy, Christian de Duve carried out seminal studies leading to the discovery of the lysosome, a critical organelle responsible for the degradation of intracellular proteins. A few years later Clark and Novikoff independently observed irregular shaped vacuoles which carried cytosolic organelles including ER, mitochondria and ribosomes[83]. Arstila and Trumps observed that these double membrane vesicles lacked hydrolytic enzymes and termed them autophagosomes (AP)[84]. Sometime later the landmark discovery of a critical set of autophagy genes (ATG) in yeast by the Ohsumi lab earned Yoshinori Ohsumi the 2016 Nobel prize in physiology and medicine[85]. These discoveries, among others, laid the foundations of a new field and generated interest within autophagy research. The rapid rise of autophagy research is also highly attributed to its associations in disease states such as alzheimer's and cancer. As such, autophagy provides promising new avenues in our understanding of health and disease.

By definition macro autophagy is a catabolic process where cytoplasmic contents are packaged into autophagosomes (AP) and delivered to lysosomes for degradation and recycling. Autophagy flux encompasses the entire autophagy process and can be separated into five primary stages: induction, nucleation, elongations/closure, degradation and recycling (Fig. 5). Compared to the vast body of literature this review will only superficially focus on proteins involved in macro autophagy however, it is worth noting that many specific subcategories of autophagy have emerged including mitophagy, aggrephagy, lipophagy, and proteaphagy to name a few[86].

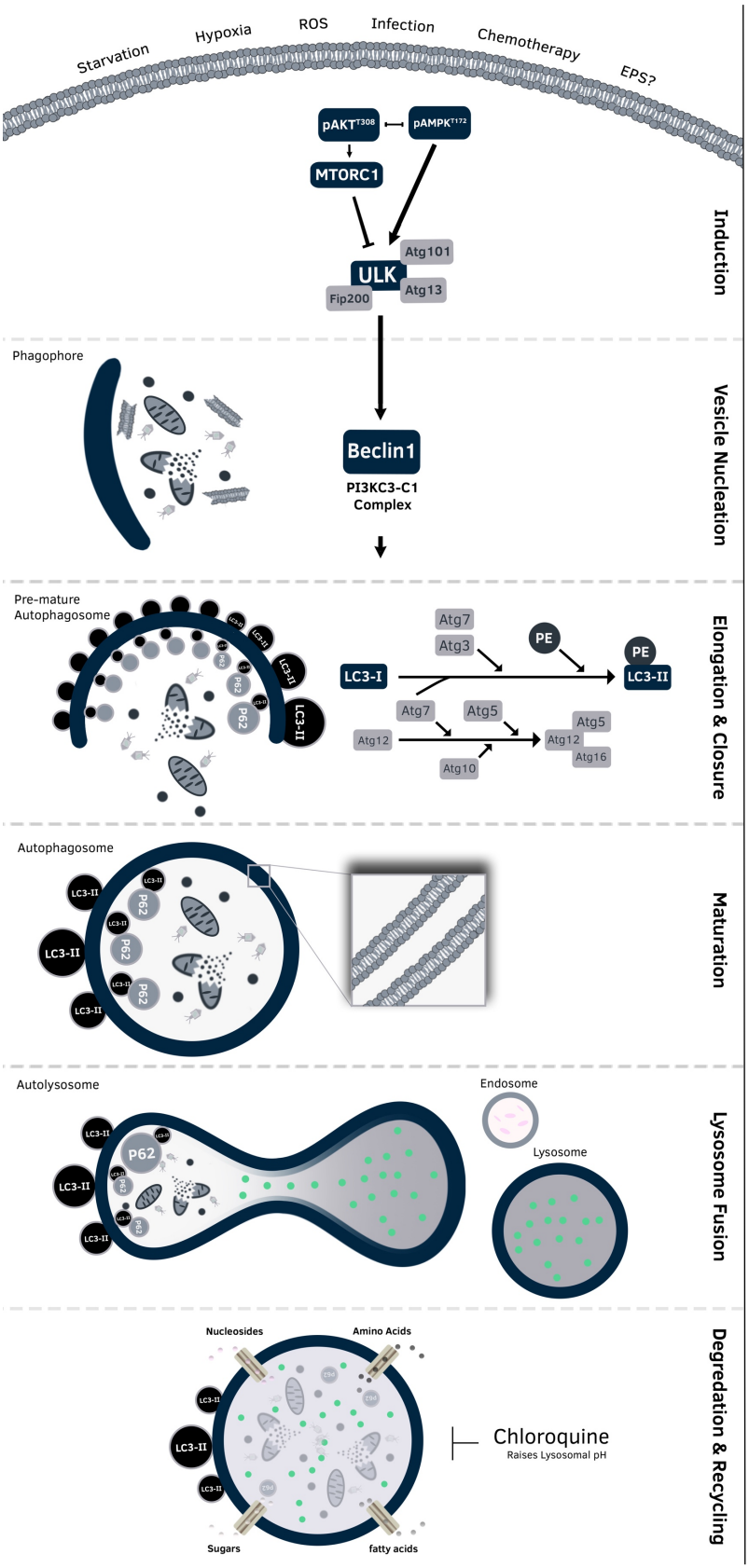

Figure 5. ***Overview of Autophagy Flux.*** Various stimuli regulate autophagy. AMPK and AKT are canonical inducers and inhibitors. Autophagy induction funnels down to the activation of the ULK complex. Critical to vesicle nucleation is the induction of Beclin-1 by ULK1. Membrane formation requires the stepwise input of multiple proteins primarily found at a specific site on the ER termed the omegasome. The lipidation of LC3 to LC3-PE is required for membrane formation and maturation of the double membrane vesical termed the autophagosome. Cargo receptors such as p62 bind and tether cargo to the inner membrane of the autophagosome. Once formed the autophagosome fuses with the lysosome creating an autolysosome. The inner contents of the Autolysosome are degraded by pH dependent hydrolytic enzymes. The contents are recycled back into the cytoplasm by specific catabolite transporters.

3.1 Induction

In response to a wide array of intra and extra-cellular stimuli, the induction of autophagy funnels down to the activation of the ULK complex (unc-51 like autophagy activating kinase 1). Upstream of the ULK complex are two canonical regulators of autophagy induction in mTORC1 and AMPK. These two proteins control autophagy depending, in part, on the energy status of the cell. AMPK responds to increases in the AMP:ATP ratio by both inhibiting mTORC1[87] and activating the ULK complex[88]. Conversely, under nutrient rich conditions the PI3K pathway phosphorylates AKT which then inhibits the TSC complex and consequently releases its inhibition of Rheb, a potent activator of mTORC1 typically localized at the lysosome[89]. Active mTORC1 is able to inhibit the lysosome[90] as well as the induction of autophagy through phosphorylation of ULK1 on S757 and ATG13 on S258[91].

The active ULK1 complex consists of ULK1 and ULK2 combined with the scaffolding protein FIP200 (FAK family kinase interacting protein) and subunits ATG101 and ATG13. When active, the ULK complex is responsible for phosphorylating an increasing list of proteins[91]. ULK1 has been repeatedly shown to facilitate the induction of autophagy through an activation of Beclin-1[92,93], a critical protein involved in the nucleation of the autophagosome[94].

Multiple intracellular and extracellular signals can alter autophagy induction. In addition to metabolic stresses, calcium can modulate autophagy induction through CAMKII activation and phosphorylation of AMPK[95]. The DNA damage response and p53 induction has been shown to both initiate and inhibit autophagy[96]. Furthermore, anti and pro-apoptotic proteins Bcl-2 and BH3-only proteins can inhibit and induce autophagy, respectively[97,98]. All together a complex array of pathways including metabolism, cell death, and cell cycle as well as multiple stressors such as hypoxia, chemotherapy, ROS, infection, and much more converge onto autophagy induction[99]. This complexity suggests autophagy plays a central role in

many cellular mechanisms. The complexity also creates a challenge in measuring and understanding its role in specific cellular responses.

3.2 Vesicle Nucleation

Beclin-1 is a central factor of the class III phosphatidylinositol 3-kinase complex (PI3KC3-C1). This complex is critical to the nucleation of the phagophore, a precursor to the autophagosome[94]. PI3KC3-C1 and the nucleation of the phagophore in mammalian cells is localized at an ER subdomain termed the omegasome. The omegasome is unique as it is concentrated with phosphatidylinositol 3-phosphates (PI(3)P) which are required for phagophore elongation[100].

Our understanding of the origins of autophagosome formation are still evolving, however new evidence has emerged that a physical interaction between organelles including the ER, mitochondria, PM and Golgi can facilitate membrane formation yet the primary source of autophagosome formation is still understood to come primarily from the ER membrane[101].

3.3 Elongation & Closure

The formation of the autophagosome requires PI(3)P (concentrated at the omegasome), WIPI 1-4, GABARAPs and approximately 15 ATG (AuTophaGy) proteins interacting in a stepwise pattern[102]. Characteristic to autophagy is the eventual addition of a phosphatidyl ethanolamine group to LC3 (LC3-II)[103]. This conversion of LC3 to LC3-II is popularly used as a biomarker for membrane formation[104]. As the phagophore elongates it engulfs cellular contents eventually reaching and fusing with itself forming the autophagosome.

Cargo receptors bind and anchor targeted proteins to LC3 or GABARAP tethering the cargo to the inner membrane of the autophagosome. p62 is one such protein which binds poly-ubiquitinated targets resulting in an aggregate of proteins termed aggresomes. Depending on the size of the ubiquitin chain these proteins are degraded through autophagy by means of the autolysosome or degraded in the proteasome[105,106,107]. p62 is often measured as an indication of mature autophagosomes however it is important to be aware that p62 has multiple functions including those within the ubiquitin proteasome system[108].

3.4 Lysosomal Fusion, Degradation & Recycling

Lysosomal function is highly dynamic and critical to the complete flux of autophagy. Autolysosome maturation and lysosomal fusion are possibly the least understood events within autophagy. Transportation of the autophagosome to the lysosome has been shown to be microtubule depended. The motor protein Dynein is also implicated in the autophagosomes transport to lysosomes. With its outer membrane the autophagosome fuses with the lysosome. The SNARE family as well as HDAC6 are emerging as proteins required in autophagosome fusion with the lysosome[109,110]. The inner membrane along with the autophagosomes cargo is then degraded by hydrolytic enzymes in the now termed autolysosome. Around 50 hydrolytic enzymes exist in the lysosome that allow it to fully degrade a diversity of substrates. These hydrolytic enzymes are dependent on the acidic pH of the lysosome which is maintained by ATP dependent proton pumps and over 20 more known membrane proteins at the lysosomal membrane[111]. Once degraded a diversity of catabolite exporters recycle the catabolic products back into the cytoplasm.

3.5 Autophagy and Cancer

Autophagy has been shown to play a diversity of roles in both yeast and mammalian cells. As such autophagy in cancer has been proposed to have a diversity of promoting and antagonizing roles in tumorigenesis[112]. On a physiological level, tumors are more exposed to autophagic stimuli such as nutrient deprivation and hypoxia yet on a molecular level autophagy related genes and proteins can also be lost or suppressed[113]. Furthermore, the roles of autophagy within cancer in regard to cell metabolism, survival and death are deeply context and cell dependent. With this in mind the current literature indicates that tumors routinely utilize autophagy, in some form, to promote cell survival[114–119]. One study of interest investigated the differing dependencies on autophagy for proliferation by comparing both cancerous and non-cancerous breast cells in response to shRNAs for critical autophagy signalling proteins. Interestingly, all cancerous cell types studied showed reduced clonogenic capacity[120]. Furthermore, degrees of sensitivity to autophagy inhibition on proliferative capacity were seen across cell types.

Patients who develop resistance to chemical therapy have been shown to have increased biomarkers for autophagy[121]. Additionally, clinical research has also supported the idea that inhibiting autophagy can lead to better patient outcomes[117,122,123]. Taken together the major focus of targeting autophagy in cancer is to inhibit rather than induce[120]. The antimalaria medicines Chloroquine (CQ) and Hydroxychloroquine (HCQ) are currently the only drugs approved to inhibit autophagy in humans. Full autophagy flux requires degradation by the lysosome. CQ accumulates specifically in the lysosome where it inhibits hydrolytic enzymes by raising pH. Early clinical trials combining CQ with radiation and chemotherapy on glioblastoma patients tripled the median survival[122]. Interestingly median survival times were also significantly improved when combining CQ with radiation alone[124,125]. The MTD of CQ for humans is unknown and an important area of future study. Additionally, better biomarkers of autophagy inhibition in human subjects are needed as the effectiveness of CQ and HCQ dosage on autophagy inhibition can be

highly variable from patient to patient. Lastly, it is important to note that although autophagy inhibition is the current focus, autophagy pathways are highly complex and context dependent. The scale of evidence weighs towards autophagy inhibition however arguments exist for and against. In contrast to above, the role of autophagy in regulating immune responses within tumors suggests the inhibition of autophagy may promote tumorigenesis[126,126]. A deeper understanding of the biological processes of autophagy regulation, as well as its context dependent roles in cancer are required to progress and safely target autophagy in cancer.

4.0 Programmed Cell Death

Homeostasis requires both controlled cell division and death. Cell death works to maintain homeostasis by removing unnecessary or harmful cells. By the 1960s, through the work of developmental biologists, morphological characteristics of cell death were established. Shortly then after the scientific community began forming the hypothesis that cell death was a programmed and regulated event[127,128].

Based on morphological criteria there are three main categories of cell death. Apoptotic cell death (Type I), Autophagic cell death (type II), and necrotic/cytoplasmic cell death (Type III)[129]. It is important to note here that although categorization is convenient it does not always reflect the reality of the cell. Cell death is the end result of multiple contributing factors. In other words, the categorization of cell death pathways does not exist independently in a dyeing cell. The integration of cell signalling pathways and cell fate decisions is still mostly unclear yet understood to be context dependent. With this in mind, the following review will cover only the fundamental aspects of cell death.

Although cancer displays a wide spectrum of heterogeneity many develop protein expression patterns designed to resist programmed cell death and promote cell survival[88,130,131]. p53 is a transcription factor critical to the cell's response to DNA damage, repair and pro-apoptotic signalling. However, p53 function is lost in greater than 50% of cancers[132]. The upregulation of the PI3K/AKT pathway is another common theme in cancer and is responsible for numerous growth and survival functions. This commonly seen upregulation of cell survival pathways and downregulation in pro-apoptotic factors allows cancers to not only proliferate more rapidly but resist death as well.

