Electrical Stimulation of Rhabdomyosarcoma Cells Induces Cell Cycle Arrest and Autophagy

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Abstract

Rhabdomyosarcoma is a family of rare juvenile cancers that express molecular factors implicated in myogenic differentiation. Cell cycle arrest is absolutely necessary for muscle maturation, while abnormal cell cycle and cell death programs are hallmarks of cancer onset and progression. The cancerous rhabdomyosarcoma cells continue to uncontrollably proliferate in part due to deficiencies in the myogenic program. Electrical stimulation is known to alter the phenotype of myogenic cells, such changes include cell cycle arrest and enhanced myoblast differentiation. Since rhabdomyosarcoma tumors are muscle-like this thesis looks at the use electrical stimulation towards these cells in an attempt to induce differentiation and prevent growth. The results indicate that the response elicited in rhabdomyosarcoma cells following electrical stimulation is different from the one observed in myoblasts, however cell cycle arrest and cell death through autophagy contribute to a large reduction in the number of cells, which represents a potential therapeutic approach.

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List of Abbreviations

ADP	Adenosine diphosphate
AIF	Apoptosis inducing factor
AMP	Adenosine monophosphate
AMPK	AMP activated protein kinase
APC	Anaphase promoting complex
aRMS	Alveolar rhabdomyosarcoma
ATM	Ataxia telangiectasia mutated
ATP	Adenosine triphosphate
ATR	ATM and rad3 related
BH3	Bcl homology 3
bHLH	basic helix-loop-helix
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
CTL	Control
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DSB	Double strand break

ERK	Extracellular signal regulated kinase
eRMS	Embryonal rhabdomyosarcoma
ES	Electrical stimulation
FACS	Fluorescence activated cell sorting
FGF	Fibroblast growth factor
FGFR	Fibroblast growth factor receptor
Fkhr	Forkhead
G_0	Gap 0 phase
G_1	Gap 1 phase
G_2	Gap 2 phase
GSK	Glycogen synthase kinase
HGF	Hepatocyte growth factor
HRP	Horseradish peroxidase
IAP	Inhibitor of apoptosis
IGF	Insulin-like growth factor
IGFR	Insulin-like growth factor receptor
INK	Inhibitor of CDK4
LFS	Li-fraumeni syndrome
LIF	Leukemia inhibitory factor
M	Mitosis
MAPK	Mitogen activated protein kinase
MCK	Muscle creatine kinase
MEF	Myocyte enhancer factor

MEK	Mitogen/extracellular signal-regulated kinase
MOMP	Mitochondrial outer membrane permeabilization
MRF	Myogenic regulatory factor
mTOR	Mammalian target of rapamycin
mtPTP	Mitochondrial permeability transition pore
Myf	Myogenic factor
NFAT	Nuclear factor of activated T-cells
PAX	Paired box
PDGFR	Platelet-derived growth factor receptor
PE	Phosphatidylethanolamine
PI	Propidium iodide
PI3k	Phosphatidylinositol-4,5-bisphosphate 3-kinase
PKB	Protein kinase B
PVDF	Polyvinylidene difluoride
R point	Restriction point
Rb	Retinoblastoma
RMS	Rhabdomyosarcoma
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RTK	Receptor tyrosine kinase
S	Synthesis phase
SAC	Spindle assembly checkpoint
SDS	Sodium dodecyl sulphate

SEM	Standard error of the mean
Shh	Sonic hedgehog
ssDNA	Single stranded DNA
STM	Stimulated
TGFβ	Transforming growth factor beta

Review of Literature

1. Mammalian Cell Cycle

During cell division the cell goes through a series of distinct and tightly regulated stages during which it replicates its DNA and then divides into two identical daughter cells (reviewed in (1)). Upon entering the cell cycle either following the completion of the previous round of division or from a quiescent state termed G_0, cells enter into the gap 1 (G_1) stage where they integrate all the extrinsic and intrinsic inputs and ready themselves for transition into S (synthesis) phase. Once in S phase, the cell initiates the replication of all chromosomal DNA. With two copies of the original DNA content the cell then enters the gap 2 (G_2) phase where it ensures integrity of the genetic material and chromosomes, and prepares for Mitosis. G_1, S and G_2 together are referred to as interphase. M phase, or Mitosis, is the final step in the process. This is when the cell partitions the chromosomes and undergoes cytokinesis, dividing all cellular contents into two new identical daughter cells (1)(See Figure 1).