4.1 Apoptosis

Australian pathologist John Kerr with the aid of A.R Currie and Andrew Wyllie coined the term apoptosis after making the observation that various tissues displayed dying cells with similar morphological characteristics[127]. The consensus of apoptotic morphology would eventually be characterized as cell shrinkage, detachment, and membrane blebbing. Pyknosis, the condensation of chromatin would also become known to be characteristic of apoptotic cell death[133]. The genetic revolution brought fresh insight into our understanding that apoptosis and cell death was indeed a controlled and highly regulated event. Studies by Horvitz et al. on worms lead to the discovery of genes controlling cell death[134]. By 1993, the landmark discovery of the cysteine-aspartic proteases (CASPase), conserved from worms to mammals,[135] launches apoptosis from the dirt and into the vast field of research seen today[128].

In humans the Caspases are a family of 12 cysteine proteases involved in functions that are fundamental to the execution of apoptosis (Fig. 6)[136]. Apoptotic caspases can be organized into initiator and executioner caspases. Downstream of apoptotic signalling events initiator caspases are activated through proximity induced dimerization and subsequent self-cleavage. Once dimerized a cascade of activating caspase cleavages ultimately leads to the activation of the executioner caspases which results in the cleavage of a larger set of cellular proteins. The wide range effects of caspase cleavage which include DNA fragmentation, chromatin condensation and nuclear and cytoskeletal degradation all occur within a span of 30-120 minutes[137]. In modern times the events leading to the execution phase of apoptosis, the initiation phase, is separated into the intrinsic and extrinsic pathways. The intrinsic pathway signals apoptosis through means of the mitochondria while the extrinsic pathway relies on the appropriately named death receptors found at the plasma membrane[129].

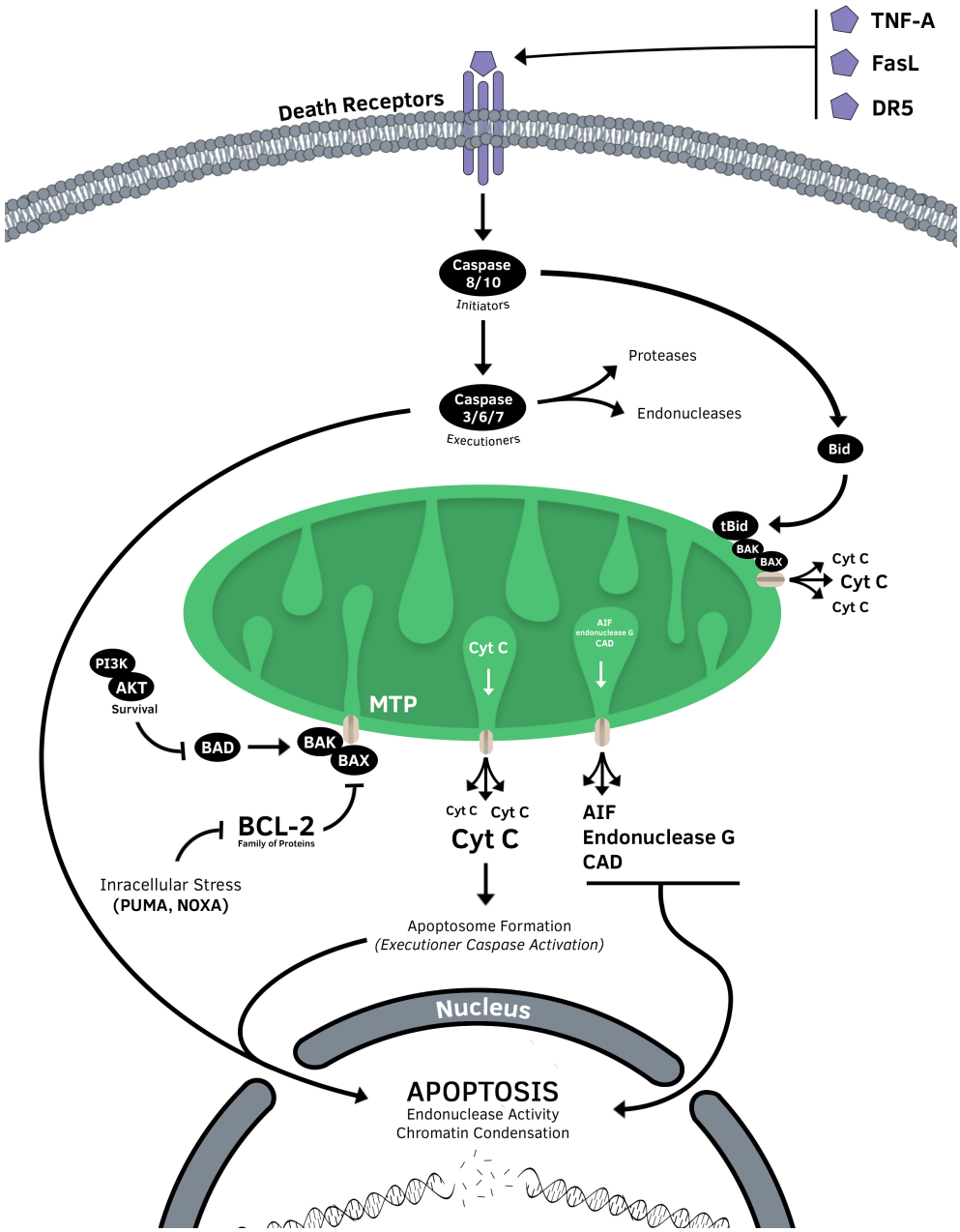

Figure 6. Pathways in Apoptosis. Apoptotic pathways funnel down to the activation of initiator caspases which further activate executioner caspases responsible for the programmed death of the cell. Caspase activation can be initiated by both intrinsic and extrinsic mechanisms. Intrinsic activation requires suppression of anti-apoptotic factors in response to cellular stressors such as DNA damage and nutrient deprivation. Once anti-apoptotic factors are sufficiently supressed MTPs open releasing multiple pro-apoptotic effectors which commit the cell to caspase dependent and or independent apoptosis. The extrinsic pathway is activated in response to ligands binding to death receptors at the PM. Once bound the intracellular end of the death receptor facilitates the activation of the initiator caspases. The extrinsic pathway integrates with the intrinsic pathway by initiator caspase mediated opening of the MTP through Bid and tBid[129,136-142].

4.1.1 Intrinsic Pathway

The intrinsic pathway of apoptosis is centralized at the mitochondria and regulates apoptosis primarily in response to intracellular cues such as ROS and DNA damage. The inter-membrane space stores proteins such as cytochrome C (Cyt C) and AIF which are individually capable of carrying out apoptosis[133]. These mitochondrial factors are always ready for liberation however constitutively active anti-apoptotic factors such as the BCL-2 family of proteins serve to inhibit this release[138]. Upon stress pro-apoptotic factors are transcribed and activated. In response to DNA damage the transcription p53 induces the transcription of pro-apoptotic factors BAD, NOXA and PUMA[139]. These pro-apoptotic factors actively suppress the anti-apoptotic factors facilitating the opening of the Mitochondrial transition pores (MTPs). The opening of the MTP allows the release of apoptotic effectors including AIF, Endonuclease G, CAD, and Cytochrome C. The release Cytochrome C is considered an irreversible step in apoptotic programing and marks the cells full commitment to cell death. Upon release Cytochrome C interacts with APAF-1 and pro-caspase 9 forming the apoptosome and subsequent activation of executioner caspases[136,140].

4.1.2 Extrinsic Pathway

The extrinsic pathway initiates apoptosis by means of extracellular cues. Death receptors at the plasma membrane bind ligands such as TNF-A, FasL, and DR5[129]. On the intracellular side transmembrane receptors play enzymatic roles facilitating the localization, dimerization, and catalytic cleavage of initiator caspases which leads to executioner caspase activation and apoptotic commitment[141]. The extrinsic pathway overlaps with the intrinsic pathway through the activation of Bid and tBid by the TNF-A R1/Fas receptor. Upon activation tBid translocates to the outer mitochondrial membrane where it oligomerizes BAX or BAK causing the release of Cytochrome C[142].

4.2 Necrosis & Necroptosis

Cell death is mostly actively programmed and controlled within cells to respond accordingly to the surrounding environment. Programmed cell death is carried out in a controlled manner with limited affect to the surrounding area. In contrast non-programmed cell death can occur in the event of irreparable damage to the plasma membrane. Unlike Apoptosis, where the cell dies in an immuno-silent manner, necrosis requires the inflammatory response in order to clear the cellular debris[143]. As such, Necrotic cell death is implicated in many pathological conditions[144]. Although Necrosis is known as a passive, energy independent form of cell death, there has been increasing evidence of forms of necrosis with defined molecular events[143]. Active necrosis entails an extensive list of emerging pathways relating to mitochondrial function, lysosomal function, NADPH-Oxidase, PARP-1, proptosis, ferroptosis and others. Active necrotic pathways are still currently being elucidated and as such overlapping mechanisms between cell death pathways still require research[144]. That said, necroptosis is likely the most well studied form of active necrosis. Initially used as a term to describe any active form of necrosis recommendations define necroptosis as any necrotic cell death that is reliant on the protein kinase RIPK3[146]. Advances in biomarkers and specific necrotic inhibitors such as nec-1s give promise to both research and future clinical application.

In cancer, the necrotic discharge of cellular contents releases intracellular signals termed damage-associated molecular patterns (DAMPs) which promotes tumor progression by means of immunosuppression, angiogenesis, proliferation, and inflammation[147]. All together necrosis is implicated in a tissue level resistance to anti-cancer drugs[148]. As necrotic signalling promotes tumor progression the inhibition of necrotic secreted signals serves as an area of interest in improving the efficacy of current and future cancer therapies.

4.3 Autophagy mediated cell death

Autophagic cell death is a somewhat poorly understood and controversial topic. The controversy stems from both a lack of standard in measuring autophagy flux and distinguishing cell death *by* versus *with* autophagy. Autophagy is widely known to be a pro-survival mechanism however, there are instances where autophagy plays direct roles in mediating cell death[149]. Cell death *by* autophagy should be reversible by two or more knockdowns and or chemical inhibitors of core autophagy machinery. Furthermore, inhibitors of other cell death pathways such as apoptosis and necroptosis should not alter the response. Autosis, studied and coined by Beth Levine et. al is one such form of autophagy mediated cell death that fits these criteria. Induced by Tat-Beclin 1 transfection and inhibited by antagonists of the Na+/K+ ATPase, Autosis is characterized by increased autophagosomes, nuclear convolution and focal swelling of the perinuclear space[150]. Although Autosis is a novel insight into autophagy mediated cell death, its place as a physiological phenomenon is yet to be established.

Autophagy has also been shown to play roles in the scaffolding of cell death machinery. Deletion of the tumor suppressor Map3k7 was shown to cause a sensitization of cells to necroptotic cell death mediated by p62 recruitment of RIPK1[151]. The recruitment of RIPK1 increased the interaction of the complementary components of the necroptosome (RIPK3, MLKI). Interestingly, knockdown of p62 led to the activation of apoptosis though the interaction of free RIPK1 with caspase 8 and FADD. Furthermore, the lysosomal inhibitor chloroquine, a compound known to cause the accumulation of autophagosomes, amplified the necroptotic response. The added autophagosomes theoretically provides more opportunity for the scaffolding of the necroptosome. Ultimately, the scaffolding functions of autophagy are a good example of how cell death pathways are becoming more established as a continuum of mechanisms rather than separate independent categories.

5.0 Electrical Pulse Stimulation (EPS)

Before electricity rapidly revolutionized society it slowly evolved as an integral force within our biology. Electricity was first studied by anatomists and physiologists within organisms including electric eels and torpedo fish. Throughout the 17th and 18th century physicists learned how to control the flow of electrons much like the torpedo fish of the past. In the early years it was found that electricity had many effects on the human body[152]. Notably, the initiation of muscular contractions, increases in heart rate, and if extreme enough death. From the 19th century to present it is clear that technology and our understanding of electricity has revolutionized our society. The medical field harnesses electricity in almost all equipment from pagers to electro-cardiograms, however, there has not been many well researched applications of electricity as a direct therapeutic for diseased states such as cancer.

The first documented use of EPS within cell culture was published in 1976 by Shainberg & Burstein who used platinum electrodes to study the effects of EPS on acetylcholine receptor regulation in chick muscle cells[153]. Following this pioneering study, the utilization of EPS has been predominantly used to study the physiological, cellular, and molecular responses of nerves and innervated tissues such as myotubes and myoblasts[154]. Prior to this paper EPS has not been studied as a breast cancer intervention.

When it comes to the usage of EPS there is an infinite amount of combinations of parameters including but not limited to: time, voltage, frequency, pulse width, and polarity. The large spectrum of possible parameters in the application of EPS creates a layer of difficulty when comparing results across studies however, the diversity of unique modes of EPS also creates exciting opportunities for its research and application.

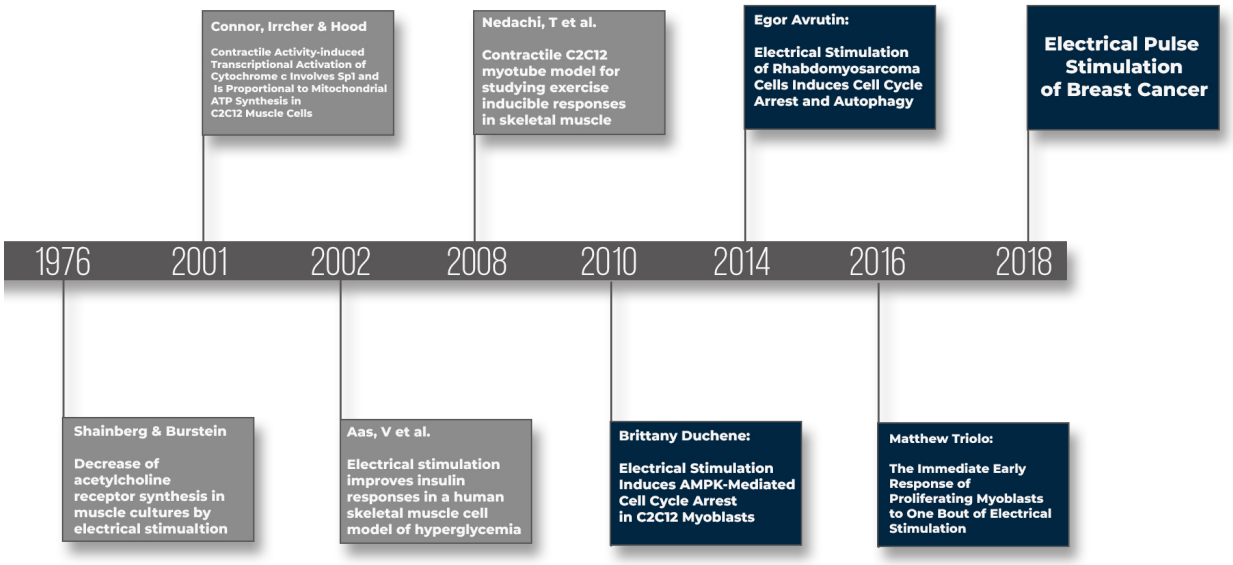

Figure 7. Historical Context of the EPS of Breast Cancer. *In-vitro* EPS has historically been used to study the response of excitable cells in cell culture. It has been used extensively on myotubes as a model of *in-vitro* exercise. More recently in cell culture EPS has been used to study the differentiation of non-excitable myoblasts. It was discovered myoblasts also respond to EPS, inducing cell cycle exit at G_1 and myotube differentiation. Further studies into rhabdomyosarcoma (RMS), a childhood muscle cancer, showed a similar result however through a differing mechanism. RMS cells arrested in G_2 and showed drastic increases in the autophagy associated membrane protein LC3-II. These results led us into our current study of EPS on MCF7s. *Darker boxes indicate work done in our lab.*

5.1 EPS of Myotubes

The physiological pathway leading to skeletal muscle contraction is a well understood phenomenon. Action potentials from nervous input or an applied electrical input are propagated through sodium channels across the T tubule. The T tubules invaginate into the muscle and ultimately cause an interaction between voltage sensitive Dihydropyridine and Ryanodine receptors. This interaction allows the release of calcium from the Sarcoplasmic Reticulum (SR). The SR is very similar to an ER however it is exclusive to muscle and stores a far larger amount of calcium than the ER. Calcium released from the SR is able to bind to troponin and facilitate the cross-bridge cycling of actin and myosin which creates the muscle contraction. The action potential within the T tubule is terminated via voltage sensitive calcium channels. Calcium is sequestered back into the SR through ATP dependent SERCA receptors[155].

Studies of *in vivo* exercise and *in-vitro* EPS of myotubes have shown many similarities across a multitude of studies. Both interventions increase the mRNA expression of PGC1α, PDK4, MYH7[156], as well as multiple myokines (IL-6, IL-8, NAMPT, ANGPTL4)[157]. Both increase Ca^{+2} transients[158,159] which has been linked to increases in the activity of AMPK[160], ERK1/2, and JNK[161]. During short term bouts of EPS, the phosphorylation of AKT has been shown to increase as well[162]. *In-vivo* and *in-vitro* studies have also shown alterations in oxidative phosphorylation, MHC I, and NF-kB. Metabolic effects of EPS on myotubes also include increases in GLUT4 translocation, glucose uptake, mitochondrial biogenesis, myokine secretion, ROS buildup, NO and decreases in glycogen[163].

5.2 EPS of Myoblasts

Skeletal muscle begins in an intermediary undifferentiated state as a myoblast. Myoblasts lack the calcium handling capabilities of myotubes yet are able to proliferate until differentiation into myotubes, a process that requires cell cycle exit. The lack of calcium handling organelles, such as the sarcoplasmic reticulum, and ability of proliferation, makes myoblasts a good intermediary model for predicting the potential responses of other non-excitable cell types. In our lab EPS (4h/day, 6 days, 5Hz 10V) was applied to C2C12 myoblasts[164]. Western blot analysis revealed that EPS induced a 6-fold increase in p27 with a correlated decrease in Cyclin E levels. Additionally, Cyclin E was also shown to have increased interaction with p27. All together this indicates a protein expression pattern typically seen in a G_1/S arrest. It was also shown that in response to EPS the phosphorylation of AMPK on T172 elevated. This phosphorylation is important for the stabilization of p27 levels necessary for the inhibition of cyclin E. By inhibiting Cyclin E, the cell is arrested at the G_1/S transition. The study concluded that in response to EPS, C2C12 myoblasts showed a protein expression pattern indicative of a G_1/S arrest.

Our lab also previously studied the early response of proliferating myoblasts to one bout of electrical stimulation[165]. In this study EPS was applied to C2C12 myoblasts for 4 hours/day (10V, 5Hz) with a 20-hour rest period. In agreement with previous studies EPS induced an increase in AKT and AMPK phosphorylation. However, total p27 protein levels were unaffected. EPS did however induce the nuclear translocation of p27. Localization of p27 to the nucleus is critical for p27 to fulfill its roles in cell cycle arrest[34,38]. Additionally, LC3-II levels were immediately upregulated and diminished 16 hours following EPS. Altogether this study concluded EPS of C2C12 myoblasts induced cell cycle arrest, potentially through AKT and AMPK signaling and altered the autophagic status of the cell.

5.3 EPS of Rhabdomyosarcoma

Rhabdomyosarcoma (RMS) is a form of malignant cancer that originates from striated muscle. It is the most common soft tissue sarcoma within childhood cancers. RMS cells contain muscle like properties however they are unable to terminally differentiate into myotubes, thus this cell type holds an interposition between non-excitable myoblasts and other non-excitable cancer cell types. In our lab, we previously applied our knowledge of muscle biology and EPS to RMS cells[166]. RMS cells were subjected 4 hours of EPS/Day (5Hz, 5V) from anywhere between 1 and 5 days. RMS cells showed proliferative arrest, cell death and morphological changes throughout the five-day time course. Contrary to previous work on myoblasts the reduction in cell number was primarily attributed to a G_2/M arrest confirmed by propidium iodine staining and subsequent FACS analyses. By the 4^{th} day Western blot analysis also revealed an increased conversion of LC3-I to LC3-II. This indicated the involvement of autophagic pathways in response to the stress of EPS. Interestingly AKT on threonine 308, a critical cell survival response and inhibitor of autophagy induction, was induced while pAMPK at threonine 172, a canonical inducer of autophagy induction was shown to decrease. Altogether RMS cells responded in similar fashion to myoblasts however, the mechanism of action differed. The arrested state of cells in response to EPS sparked interest into the ability of other non-excitable cancer cell types. Furthermore, as autophagy is a relatively new and evolving field the intersection of autophagic activity, cell cycle arrest and cell death serve as an emerging area of interest.

6.0 Hypotheses & Research Objectives

This research project is focused on *in-vitro* EPS of MCF7 breast cancer cells. The ultimate goal is to characterize and investigate the response of MCF7s to EPS. The following objectives and hypotheses are based off the prior work detailed above. In accordance with the response of EPS to RMS cells EPS will attenuate cell growth eliciting a G_2/M cell cycle arrest, induce cell death and upregulate autophagy associated proteins. Since non-excitable myoblasts have previously been shown to have calcium dependent EPS responses we also hypothesize that chelating calcium will attenuate the EPS response in MCF7s. In line with these hypotheses my objectives are to quantify cell proliferation and viability, investigate potential mechanisms of cell cycle arrest, measure markers of autophagy, and compare results with the presence and absence of the calcium chelator BAPTA-AM. Additionally I aim to investigate whether a time and or dose (voltage) dependent response to EPS within MCF7s exists.

7.0 Manuscript

Electrical Pulse Stimulation of MCF7 Breast Cancer Coordinates Autophagy Reprogramming and Proliferative Failure Leading to Cellular Senescence

Mitchell A. Shiell, and Michael K. Connor

School of Kinesiology and Health Science

York University, Toronto, Ontario, M3J 1P3, Canada

Manuscript Author Contributions

Mitchell A. Shiell: performed all experiments; data analysis; interpretation; and wrote the manuscript.

Dr. Michael K. Connor: supervised this project as the principal investigator; and contributed to the writing and editing of the manuscript.

7.1 Abstract

Electrical Pulse Stimulation (EPS) has been predominantly used to study the physiological, cellular, and molecular responses of excitable cells such as nerves and skeletal muscle. However, it has been recently shown that non-excitable cells, including myoblasts and rhabdomyosarcoma cells, slow proliferation in response to EPS. Thus, we hypothesized that EPS would also elicit favorable growth inhibitory responses in breast cancer (MCF7) cells. MCF7 cells were subjected to EPS (5 Hz, 6-10 V, 4 hrs/day) for up to 2 days in cell culture and changes in morphology, protein expression and proliferation were evaluated. EPS resulted in dramatic reductions in cell numbers, that were not a result of necrosis, and stark morphological changes associated with mitotic catastrophe and cellular senescence. Prior to cellular senescence EPS arrested cells in G_2/M while concurrently increasing the expression of the cell cycle inhibitor p21, in what may be a p53 independent manner. In addition to these large and rapid reductions in cell numbers, EPS radically increased the protein levels of the autophagy markers LC3(I/II) and p62 in a calcium-dependent fashion, while also potentially causing an inhibition in the late stages of autophagy in a calcium-independent manner. Furthermore, it appears that p62 elevations following EPS play a role independent of the autophagosome that may potentially serve as a mechanism of cell survival and proteostasis. 6-fold increases in the activation of the AKT pathway also suggest a strong cell survival response and a hypertrophic phenotype that may contribute to cellular senescence through mTORC1 activation. Taken together these results provide a framework for the development of EPS as a potential novel localized therapy to breast and other cancer cell types.

7.2 Introduction

Cancer is a disease of perpetual uncontrolled and unwanted cell division. If left untreated, from a single cell this unwanted cell division can accelerate, aggregate, and spread thus, expanding its territory and further disrupting health and homeostasis[2]. The control of cell division is comprised of a complex and tightly regulated series of checks and balances known as the cell cycle; a process fundamental to preventing aberrant cell growth[24]. In cancer, when proliferative control has been lost, the ability to force cell cycle arrest can represent a major step in halting disease progression[27]. Cell division depends on a balance between cell cycle accelerators (i.e. cyclins/CDKs) and cell cycle inhibitors (i.e. p27, p21)[32,38,42,167]. In order to reign in proliferation, a shift in the balance between these cell cycle regulatory proteins that favors quiescence is necessary. There are multiple avenues to achieve cell cycle withdrawal, including decreased cyclin/CDK activity and/or increased activity of cell cycle inhibitory pathways, all of which can be invoked by both internal and external stimuli[34,53].

Originally autophagy was primarily identified as a metabolic process yet more recently it has been implicated in the regulation of an expanding range of functions[86]. Autophagy is a catabolic process where cytoplasmic contents are packaged into double membraned vesicles, termed autophagosomes, which are ultimately delivered to lysosomes for the enzymatic degradation and recycling of their contents[82]. Autophagy has been implicated in the regulation of a wide range of functions including proliferation and cell death[96,120,150,151]. Furthermore, autophagy induction has been elucidated as an important mechanism for adaptive chemotherapy resistance in tumor cells[168]. A common marker of autophagic activity is the formation of the double membraned mature autophagosome which is dependent, in part, on the microtubule-associated protein light chain 3 (LC3)[169]. During autophagosome maturation LC3-I is converted to LC3-II by the addition of a phosphatidyl ethanolamine group[103]. This conversion is often used as a general indication of the autophagic activity within the cell. However, alterations in LC3 protein content alone has limitations as it does not account for autophagic flux which encompasses the

entire process of autophagy from induction to protein degradation. Since variations in LC3-II content can be attributed to alterations across and within multiple stages of autophagy, it is important to consider the entire process of autophagy flux including both synthesis and degradation events when contextualizing autophagy markers such as LC3-II. Selective autophagy is a subdivision of autophagy which requires specific cargo receptor proteins such as p62, a commonly used autophagy marker that is typically localized within the autophagosome and destined for degradation following lysosomal fusion[170]. p62 reductions are used as an indication of increased degradation within the autolysosome due to an increased flux, while elevations in p62 typically imply an inhibition of degradation within the late stages of autophagy which would cause an accumulation of autophagosomes. It is also important to consider changes of LC3-I and p62 protein content can be indicative of events outside of the autophagosome including the p62 dependent accumulation of LC3 in protein aggregates[171–173], a process that can be implicated in autophagy, the ubiquitin proteasome pathway and cell survival[105].

Electrical Pulse Stimulation (EPS) has been used for decades as a means to elicit cellular adaptations *in vivo* by altering membrane polarity. Shainberg & Burstein first used EPS in cell culture in 1976 as a method for studying the regulation of electrical activation of chick muscle cells[153]. EPS of differentiated myotubes in cell culture elicits similar responses to those seen in skeletal muscle stimulated by the motor nerve *in vivo*, showing its utility as a suitable functional model[156]. Thus, using EPS in cell culture has provided a reliable and representative means to control and isolate the specific molecular and cellular events responsible for the adaptations to muscle contraction. Recently, it has been shown that the EPS of non-excitable myoblasts induces cell cycle arrest and subsequent differentiation into myotubes[164,165]. The molecular signaling events and reductions in the proliferative capacity of myoblasts necessary for terminal differentiation suggested that EPS could represent a way to induce novel phenotypic alterations in other non-excitable cell types, including cancer cells. Rhabdomyosarcoma (RMS) is an aggressive childhood cancer whose cells demonstrate muscle gene expression profiles similar to those seen in myoblasts/satellite cells[174]. When subjected to EPS, RMS cells arrested in G_2/M and demonstrated an

increase in the autophagy markers LC3-I and LC3-II[166]. Since EPS was able to elicit these potential anti-proliferative effects in non-excitable RMS cells, the possibility existed that EPS could elicit similar phenotypic alterations in other non-excitable cancerous cell lines.

In the current study MCF7 breast cancer cells were subjected to EPS at multiple intensities in cell culture. We report herein that EPS sets in motion a program of proliferative failure, indicative of senescence, that arrests the cell in G_2/M in what may be a p53 independent manner. EPS also markedly increases the autophagy markers LC3 and p62 in a mechanism that suggests an increased induction of autophagy in a calcium dependent fashion, while concomitantly inhibiting the late stages of autophagy through a calcium independent mechanism. Thus, given that the upregulation of autophagy has been identified as a critical adaptation in tumors that leads to chemotherapy resistance[119], inhibiting the late stages of autophagy with EPS might be a novel method in sensitizing tumor cells to chemotherapy while also facilitating the failure of tumor growth. Taken together, these results provide a framework for the application of EPS to other cancer cell types with the ultimate goal of developing a novel localized cancer therapy.