Progression through the cell cycle is controlled by a family of proteins known as cyclin-dependent kinases (CDKs) (reviewed in (2)). CDK activity is reliant on their interaction with a class of proteins referred to as cyclins. A number of cyclin/CDK complexes regulate the various stages of the cell cycle, each playing a specific role. CDK4/CDK6 complexes regulate the G_1 stage, while CDK2 and its cyclins are responsible for G_1/S transition and S phase (2). CDK1 activity is actuated throughout the G_2 phase and regulates the initiation of Mitosis (2). CDK7 is thought to participate in the activation of the other cyclin/CDK complexes (2). Knock-out studies of the cyclins, and their corresponding CDKs, indicate that the remaining proteins are often able to compensate for the missing ones (3). Reports claim that in most cases CDK1 is sufficient for

the full completion of the cell cycle when all other interphase CDKs are absent (4). CDK1, as well as Cyclin A2 and B1, appear to be indispensable for the cell cycle process (4).

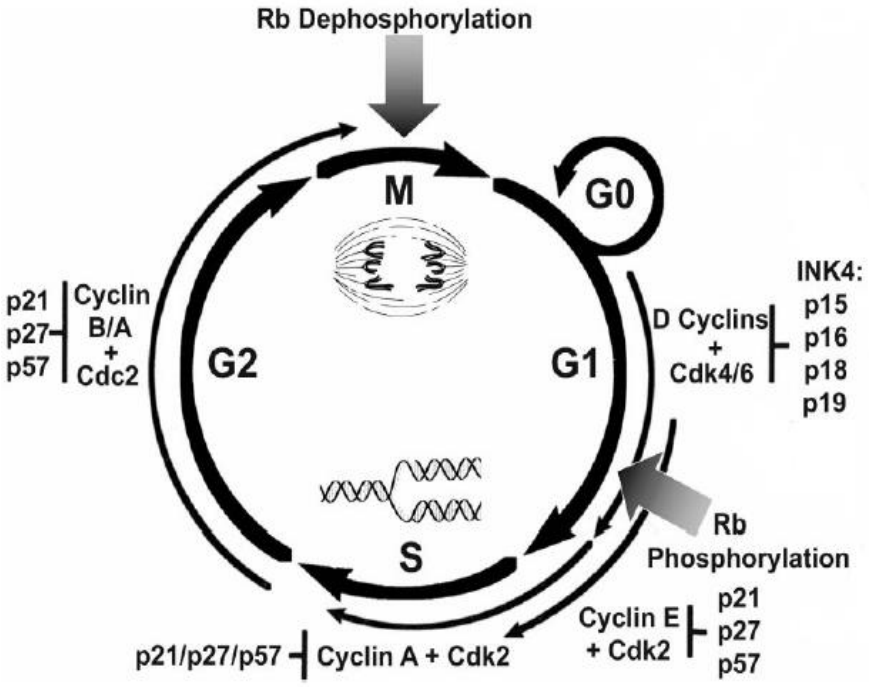

Figure 1. The cell cycle consists of 4 stages: G1, S, G2, and M. The transitions from one stage to the other are regulated by the cyclin/CDK complexes. INK4 and CIP/KIP proteins inhibit the cyclin/CDK complexes to allow strict control of cell cycle progress. Modified from Donovan J, Slingerland J. Transforming growth factor-ß and breast cancer: Cell cycle arrest by transforming growth factor-ß and its disruption in cancer. Breast Cancer Res. 2000;2(2):116-24.

Throughout the cell cycle CDK protein levels tend to remain relatively constant. Thus, the major forms for their regulation are protein-protein interaction and post-translational modifications (5). A number of mechanisms are responsible for the control of CDK activation. One of these is the binding of the cyclin proteins to the appropriate CDK. Cyclin protein levels oscillate throughout the cell cycle, and their interaction with the CDKs is required for the activity of the complex (5). The changes in the protein levels of the cyclins are dependent upon the balance between synthesis and ubiquitin mediated proteolysis (5). Cyclin D expression is

induced during G_1 downstream of mitogenic signals. As cells near the G_1/S transition cyclin E expression is activated by E2F transcription factors (3). Cyclin A is synthesized as cells are replicating their DNA material and it regulates the exit from S phase (3). In the G_2 phase, Cyclin A is degraded while Cyclin B is synthesized, the latter is responsible for the transition into mitosis. Prior to anaphase the Cyclin B is degraded by the anaphase promoting complex (APC) which allows the daughter cells to split (2). Cyclin protein levels and activities of the CDK complexes are subjects to strict and time dependent oscillations, which are necessary to prevent an aberrant cell cycle. Excessive levels of cyclins and CDKs drive the oncogenic transformation observed in many cancers (6)(see Cancer section for further discussion).