7.3 Materials and Methods

Cell Culture MCF7 breast cancer cells were incubated at 37°C and 5% CO_2 in Minimum Essential Medium Eagle, Alpha Modification (AMEM; Sigma Mississauga, ON), supplemented with 5% FBS (Wisent, Montreal, QC) 3% antibiotic antimycotic, 1% sodium pyruvate, 1% Non-essential amino acids and 10µg/ml of human insulin (Sigma). For LDH experiments phenol free MEM (Gibco Fisher Scientific, Mississauga, ON) was used as described for AMEM above with the exception of sodium pyruvate.

Electrical Pulse Stimulation MCF7 cells were cultured in 6-well plates and stimulated using a lid which was modified with two parallel platinum wire electrodes extending into the media. A total volume of 5.5ml per well was used to ensure consistent delivery of EPS to the media. Cells were stimulated using a Harvard Apparatus Stimulator CS System (Harvard, MTL, QC). 6, 8 or 10 Volts applied at a frequency of 5 Hz with alternating polarity of current for 4 hours with a 20-hour rest period for either 1 or 2 days.

Cell Collection Cells were harvested at various times and washed with cold Phosphate Buffered Saline (PBS, Wisent), scraped, and centrifuged (2320 x g at 4°C). The pellet was resuspended in TENT buffer (0.2% TENT – TRIS, EDTA, NaCl, 0.2% Triton x-100), and 1% phosphatase inhibitor (Phos-Stop, Roche Diagnostics, Montreal, QC). Cell were sonicated (20% Max) and centrifuged (16168 x g at 4°C). The resultant supernatant was removed and stored at -80°C for future analyses.

Immunoblotting Protein concentrations of collected samples were determined using a Bradford assay and 25µg of protein were separated using an SDS polyacrylamide gel electrophoresis (PAGE, 12% and 16% gels). Following electrophoresis proteins were transferred overnight (4°C, 40V) onto a PVDF membrane. Quality of transfer was confirmed using amido black staining and membranes were blocked for 2 hours in 10% milk at room temperature. Membranes were probed overnight (4°C) with primary antibodies for: AMPK, $pAMPK^{T172}$, AKT, $pAKT^{T308}$, LC3I/II, p53, Beclin-1, (Cell Signaling, Whitby, ON), p27 (BD

Biosciences, Mississauga, ON), $p27^{T198}$ (R&D Systems, Minneapolis, MN), Cyclin E, p62, COXIV, β-actin (Abcam, Cambridge, MA), and p21 (Santa Cruz, Dallas, TX). Following incubation with primary antibodies, membranes were rinsed 3 times using a Tris-Buffer Saline with Tween-20 (0.05%) (TBST) and incubated with HRP-linked anti-rabbit and anti-mouse secondary antibodies (Cell Signaling, Whitby, ON) in 5% milk with shaking at room temperature for 1 hour. Membranes were subsequently washed 3 times in TBST at room temperature and protein levels were visualized using enhanced chemiluminescence substrates (Millipore, Whitby, ON) with a Kodak In Vivo FX Pro imager. Images were quantified using Carestream software (Marketlink Scientific, Burlington, ON). β-actin was used to control for protein loading.

Assessment of cell Viability and Growth Inhibition Cell viability was quantified using a modified LDH assay[175,176] to measure the activity of lactate dehydrogenase (LDH) released as an indication of cell death. Briefly, free floating LDH released from dead cells into the media is able to reduce NAD+ to NADH. Newly synthesized NADH facilitates the production of formazan which can be measured spectrophotometrically. The amount of formazan was measured using a microplate reader (BIOTEK, Vermont, MN; 490nm). 1ml of media was removed for the measurement of the released LDH (sampled media). LDH release values were compared to a condition of complete cell death which was elicited by exposing the well to a 10x Lysis Buffer (10% Triton X-100) for 30 minutes. Following the 30 minutes 1ml of this complete lysis media was collected as a representation of the total LDH derived from outside and inside of the cells. Media alone, absent of cells, was used to measure background LDH values. After subtraction of background, LDH release values (sampled media) were subtracted from maximum LDH release values (lysis media) and this value represented the amount of LDH within the adherent living cells. The LDH within living cells was measured for controls and EPS cells before as well as 4, 24, 28, 48 and 72 hours following the onset of EPS. Total live cell LDH measurements were expressed as a ratio between EPS and controls. This value

represents the percentage growth inhibition of the treatment condition. More details of calculations and methods used were published previously[175,176].

Cell cycle Analysis MCF7 cells were collected via tyrpsination, pelleted and washed twice in cold PBS. Following washing cells were fixed by the dropwise addition of ice cold 70% ethanol and stored at -20ºC. The cells were incubated overnight in staining solution (750µl of PBS, 200 µl of 50 µg/mL propidium iodide Bioshop, Burlington, ON) and 50 µl of 10 µg/mL RNase (Sigma Mississauga, ON)). DNA content was measured using an Attune NxT Flow cytometer (Fisher Scientific, Mississauga, ON) and cell cycle profiles were determined using FlowJo software (BD Biosciences, Mississauga, ON).

Pharmacological treatments *BAPTA-AM,* a chelator of intracellular calcium ions, was used to assess the role of Ca^{2+} in the cell when responding to EPS. DMSO was used as a reliable control. BAPTA-AM was added to the media at concentrations ranging from 4 µM to 12 µM. Chloroquine (CQ), a lysosomotropic compound that is routinely used to investigate late stage autophagy flux, was added to media at concentrations ranging from 2.5 µM to 20 µM with DMSO used as a control. CQ accumulates in lysosomes where it raises the lysosomal pH thus inhibiting the pH dependent hydrolytic enzymes required for lysosomal degradation. LC3 and p62 levels were measured via western blotting to infer changes in autophagosome formation and degradation, respectively.

Senescence Associated β-Galactosidase (SA-β-Gal) Staining Cellular Senescence was assessed using a SA-β-Gal staining kit (Cell Signaling, Pickering, ON). Cells were washed with PBS and fixed for 15 minutes. Following fixation cells were washed again with PBS and subsequently placed in a dry 37℃ incubator (0 % CO_2) overnight. SA-β-Gal activity was indicated by blue staining and this was qualitatively assessed via brightfield microscopy (10x magnification).

Statistical Analyses Statistical analyses were done using GraphPad Prism 5 software. One and two-way ANOVAs followed up by a Tukey's post-hoc test were used to determine differences between individual groups. Error bars are represented as ±SEM and statistical significance was determined when $p \leq 0.05$.

7.4 Results

EPS results in growth inhibition and not necrosis EPS (2 d x 4 hrs/d) resulted in a noticeable reduction in the number of cells visible using light microscopy (Fig. 1A, panel 1 vs. 3). This phenomenon was even more evident 72 hours after the onset of the first bout of EPS (Fig. 1A, panel 2 vs. 4). Given that this EPS dependent decrease in cell number could be a result of decreased proliferation and/or increased cell death, we assessed cell viability and proliferation using a modified LDH assay[9,175]. LDH is released into the media from cells with cytoplasmic damage caused by necrotic cell death. The amount of LDH released into the media from cells exposed to EPS was measured and compared to LDH released into the media of non-stimulated control cells. In addition to measuring LDH released into the media, total LDH was measured by lysing cells and collecting the resulting media. This total LDH value was used to calculate the percentage of LDH released for each condition and timepoint. Measurements were taken before and after both bouts of EPS (0, 4, 24, and 28 hrs) as well as 48 and 72 hours following the onset of EPS. LDH release from EPS cells was not increased 72 hours after the onset of EPS (Fig. 1B) and no change in the difference between EPS and non-stimulated controls over time were evident (Fig. 1C). While there was a statistical difference between EPS and non-stimulated control cells at 72 hrs, this difference is attributable to LDH released from control cells being less than the background values. These results suggest that EPS does not induce necrosis and that the vast majority of cells remained viable after EPS. By lysing the attached cells, measuring LDH activity and comparing this activity in EPS and non-stimulated cultures, an assessment of relative cell number was evaluated. Following cell lysis, total LDH activity was measured at all timepoints with the 0 hour non-stimulated control values used as a reference point. Total LDH did not differ between the EPS and control cultures for the first 28 hours. After the 2nd bout of EPS (28 hrs) total LDH in non-stimulated controls progressively rose while EPS conditioned cells remained unchanged (Fig. 1D). By 48- and 72-hours post onset, the total LDH activity of EPS conditioned cells was 54% and 83% lower than that of non-stimulated controls (Fig. 1E). In agreement with light microscopy images (Fig. 1A), this differential in total

LDH activity is a strong indication and representation of the reduction in cell number and growth inhibition seen in response to EPS. Taken together these data indicate that EPS induces drastic and sustained decreases in proliferation.

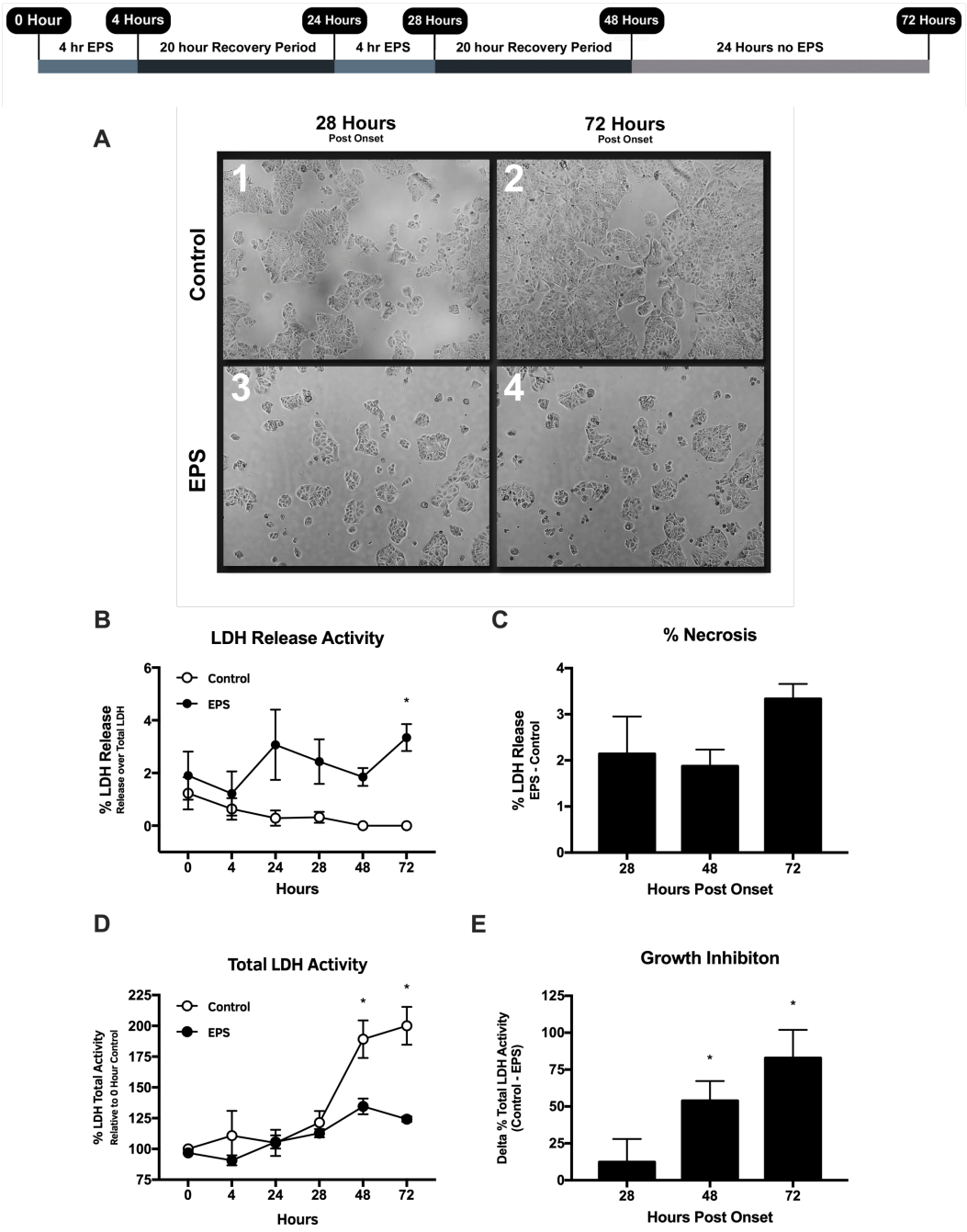

Figure 1. EPS results in growth inhibition and not necrosis. **(A)** brightfield microscopy images (4x magnification) of control (panels 1,2) relative to EPS conditioned cells (8V) (panels 3, 4) taken 28 and 72 hours following the onset of EPS. **(B)** LDH release activity over 72 hours. Following background subtraction all LDH release values are calculated as the percentage of the total LDH activity. **(C)** LDH release activity represented as the percentage of necrotic cells. The percentage of necrosis in EPS conditioned cells was subtracted from the percentage of necrotic cells of parallel controls. **(D)** Total LDH activity over 72 hours. Total LDH activity attained from measuring LDH activity in cultured cells under a condition of complete cell lysis. **(E)** Represented as the relative inhibition of growth, the total LDH activity of controls was subtracted from respective total LDH values from EPS conditioned cells. For (B-E) all values are represented as the mean ± S.E.M.; * $p \leq 0.05$ (N = 4-6)

EPS induces cellular senescence LDH experiments indicated that EPS induced reductions in proliferative capacity. In order to assess whether the EPS dependent changes in proliferative capacity were transient or permanent, cells were stimulated (4hr/d, 2d, 10V) and re-plated with fresh media. Cell morphology and growth was monitored over both days of EPS exposure (Fig. 2A) as well as 4 days following re-plating (Fig. 2B). Immediately after the first bout of EPS the cells were morphologically identical to non-stimulated cells (Fig. 2A, 4 hrs) however, 24 and 48 hours following the onset of EPS distinct morphological differences emerged (Fig. 2A). Cells undergoing EPS had a noticeable population with a small and rounded appearance (Fig. 2A, Arrows). Differences were magnified over time with cells undergoing EPS attaining an enlarged flattened morphology (Fig. 2B). Furthermore, EPS cells also contained puncta both at the plasma membrane and clustering towards the nucleus (Fig. 2B, Arrows). During the 4-day period following re-plating, cells exposed to EPS showed no discernable increase in confluence while non-stimulated control cells reached 100% confluence and required re-plating every 48 hours (Fig. 2B). Thus, EPS appeared to induce a permanent alteration in proliferative capacity. Additionally, the enlarged flattened morphology further suggested EPS may have induced cellular senescence. To assess whether EPS indeed activated a senescence program, cells were stained for senescence associated β-Galactosidase (SA-β-Gal) activity (Fig. 2C). SA-β-Gal staining showed a distinct increase in positive staining in the vast majority of EPS cells (Fig. 2C, panel 2) relative to the control cells (Fig. 2C, panel 1) demonstrating an increase in cellular senescence indicated by the appearance of distinct black nuclei (Fig. 2C, Arrows).

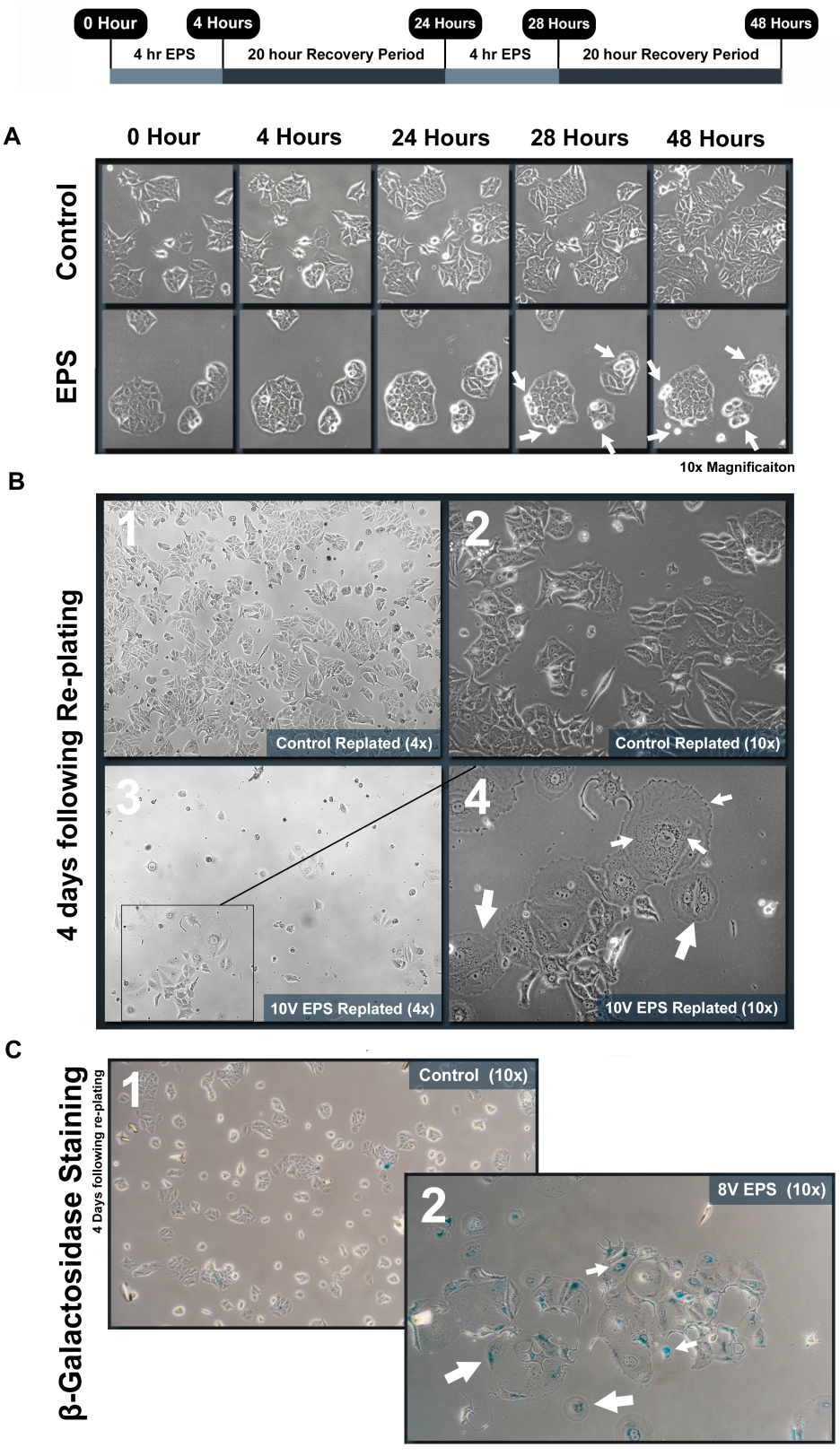

Figure 2. EPS induced morphology and senescence. (A) Brightfield microscopy images of control and EPS conditioned cells over 48 hours. Arrows indicating cell rounding. (B) Brightfield microscopy images of control (panels 1-2) and EPS conditioned cells (panels 3-4), 4 days following re-plating (6 days following the onset of EPS). Larger arrows indicating double nucleated cells, smaller arrows indicating puncta radiating from nucleus of enlarged flattened cells. (C) Brightfield microscopy images of SA-β-Gal stained control conditioned (panel 1) and EPS conditioned (panel 2) cells taken 4 days following re-plating (6 days following the onset of EPS). Arrows indicate examples of positive staining. 10x magnification for all images with exception to B panels 1 and 3 (4x magnification).

EPS does not cause a G_1 arrest but rather a G_2/M arrest Cellular senescence is classically associated with a G_1 arrest[177–180] therefore, we measured the effects of EPS on proteins known to control the G_1-S transition. pAMPK ^{T172} phosphorylation is commonly associated with a G_1 arrest in response to metabolic stress[181]. Compared to controls pAMPK^{T172} protein levels were decreased by approximately 53% with EPS. At the maximum intensity of EPS (10V) a time dependent relationship was evident, with a further reduction in pAMPK^{T172} in EPS cells from 56% to 30% between 1 and 2 days, respectively (Fig. 3B). In contrast, pAKT^{T308}, a phosphorylation site known to drive cell growth and G_1/S phase progression[34], showed large elevations in protein levels, reaching values that were 370% higher in EPS cells compared to non-stimulated control cells (Fig. 3C). No differences in total AMPK or AKT were evident. EPS also had no effect on the protein levels of the CDK inhibitor p27 (Fig. 3D). Furthermore, cyclin E protein demonstrated decreases of up to 37% in protein levels compared to non-stimulated cells (Fig. 3E). Taken together, the effects of EPS on AMPK, AKT, p27 and cyclin E do not indicate arrest in G_1, but actually suggest that cells have progressed through the G_1/S transition after EPS. In order to assess the cell cycle profiles of EPS (8V, 48hrs) and non-simulated cells we stained the cells with propidium iodide and subsequently measured for DNA content using flow automated cytometry (FACS) (Fig. 3F-H). These cell cycle analyses revealed that EPS caused a significant reduction of cells in G_1 with a proportional rise of cells in G_2/M. The proportion of cells within S phase remained unaltered by EPS.

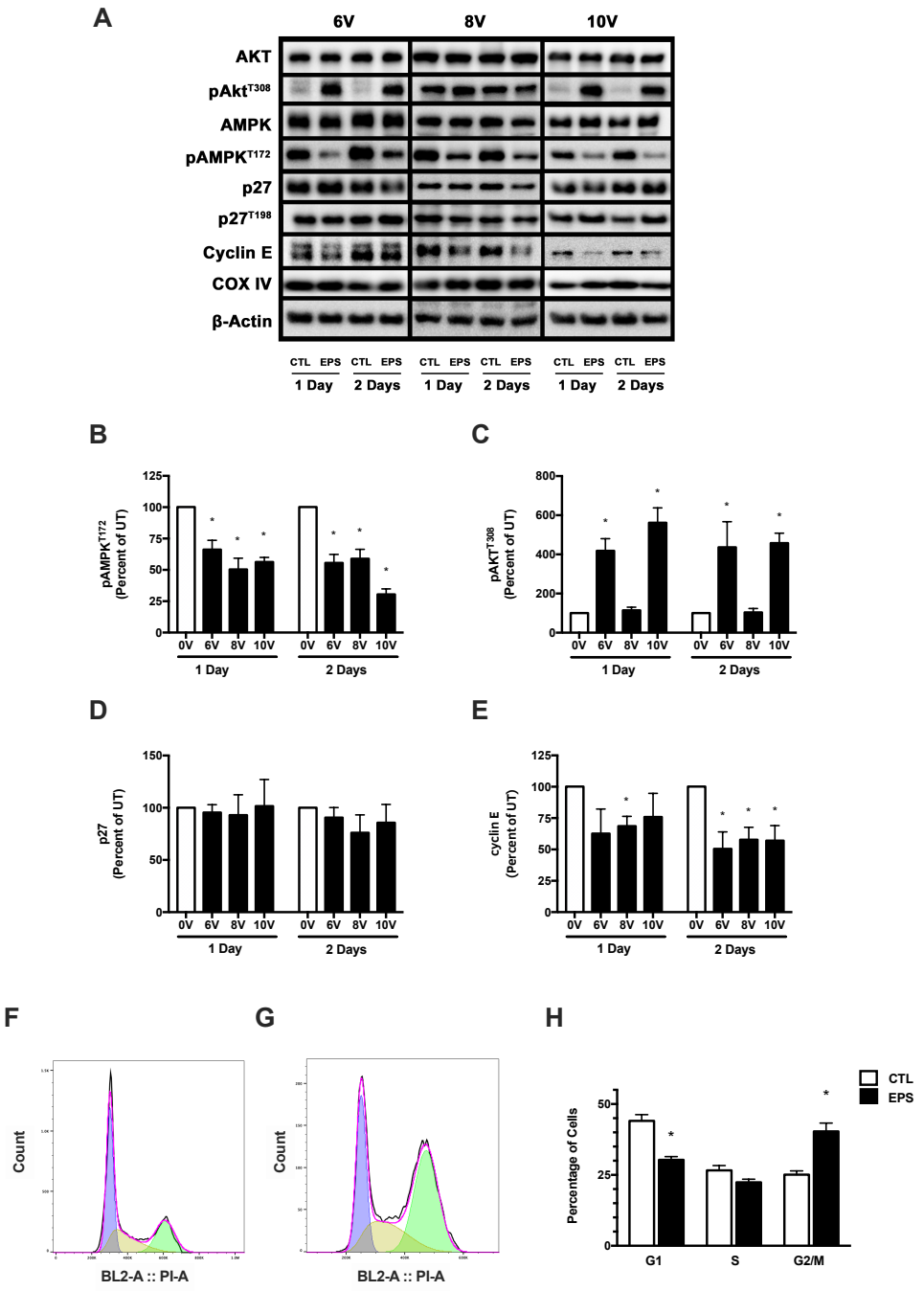

Figure 3. EPS does not cause a G_1 arrest but rather a G_2/M arrest. **(A)** Western blots of proteins implicated in G1/S phase signaling with exception to COX-IV a marker of mitochondria and β-actin which was used as the loading control. **(B-E)** protein expression relative to non-stimulated controls. Non-stimulated controls were standardized as 100%. All values are represented as the mean ± S.E.M.; * $p<0.05$ relative to non-stimulated (N = 3-8) **(F)** Histogram from FACS analysis of PI stained non-stimulated cells. **(G)** Histogram from FACS analysis of PI stained EPS conditioned cells. **(H)** Comparison of the proportion of cells within G_1, S and G_2/M between non-stimulated and EPS conditioned cells. All values are represented as the mean ± S.E.M.; * $p\leq0.05$ (N = 4)

Markers of autophagy are increased following EPS Given the distinct reduction in cell number following EPS we analyzed whether cells were entering non-necrotic cell death programs. Prior work on RMS cells showed that EPS elevated LC3 protein levels. Since there was no sub-G_1 population evident in EPS cells measured by FACS (Fig. 3G) initial experiments focused on LC3 and potential modes of cell death mediated by autophagic programming. We found that MCF7s exposed to EPS caused drastic increases in autophagy markers. EPS increased LC3-I up to 484% after 2 days (10V), an indication of autophagy induction (Fig. 4A, upper band, Fig. 4B). Upon autophagy induction LC3-I is converted to LC3-II (lower band). This conversion is essential for the membrane formation required to form mature autophagosomes. EPS drastically increased LC3-II at all voltages reaching a maximum of 746% following two days of EPS (Fig. 4C). LC3-I to LC3-II conversion rates (LC3-II/Total LC3) relative to controls were also elevated following EPS (Fig. 4D). During autophagy cytoplasmic contents are engulfed inside the autophagosome. This engulfment can, and commonly does include the ubiquitin cargo binding protein p62. EPS caused a large and rapid increase in p62 protein reaching levels that were 579% greater than those in non-stimulated cells after only 1 day of EPS (Fig. 4E).