Additional mechanisms that regulate CDK activation include a group of proteins, which consists of two families known together as CKIs (cyclin-dependent kinase inhibitors), which prevent the catalytic activity of the cyclin/CDK complexes through direct binding. The INK4 family of proteins inhibits specifically the CDK4/6 complexes, while the CIP/KIP proteins (p21, p27 and p57) inhibit most CDKs (5). Also, two phosphorylation sites also regulate the activity of the cyclin/CDK complexes. The kinase Wee1 phosphorylates the CDK protein near its amino terminus and this modification is inhibitory to the activity of the complex. The phosphatase Cdc25 removes this phosphate group, thus promoting CDK activity (5). Another phosphorylation required for the optimal activity of a cyclin/CDK complex is mediated by CDK activating kinase (CAK), which consists of CDK7 bound to Cyclin H (5). Altogether, these four mechanisms allow for precise regulation of progression through the cell cycle.

Cells integrate various internal and external cues in order to gauge their progress and readiness throughout the various stages of the cell cycle. Growth factors promote cell cycle entry, and when withdrawn lead to cell cycle arrest before the onset of S phase (1). Most of the cell cycle regulation during the G_1 phase and G_1/S transition converges on Rb interaction with

E2Fs (7). E2Fs, a family of transcription factors, control many of the genes responsible for the replication of cellular DNA (8). Those transcription factors are bound and inhibited by the retinoblastoma protein (Rb), as well as Rb related proteins p107 and p130 (9). The interphase CDK complexes phosphorylate Rb at multiple residues, alleviating its inhibitory actions towards E2Fs (2). Once a cell begins the DNA replication process it is irreversibly committed to a round of replication and must complete the cell cycle to preserve its integrity. Errors in vital areas such as DNA replication and chromatin assembly among others will result in abrogation of the subsequent cell cycle steps, prompting damage repairs or cell death (10).

1.1 G_1/S Transition

The G_1 phase is driven by Cyclin D/CKD4/6 complexes, while G_1/S transition is completed by Cyclin E/CDK2 (7)(See Figure 2). In somatic cells mitogenic signals are a major contributing factor to the increase in Cyclin D protein levels during the early G_1 phase (7). Increased transcription and inhibition of the degradation process lead to accumulation of Cyclin D which then binds CDK4 (7). Active CDK4 phosphorylates Rb, prompting the release and disinhibition of the E2F transcription factors (7). E2Fs in turn promote the expression of Cyclin E and other proteins necessary for S phase progression (8). The newly synthesized Cyclin E binds CDK2 which then phosphorylates Rb, in a positive feedback manner enhancing its own expression (7). During this phase the cell must ensure all essential factors required for a successful round of division are in place, these include adequate supply of nutrients and readiness of the intrinsic machinery required for DNA replication. Failure to do so will compromise the survival of the dividing cell. In order to proceed into S phase the Cyclin E/CDK2 complexes must overcome an inhibitory threshold imposed by the CIP/KIP proteins,