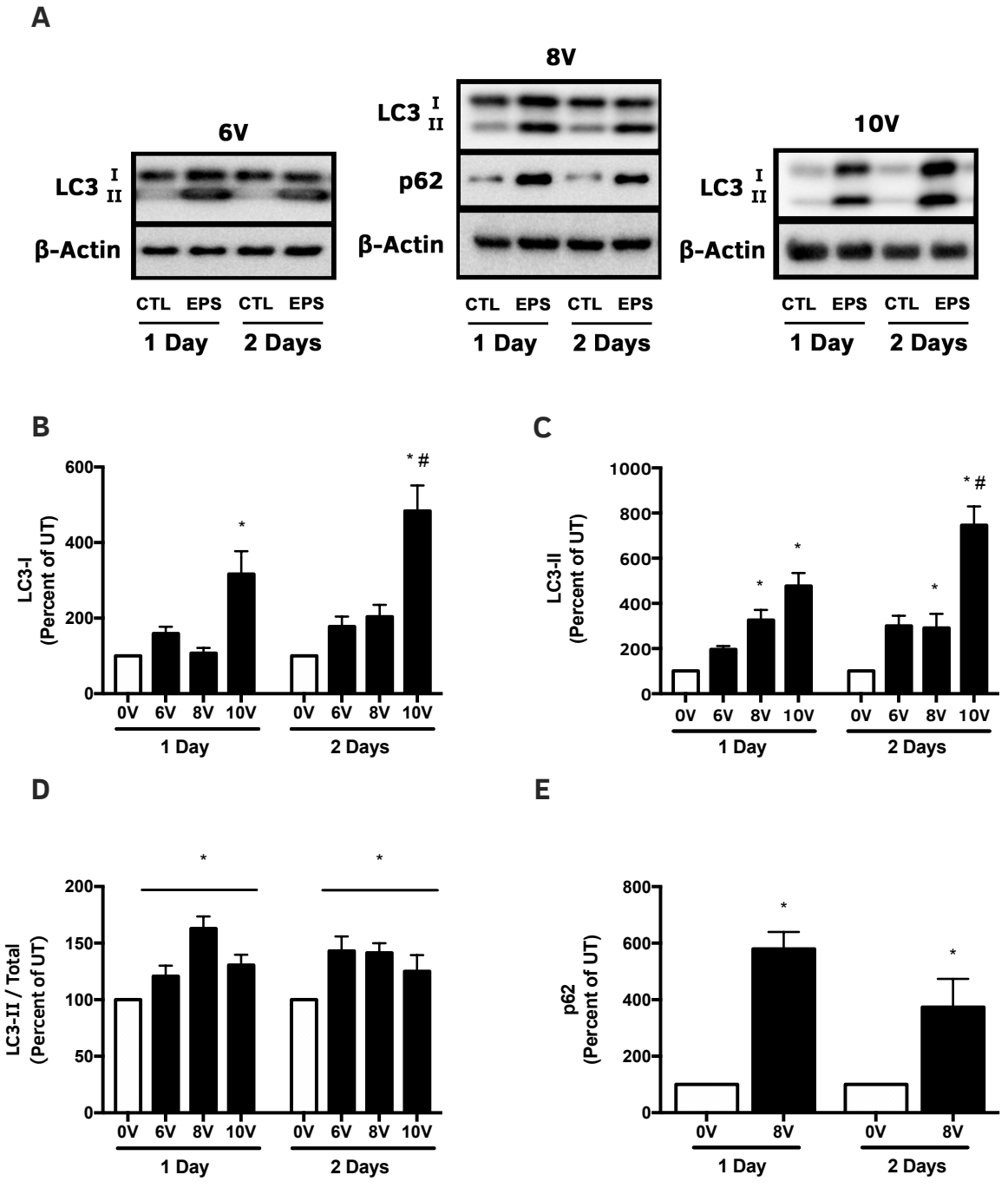

Figure 4. Markers of autophagy are increased following EPS. **(A)** Western blots of proteins implicated in autophagy with exception to β-actin which was used as the loading control. **(B-D)** protein expression relative to non-stimulated controls. Non-stimulated controls were standardized as 100%. All values are represented as the mean ± S.E.M.; * $p \leq 0.05$ relative to non-stimulated, # $p < 0.05$ relative to all voltages (N = 4-8).

EPS may inhibit late stage autophagy Elevations in both of the autophagy markers LC3-II and p62 suggested that an inhibition of late stage autophagy was occurring and causing a build-up of autophagosomes. The lysosomal inhibitor chloroquine (CQ), routinely used to assess autophagic flux, was used to assess the status of late stage autophagy. CQ inhibits autophagosome degradation by accumulating inside lysosomes and elevating the pH of the otherwise acidic organelle. This elevation inhibits pH dependent hydrolytic enzymes required for lysosomal degradation. This allowed us to compare autophagic flux in control and EPS cells and assess the contribution of lysosomal degradation to the increase in LC3-II and p62 observed with EPS. Initial dose response experiments showed that, as expected, CQ increased LC3-II proteins levels in non-stimulated cells at 15 μM and 20 μM attaining values that were 395% and 597% higher than those in DMSO treated cells, respectively (Fig. 5A-B). Surprisingly, CQ failed to increase p62 protein levels (Fig. 5C-D), potentially an indication that the autophagy pathway is altered in MCF7 cells. In addition, CQ had no effect on p62 protein levels in EPS cells (Fig. 5D). At 20 μM by comparing the CQ treated EPS group to the CQ treated control group we see that with reduced lysosome function the relative difference in LC3-II between EPS and controls is greatly decreased, attributed to the increase in LC3-II in non-stimulated CQ treated cells (Fig. 5E-F). In addition, an additive effect of EPS with 15 μM and 20 μM of CQ was also evident.

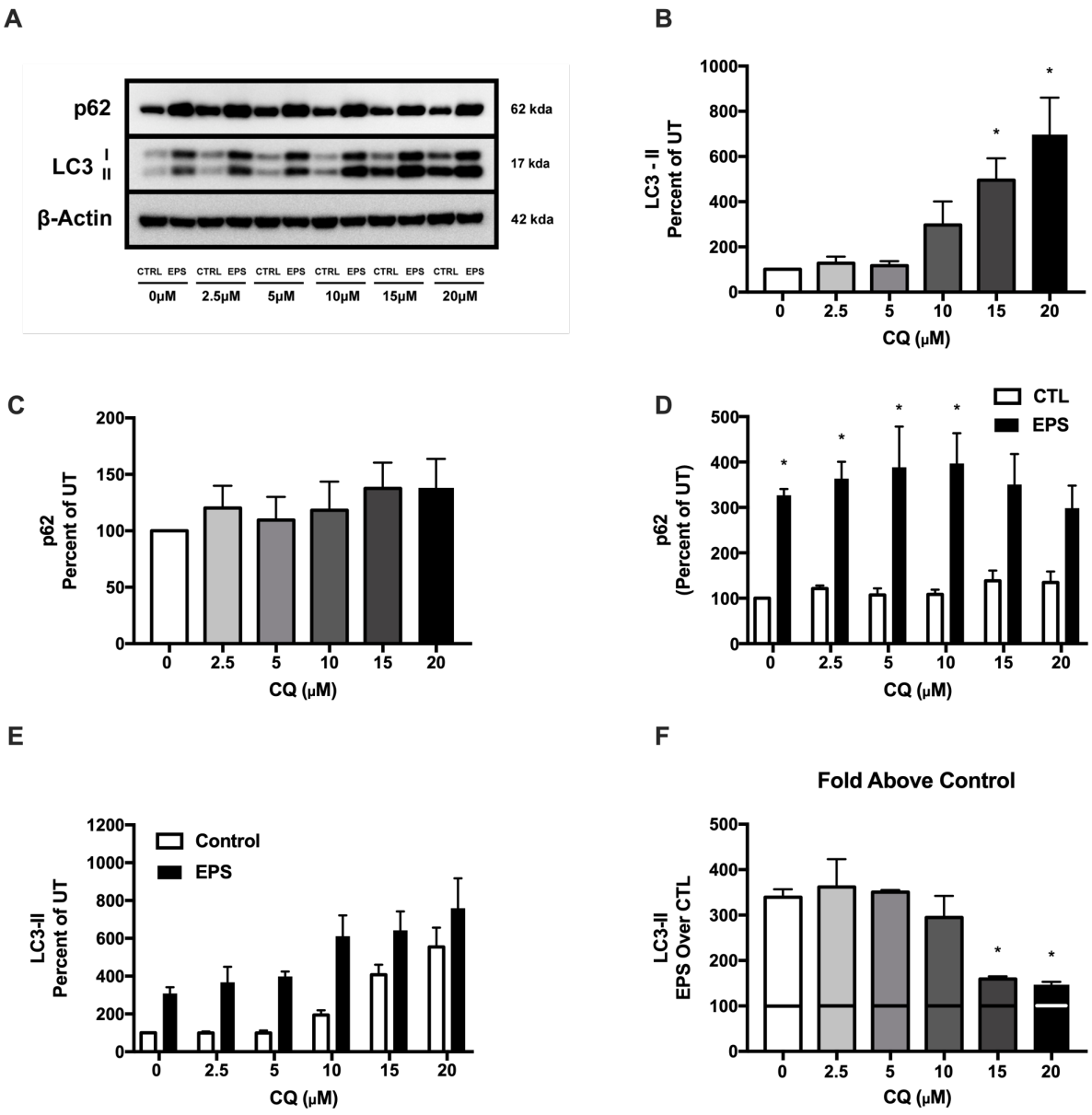

Figure 5. EPS may inhibit late stage autophagy. **(A)** Western blots. β-actin used as the loading control. **(B-C)** relative protein expression in response to increasing concentrations of CQ. All values are relative to the untreated control condition which was standardized as 100%. All values are represented as the mean ± S.E.M.; * $p<0.05$ relative to untreated (N = 5-6) **(D-E)** relative protein expression in response to increasing concentrations of CQ with or without EPS. All values are relative to the untreated control condition which was standardized as 100%. All values are represented as the mean ± S.E.M.; * $p<0.05$ relative to untreated (N = 4) **(F)** Relative fold above control between the EPS condition and its respective control. All values are relative to the respective control condition which was standardized as 100% and represented as a line within each bar. All values are represented as the mean ± S.E.M.; * $p\leq0.05$ relative to the untreated EPS condition (white bar) (N = 3)

EPS induced responses in MCF7 cells are Ca+2 dependent Given the importance of Ca^{2+} signaling in excitable cells, the intracellular Ca^{+2} chelator BAPTA-AM (BAPTA) was used to determine the role of Ca^{2+} in the response of MCF7 cells to EPS. BAPTA appeared to be highly effective in chelating Ca^{2+} as inhibitory effects were visible at concentrations as low as 4 μM. While non-stimulated cells exposed to 12 μM BATPA alone were able to proliferate and survive, all cells subjected to both EPS and BAPTA exhibited obvious cell death by 48 hours post EPS (Fig. 6B). Given that Ca^{2+} is known to activate AMPK, unexpectedly we found that BAPTA caused a dose-depended increase in $pAMPK^{T172}$ levels (Fig. 6C). In addition, Ca^{2+} chelation was able to completely abolish the EPS-induced decreases in $pAMPK^{T172}$. Furthermore, BAPTA treatment blunted the EPS-induced reduction in cyclin E protein levels at a concentration of 12 μM (Fig. 6D), thereby illustrating a role of Ca^{2+} in this response.

The most dramatic effects of EPS and BAPTA were evident for the autophagic proteins LC3-I and p62 (Fig. 4). Ca^{2+} chelation with BAPTA prevented the EPS-dependent increases in LC3-I and p62 protein levels in a dose-dependent manner (Fig. 7A-C). These Ca^{2+}-dependent effects were only observed in the adaptive response to EPS as BAPTA alone did not alter p62 or LC3 levels in non-stimulated control cells (Fig. 7B-D). This suggests that EPS requires Ca^{2+} for the elevations of p62 and LC3-I. Furthermore, BAPTA increased the conversion of LC3-I to LC3-II in response to EPS at 8 and 12 μM demonstrating 98% and 79% increases above controls, respectively (Fig. 7D). This suggests that EPS and Ca^{2+} plays a role in the processing of LC3 in MCF7 cells in response to EPS. Given that adding CQ did not affect p62 in non-stimulated control cells (Fig. 6B), CQ did not alter the p62 response to BAPTA and EPS, as expected. This further confirmed that the changes in p62 in response to EPS and BAPTA are independent of lysosome function.

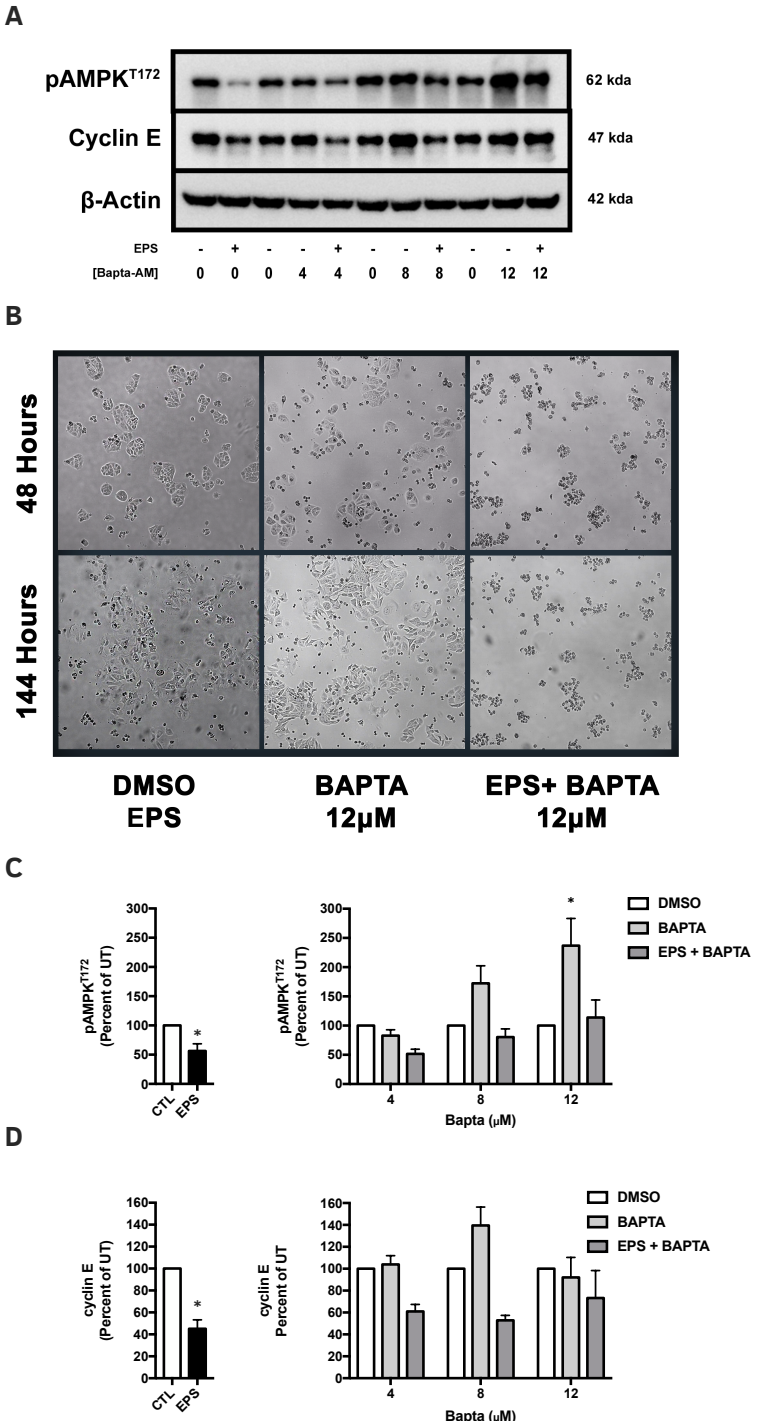

Figure 6. Ca^{+2} dependent responses of EPS: cell cycle regulatory proteins. **(A)** Western blots of proteins implicated in autophagy and G1/S phase signaling with exception to β-actin which was used as the loading control. **(B-C)** protein expression relative to parallel non-stimulated controls. Non-stimulated controls were standardized as 100%. All values are represented as the mean ± S.E.M.; * $p \leq 0.05$ relative to untreated (N = 3-5) **(D)** Representative brightfield microscopy images of EPS conditioned cells with or without 12 μM BAPTA treatment 48 hours following the onset of EPS.