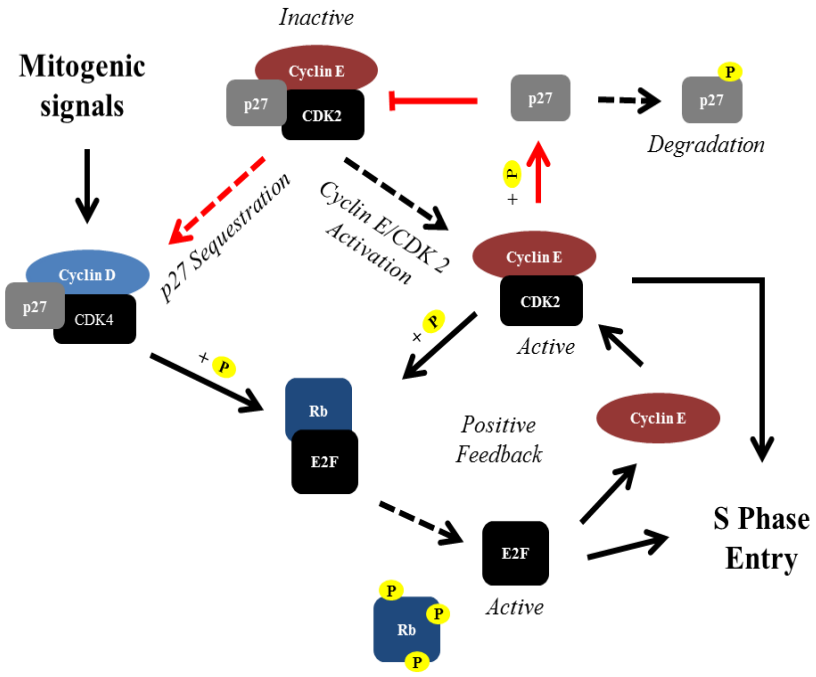

Figure 2. Regulation of the G_1/S transition. Mitogenic signals increase protein levels of Cyclin D which then binds and activates CDK4. The Cyclin D/CDK4 complexes phosphorylate Rb to cause the release and disinhibition of the E2F transcription factors. The newly synthesized Cyclin E once bound to CDK2 causes further phosphorylation of Rb, thus in a positive feedback manner enhancing its own expression. Cyclin E/CDK2 complexes are inhibited by p27 which must be degraded or sequestered to allow S phase entry. Modified from Sherr CJ, Roberts JM. CDK inhibitors: Positive and negative regulators of G 1-phase progression. Genes Dev. 1999;13(12):1501-12.

p27 amongst those (11). p27 actions are counteracted by numerous mechanisms. CDK2 itself phosphorylates p27 on threonine 187 promoting its ubiquitination and subsequent degradation. Serine 10 phosphorylation also leads to nuclear export and its cytoplasmic degradation (11, 12). Threonine 157 phosphorylation causes cytosolic retention, while phosphorylation on tyrosine 88 disrupts the ability of p27 to inhibit Cyclin E/CDK2 (11). Unlike Cyclin E/CDK2, p27 binding to Cyclin D/CDK4/6 positively regulates the activity of the complex (7). This interaction helps sequester the protein away from Cyclin E/CDK2 complexes, thus alleviating the inhibitory effects of p27 (7). If the mitogenic input is withdrawn Cyclin D expression is reduced and INK4 activity is upregulated, Cyclin D/CDK4 complexes no longer titrate the CIP/KIP proteins and

this in turn limits Cyclin E/CDK2 activation (7). Ultimately, once the cyclin/CDK complexes overcome the inhibition by the CKIs and hyper-phosphorylate Rb, the E2F factors are free to express their target genes and initiate DNA synthesis. Throughout S phase, the ubiquitin ligase FBW7 tags Cyclin E for degradation, while Cyclin A levels accumulate. Cyclin A binds to CDK2 and promotes S phase exit once all the genetic material is properly replicated (2). The timing and complexity of molecular mechanisms involved in the regulation of the transition from G_1 into S accentuates the importance of this stage in the decision to commit to a round of division and the proper completion of the process.

1.2 G_2/M Transition

As cells enter the G_2 phase Cyclin A associates with CDK1. However, as cells near the G_2/M transition Cyclin A is degraded and the newly synthesized Cyclin B binds with CDK1. When activated, the Cyclin B/CDK1 complex is reported to phosphorylate a large number of proteins that regulate various structural processes essential for mitosis (reviewed in (13)). These events include nuclear envelope breakdown, chromosome condensation, centrosome separation and spindle assembly (13). Additionally, Cyclin B/CDK1 complexes contribute to the activation of the ubiquitin ligase APC. In turn, APC signals for the degradation of the anaphase inhibitors securins as well as the cyclin proteins, a step required for the further progression through mitosis (13). In anaphase the sister-chromatids are held together by a protein complex known as cohesin. In order to allow the separation of the chromatids cohesin has to be cleaved by the protease separase. Securins inhibit separase and hence block further separation of the daughter cells, an effect alleviated once APC signals for the degradation of securins (13). Once the binding action

of cohesin is removed the sister-chromatids are pulled apart then the cell begins cytokinesis and eventually divides into daughter cells.