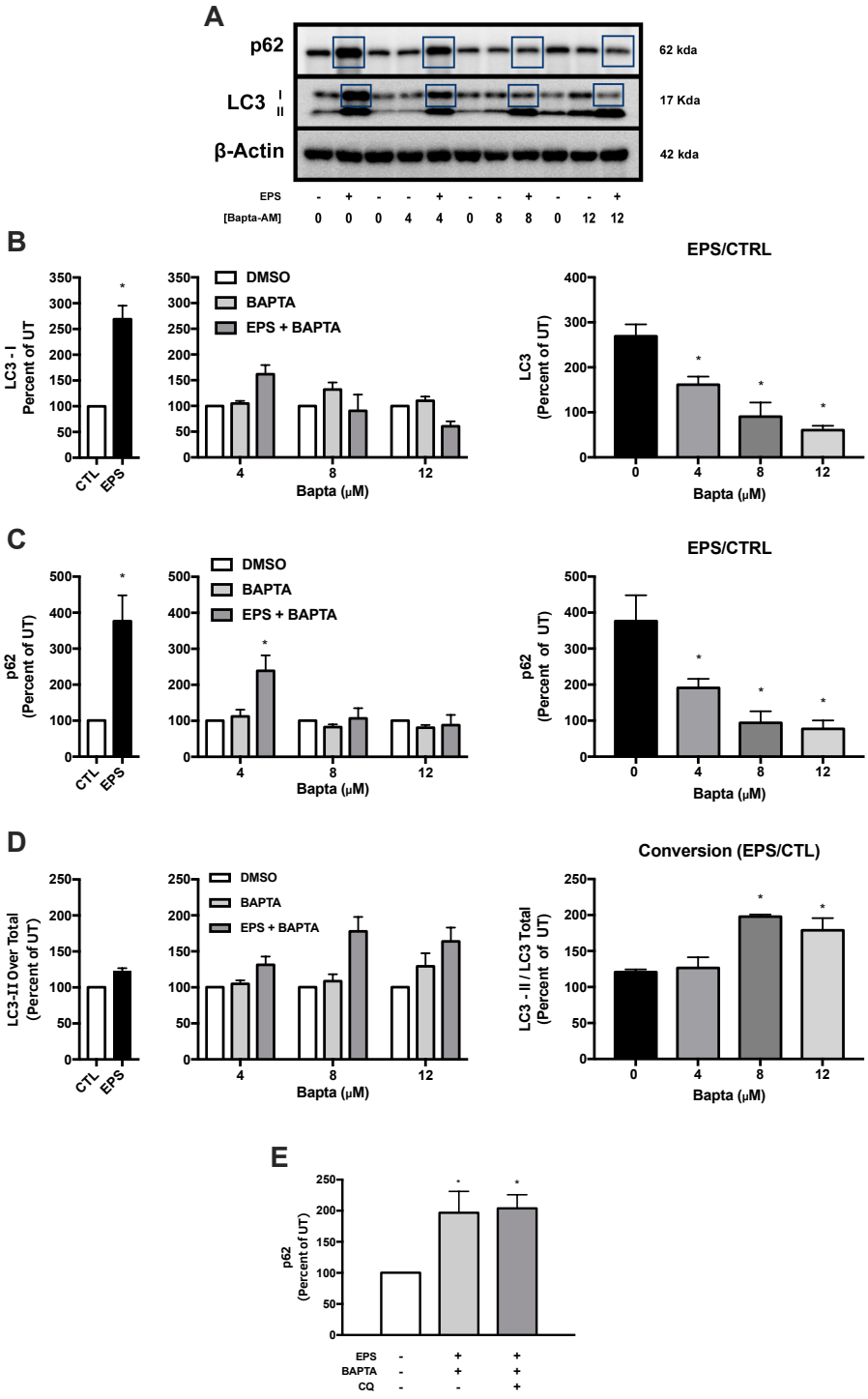

Figure 7. Ca^{+2} dependent responses of EPS: Autophagy regulatory proteins. **(A)** Western blots of proteins implicated in autophagy signaling with exception to β-actin which was used as the loading control. **(B-D)** protein expression relative to parallel non-stimulated controls. Non-stimulated controls were standardized as 100%. All values are represented as the mean ± S.E.M.; * p≤0.05 relative to untreated (N = 4-5) **(E)** Relative p62 expression in response to EPS, CQ and BAPTA. No effect of CQ (20 μM) on blunted EPS elevation of p62 in the presence of BAPTA (6 μM) (N = 4).

EPS may induce G2/M arrest via p21 independent of p53 p21 induction has been more recently associated with autophagic pathways, specifically via Beclin-1 mediated induction[182], as well as cell cycle arrest typically associated with p53[60]. Although Beclin-1 was unaffected by EPS (data not shown), we saw 2-fold increases in p21 (Fig. 8A-B). Despite these increases in p21, there were no effects of EPS evident on p53 protein levels (Fig. 8C). This suggests that the increase in p21 may not be due to increased transcriptional activation by p53. Additionally, this induction of p21 is unaffected by BAPTA or CQ (Fig. 8B). Given that EPS induces a G_2/M phase arrest we decided to measure the activation of CDC2 through phosphorylation on threonine 161 ($CDC2^{T161}$), a necessary step for G_2 progression which has been shown to be mediated through a p21 dependent mechanism[62] (Fig. 8D). There were no observable effects of EPS, BAPTA, or CQ on the protein levels of $CDC2^{T161}$ (Fig. 8E).

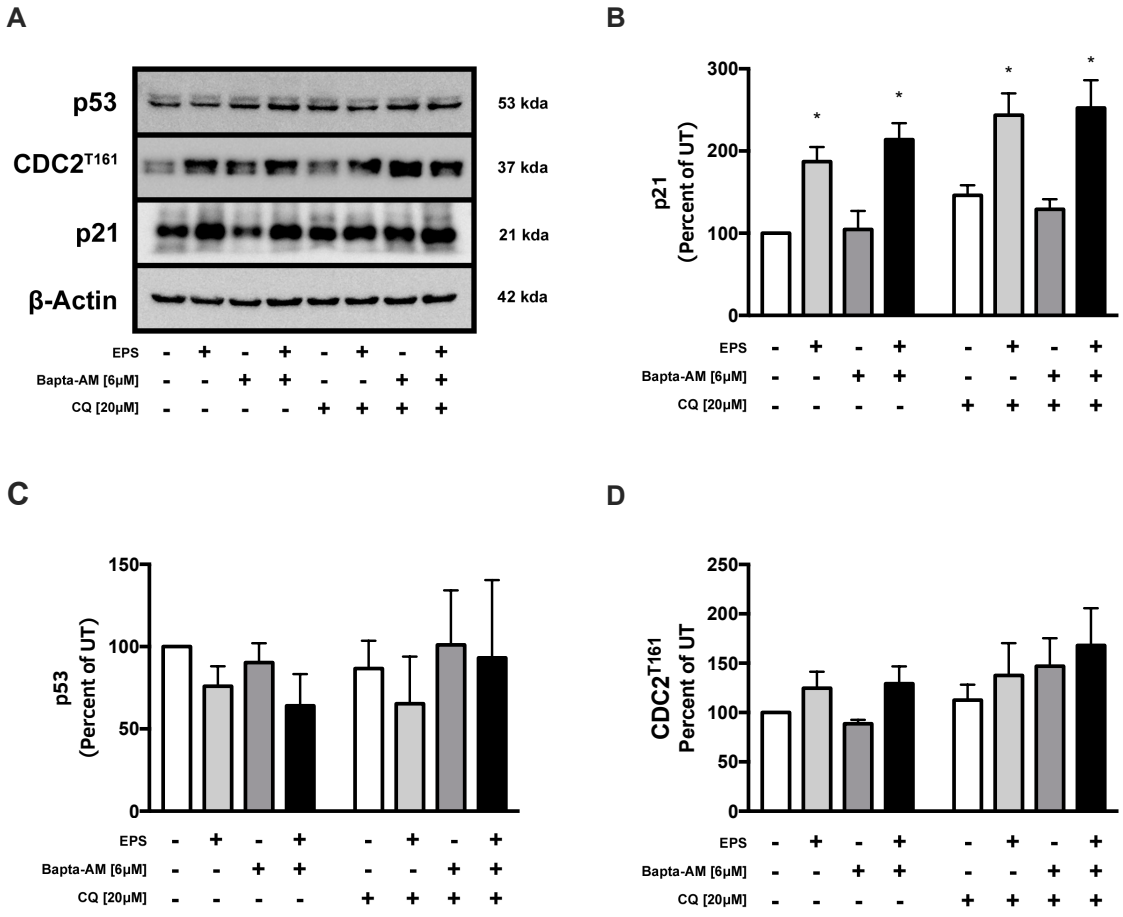

Figure 8. EPS may induce G_2/M arrest via p21 independent of p53. **(A)** Western blots of proteins implicated in G_2/M phase signaling with exception to β-actin which was used as the loading control. **(B-D)** protein expression relative to the non-stimulated control. Non-stimulated controls were standardized as 100%. All values are represented as the mean ± S.E.M.; * $p \leq 0.05$ relative to untreated (N = 4-6)

7.5 Discussion

The application of EPS is primarily understood as a mechanism that provokes membrane depolarizations in excitable cell types[154]. However, processes beyond membrane depolarizations within excitable cell types are also affected through the application of electrical charge[183,184]. Furthermore, our lab has previously shown both anti-proliferative as well as autophagic effects following the application of low-frequency alternating currents to non-excitable myoblast and cancerous RMS cells[166]. These studies lay the foundation for our current study where we show that the EPS of MCF7 cells induces novel functional and molecular events leading to cell cycle arrest, autophagy reprograming and ultimately the induction of proliferative senescence.

The morphology and reductions in cell numbers following EPS, led us to examine the role of non-programmed cell death through necrosis. Measurements of LDH within the media affirmed that EPS did not elicit significant cytoplasmic damage or necrosis. Furthermore, total LDH measurements following cell lysis confirmed that the reductions in cell number following EPS were a result of a decline in cell proliferation. EPS did not seem to activate apoptosis however our observations did show drastic alterations in autophagy status. Additionally, there was a clear indication that EPS leads to senescence. Cellular senescence is more commonly associated with an initial G_1 arrest[177–180] however, our results indicated that EPS causes a G_2/M phase arrest. Within the M phase spindle formation and chromosome separation rely heavily on charge dependent processes[185,186]. In agreement with this, mitotic catastrophe has been observed in glioblastoma cells following the application of high-frequency alternating currents[183,187]. Cells undergoing mitosis during and or following EPS may be unable to complete mitotic division leading to the stall in mitosis. A stall within M phase agrees with the cell rounding and double nucleated cells seen following EPS (Fig. 2). That said, most of the distinct morphological characteristics were manifested 48-72 hours following the onset of EPS suggesting that EPS sets in motion a programmed response in MCF7

cells. Within this timeframe EPS cells enlarge and flatten greatly compared to non-stimulated controls. This morphology matched that associated with senescence. Furthermore, after re-plating 48 hours following the initiation of EPS, no appreciable cell growth was observed and over the subsequent 4 days monitored, regardless of re-plating and the replenishment of media, cell proliferation was absent. Therefore, EPS elicited a permanent arrest and loss of re-proliferative potential both of which are characteristic of senescence. Thus, the enlarged and flattened morphology, proliferative arrest and increased positive SA-ß-gal staining indicate strongly that the proliferative failure seen in cells initiated by EPS does indeed lead to cellular senescence. Specific prerequisites to cellular senescence have been shown to require cell enlargement and cell cycle arrest in a process that has been recently termed geroconversion[188,189]. This process involves a loss of re-proliferative potential and the formation of a hypertrophic phenotype. The associated hypertrophic phenotype (enlarged and flattened) and loss of re-proliferative potential has been shown to be driven by mTOR activity during arrest[188]. EPS induces robust increases in AKT activation, which strongly indicates the downstream activation of mTOR that could drive the hypertrophic phenotype observed in EPS cells. Furthermore, it has been shown that in some cell types AMPK suppresses geroconversion in an effort to maintain quiescence through mTOR inhibtion[190]. Here we show substantial reductions in AMPK activation following EPS. This further supports a mechanism of mTOR activation leading to the hypertrophic phenotype observed following EPS.

To attain senescence the cell also requires an initial arrest. One of the main biomarkers in cell cycle arrest leading to senescence is p21 induction[191]. Here we show sustained two-fold increases in p21 following only a single bout of EPS. In agreement with results in RMS cells and the protein expression patterns observed in this study (no change in p27 and decreases in cyclin E protein levels) cells subjected to EPS demonstrated a phenotype suggestive of a transition through G_1/S. Additionally, AMPK activation has been shown to arrest MCF7 cells in G_1[181] thus the EPS dependent reductions in AMPK activation further supported the idea that EPS did not elicit a G_1 arrest and thus further suggested a G_2/M arrest prior to senescence. FACs analysis confirmed this theory showing an accumulation of cells in G_2/M following EPS.

That said, more work is needed to fully elucidate the precise location and mechanism of EPS induced cell cycle arrest within G_2/M.

Autophagy reprogramming in response to EPS was initially observed in experiments using RMS cells with elevations in the autophagy protein marker LC3[166]. Here we confirmed these results, with EPS yielding dose dependent elevations in total LC3 protein content across voltages with 3-fold increases in LC3-I and 7-fold increases in LC3-II, respectively. LC3-I conversion to LC3-II is a critical event in autophagosome formation and is a commonly used marker for this process[104]. However, elevations in LC3-II and autophagosome content can be attributed to a complex balance of multiple synthesis and degradation events and are not solely indicative of changes in autophagy flux. By using the lysosomal inhibitor CQ, we were able to assess the status of autophagy flux following EPS. In response to EPS the pool of LC3-II rose 2.4-fold above non-stimulated controls. This relative 2.4-fold increase in LC3-II was reduced with the inhibition of the lysosome. The compression of the relative difference between control and EPS conditioned cells in the presence of CQ indicates that the pool of LC3-II formed in response to EPS may be the result of a similar inhibitory event within the autophagy pathway. However, EPS consistently showed an additive effect to pharmacological lysosome inhibition with a consistent absolute difference of approximately 200% across all CQ concentrations. Higher concentrations of CQ are required to ensure complete inhibition of the lysosome in order to gain a clearer understanding of specifically how EPS-dependent effects on autophagy flux are elicited.

Autophagy has been shown to be upregulated within chemotherapy resistant tumors[121], furthermore, the late stage inhibition of autophagy through the use of CQ has been shown to improve patient outcomes by eliminating the chemotherapy resistance conferred through autophagy[116,117,122–125]. Therefore, EPS may then serve a similar purpose via a localized stimulus, which can potentially mitigate the systemic issues associated with drug treatments such as CQ. Additionally, we show an association of proliferative failure and autophagy inhibition that agrees with prior observation of various breast cancer cell lines exposed to

silencing mRNA of autophagy regulating genes[119]. Interestingly, most cancerous cell lines showed more drastic reductions in cell number compared to non-cancerous breast cell lines suggesting a potential cancer-specific targeting effect of EPS.

Surprisingly, the cargo-sequestering protein p62, which can be localized inside autophagosomes, responded to EPS but not CQ in MCF7 cells. The lack of response of p62 to CQ implies that elevations in p62 following EPS are independent of the autophagosome, supporting the idea that p62 and/or autophagy regulation is somehow altered in MCF7 cells. It is worth noting that p62 has multiple functions outside of autophagosomes including the regulation of CDC2[71,193,194], influence within proteostasis[108,195] and an additional mechanism of mTORC1[196] activation. The elevation of p62 in the EPS response may then serve any number of these additional functional roles.

The established effects of low frequency electrical current point to membrane depolarization events leading to the release of calcium stores into the cytoplasm[155]. Although MCF7 cells lack the vast sarcoplasmic calcium storage capacity of myotubes, they still do contain substantial stores, the most notable of which being within the endoplasmic reticulum[197]. Thus, we assessed the role of Ca^{2+} using the membrane permeable calcium chelator BAPTA-AM. MCF7 cells were highly sensitive to BAPTA with effects observed at concentrations as low as 4 μM. Following EPS, BAPTA did not mitigate the anti-proliferative effects of EPS but did cause distinct morphological changes associated with cell death which were most prominent at 12 μM BAPTA. While non-stimulated cells exposed to 12 μM BAPTA alone were able to proliferate and survive, all cells subjected to both EPS and BAPTA exhibited obvious cell death by 48 hrs. Interestingly, we also found a dose dependent decline in the EPS dependent elevations of LC3-I and p62. No such effect was seen in non-stimulated cells exposed to BAPTA alone suggesting that Ca^{2+} is involved in the adaptive but not baseline regulation of LC3-I and p62. The lack of effect of co-treatment with BAPTA and CQ further confirmed that the p62 protein response is autophagosome and lysosome independent. Previous studies have shown that the accumulation of protein aggregates containing p62 and LC3-I form

under conditions of autophagic impairment that are implicated in cell survival and senscence[105,171–173,198], a response similar to that described herein following EPS. Our results suggest that Ca^{2+} EPS-dependent p62 and LC3-I elevations may be associated with a pro-survival response since EPS in the presence of BAPTA elicits massive cell death. However, the precise mechanism of action requires further study to completely map out the potential roles of p62 and LC3-I in cell survival. With EPS, we observed an overall increase in conversion rates between LC3-I and LC3-II which were further increased following calcium chelation. CQ experiments revealed that LC3-II looks to be accumulating due to an inhibition of late stage autophagic degradation however the additive effect of EPS in the presence of CQ may be contributed to EPS-dependent elevations in LC3 conversion. Calcium induced events following EPS look to elevate LC3-I in an autophagy independent manner which may be masking the elevation in conversion rate under EPS only conditions (Fig. 9).

Overall, we show that EPS sets in motion a programmed proliferative failure leading to senescence which requires cell cycle arrest in G_2/M, in what may be a p53 independent manner. We also show that EPS drastically increases the autophagy markers LC3 and p62 in a mechanism that is mediated in a calcium-dependent fashion, while also inhibiting the late stages of autophagy through a calcium-independent mechanism. The simultaneous reprogramming of autophagy and proliferative pathways makes the EPS of MCF7 cells an interesting model of study in the intersection of these two emerging mechanisms. Both mTOR signaling and proteostasis serve as particular points of interest for future investigation as they may serve as a central point of regulation between autophagy, growth signaling, and cell survival in senescent programming. These results also provide a framework for the application of EPS to other cancer cell types, with the ultimate goal of developing a novel localized cancer therapy that can both induce permanent arrest while sensitizing tumors to chemotherapy.

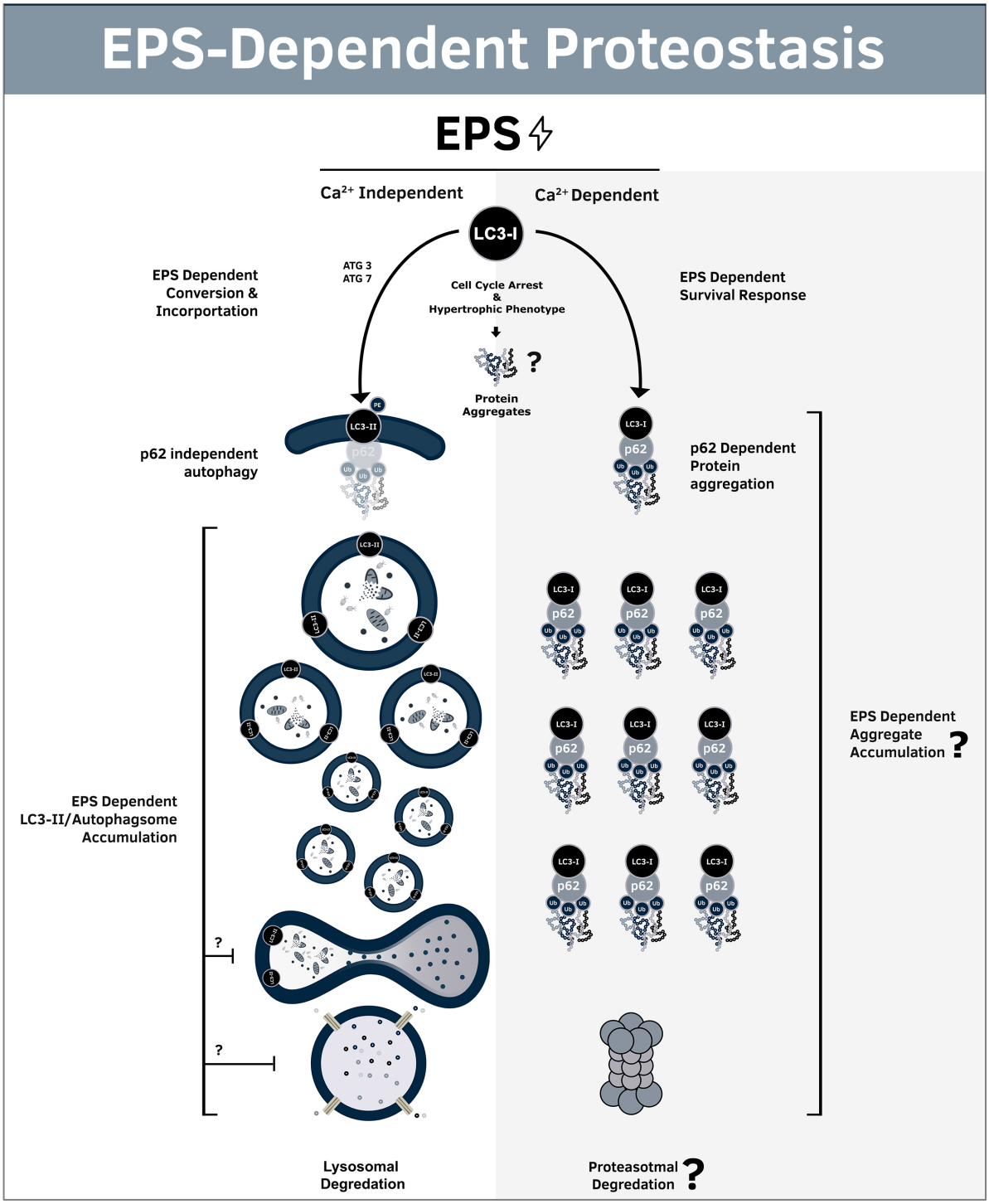

Figure 9. Working Model of EPS-Dependent Proteostasis. EPS elicited both calcium dependent and Ca^{2+} independent effects. Ca^{2+} dependent effects included the elevation of LC3-I and p62. In the presence of Ca^{2+} chelation LC3-I and p62 elevations are abolished. Our results are in line with the current literature that details p62 dependent LC3-I binding, independent of autophagosomes within protein aggregates, in the presence of autophagic impairment[105,170–172]. Ca^{2+} independent EPS effects looks to include an impairment of the late stages of autophagy leading to the accumulation of autophagosome associated LC3-II. Excessive protein aggregate formation may be the result of cell cycle arrest in the presence of a hypertrophic phenotype, both of which have been shown as critical events in cellular senescence[187]. (?) indicate areas of interest for future investigation.

8.0 Limitations & Future Directions

Cell survival and the integrated response Our study indicates that in response to EPS, p62 may play roles outside of autophagosomes. The elevations of p62 in response to EPS may also serve as a survival mechanism. This hypothesis is based off the distinct elevations in cell death seen in cells treated with EPS and BAPTA; a treatment condition which concomitantly abolishes the p62 EPS response. Furthermore, the silencing of p62 has been shown to cause conditions of cell death in various cell types[105,198]. The mechanism of cell death under EPS and BAPTA is still unknown however an assessment of caspase cleavage products via western blotting are a good initial point of assessment for future research. It is becoming increasingly clear that mTOR and p62 are likely to have a critical impact on the functional effects observed here. mTOR has been shown to be activated in response to p62[196] and AKT[89] elevations, both of which are involved in cell survival, thus mTOR serves as a central point of regulation. mTOR not only links cell growth, survival and senescence but it also has localized effects at the lysosomal membrane with its co-activator Rheb[90,199]. Thus, the lysosome as well as its function serves as another point of contact between autophagy, survival and senescent programming that may be linked through mTOR.

Additional experiments of interest include the use of a proteasome inhibitor to assess if the regulation of p62 following EPS as well as EPS and BAPTA-AM is mediated though the ubiquitin proteasome pathway. Lastly, the source of calcium induced events is an area of interest. The largest calcium store in MCF7s is the ER. ER calcium channel inhibitors such as thapsigargin serve as an interesting experiment to initially assess the role of the ER in the calcium induced events of EPS.

Cell Types and *In-Vivo* Studies – MCF7s contain some unique characteristics in both autophagy and calcium signaling regulation. MCF7s contain a single chromosome deletion of beclin-1[200] creating further inquiry into whether MCF7s contain any other dysfunctions in autophagy pathways. Furthermore, the sarco-/endoplasmic reticulum Ca^{2+}-ATPase (SERCA3), a critical regulator of the influx of calcium into the ER is greatly reduced in MCF7s suggesting the calcium handling capabilities at the level of the ER may have an attributable contribution to the autophagy effects seen in response to EPS[201–203]. A previous study compares the growth rates of both normal and cancerous breast cell lines with the added inhibition of autophagy[120]. Interestingly, non-cancerous MCF10As had relatively unaffected proliferation rate compared to MCF7s. Furthermore, MCF7s in comparison to other breast cancer cell lines were far less affected by autophagy reprogramming. Thus, whether or not the responses of EPS outlined here are unique to MCF7s or carries over to other non-excitable cell lines such as non-cancerous MCF-10As and other cancerous cell types such as prostate LNCaP cells is an area of interest for future study. Additionally, whether or not these effects can be carried over into 3D cultures and *in-vivo* models is another area of interest. The groundwork for such a study has yet to be developed. Questions as to how EPS can be administrated *in-vivo* and the effects of electrical impedance while stimulating a three-dimensional tumor remain to be answered. That said, *in-vivo* electrical stimulation has been applied and well documented within a live rat model to study phenotypic alterations in response to chronic contractile activity in skeletal muscle[204]. Additionally, developing and subjecting chemo resistant cell lines to EPS is another avenue of interest for future studies. The effects of late stage autophagy inhibition in response to EPS provide an exciting avenue for the potential ability of EPS to sensitize cells to chemotherapy as it has been shown that tumors are able to induce autophagy as a mechanism to resist chemotherapy. Future experiments of interest would combine EPS and chemotherapy with the hypothesis that cells exposed to EPS will be more sensitive to lower doses of chemotherapy than non-stimulated cells.

The Mechanism of Action and EPS Conditioned Media – The exact EPS mechanism of action is yet to be understood. At the conclusion of experiments, it was brought to our attention the contribution of EPS conditioned media versus the effects of EPS alone[205]. Following EPS, it became noticeable that the media was discolored relative to controls. The interest in the effects of EPS on the media conditions became fully apparent when measuring background LDH values. Our media is supplemented with FBS that contains LDH. Naturally this background LDH needed to be measured and subtracted. Due to the discoloration in the EPS conditioned media we decided measure two backgrounds, one of the media and the other of media stimulated in the absence of cells. The LDH values from the conditioned media stimulated without cells showed an approximant 50% reduction in LDH content on each stimulation. This further brought into question the contribution of the potential denaturation and or cytotoxicity of the media on the cells following EPS. Initial experiments were done using the established protocol of a 4-hour EPS at 8V with a 20-hour rest period however, this time the media was replaced immediately following EPS. Furthermore, a small subset of experiments was performed where media alone was stimulated and then added to cell cultures. These cells were monitored for 72 hours and collected and analyzed via western blotting. Within the EPS conditioned media group (cells cultured in stimulated media), the 'hallmarks of EPS', reduced growth rate and enlarged flattened morphology were present. The EPS conditioned media group also saw elevations in LC3 and p62 as well as a reduction in pAMPK levels relative to untreated cells. Additionally, the previously observed effects were reduced with the replacement of media following EPS. These initial experiments indicate a definite effect of EPS conditioned media however, cell exposed to EPS conditioned media seem to recover whereas cells exposed to EPS do not. This has obvious implications on the practical applications of EPS as denaturation and or cytotoxicity of the media due to EPS can be attributed as an artifact of culture conditions that may not have relevance *in-vivo*. That said, studies on the effects of EPS conditioned media requires further study to clearly dissociate the contribution of EPS mediated effects from EPS conditioned media effects and elucidate the mechanism of action occurring with EPS.

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