1.3 Cell Cycle Checkpoints

Maintaining the integrity of the genetic material during replication is crucial in preventing oncogenic transformation or ensuring the survival of a cell. A complex network of safeguard mechanisms termed checkpoints ensure orderly progression of the cell cycle, which includes successful completion of each stage prior to further progress as well as a response to DNA damage and an attempt to repair it (14). When activated by both extrinsic and intrinsic stimuli checkpoints can arrest the G_1/S or G_2/M transitions or prevent further progress during S or M phase (14). Extrinsic signals are primarily integrated in the G_1 stage. Early work identified a point termed R (restriction) point in the cell cycle past which the division process seems to proceed independently of further outside influence. Once past R point the cells are committed to a round of replication. Conversely, when mitogens are withdrawn prior to the R point the cell will not initiate DNA synthesis (1). The cellular signalling cascades involved in the regulatory step ultimately converge on the opposing actions of Cyclin E and p27, which regulate the G_1/S transition (7).

Once into S phase the cells are committed to completing the cell cycle, and the intrinsic mechanisms are largely in place to prevent errors or damage and ensure the timely order of the stages. Possible culprits for checkpoint activation include DNA damage, replication fork disruptions and faulty chromatin assembly (15). Many stressors can damage the DNA helix, often culminating in either a double strand break (DSB) or exposed single stranded DNA (ssDNA) (15). The response to these events is initiated by two enzymes which once recruited to

the damaged site, initiate the signalling pathways that will ultimately attempt to repair the damage and delay further cell cycle progress. ATM (ataxia telangiectasia mutated) is shown to be active in the presence of DSB, while ATR (ATM and rad3 related) is recruited in the presence of ssDNA (15). ATM and ATR will in turn activate various mediators that participate in the repair process, also they will trigger a signalling sequence that involves CHK1 and CHK2 (15). CHK1 is reported to inhibit cdc25 activation and thus limit CDK activation, namely the Cyclin E/CDK2 and Cyclin B/CDK1 complexes during the G_1/S and G_2/M transitions respectively (15). CHK2 is an activator of p53, a transcription factor whose target gene products mediate DNA damage response including the CDK inhibitor p21 (15). p53 arrests growth and proliferation, contributes to DNA repair and induces cell death pathways in response to genomic damage (16). The activity of p53 is central in preventing oncogenic transformation, it is one of the most frequently mutated genes in cancers which allows those malignant cells to evade checkpoint mediated regulation (16, 17).

During S phase the cells perform extensive DNA synthesis, where possible aberrations besides the aforementioned DNA damage can occur, including a stalled replication fork. Once again an ATM/ATR regulated response will help preserve the integrity of the stalled replication forks, the initiation or firing of new ones, and as discussed above repair any genomic damage, thus attempting to recover DNA replication all while delaying further cell cycle progression (15). While in mitosis, the spindle assembly checkpoint (SAC) will arrest compromised cells prior to anaphase. As the name implies SAC ensures correct spindle assembly which in turn mediates proper chromosome segregation (18). SAC pathway proteins form the mitotic checkpoint complex which binds and inhibits cdc20, an activator of the APC/C complex, thus preventing the degradation of Cyclin B and securin and the subsequent progression through mitosis (18). This

highly regulated network of checkpoints is essential for proper cell cycle completion and the maintenance of cell integrity, and when this network is compromised severe malignancies can arise (see Cancer section).

2. Programmed Cell Death Pathways

Cell death pathways are a set of evolutionary conserved mechanisms implicated in a range of functions in multicellular organisms such as development, tissue patterning, and homeostasis by regulating the removal of unwanted cells (19). Cell death pathways were originally classified based on the apparent cellular morphological features, but research has elucidated the biochemical properties that characterize the underlying molecular events (19). When deregulated, cell death pathways help shape disorders such as developmental defects, degenerative disorders and cancer (19). Apoptosis and autophagy are well-defined forms of programmed cell death which, unlike necrosis, are regulated via molecular signalling mechanisms influenced by both external and internal stimuli. In contrast necrosis is less ordered and occurs as a result of cellular edema and disruption of the plasma membrane (19). Even though apoptosis and autophagy are distinct molecular processes there exists a well-documented cross talk between the two pathways to ultimately render the cell capable of adequately responding to multiple stressors (20).

2.1 Apoptosis

Apoptosis was first defined by DNA fragmentation, condensation of the nucleus and plasma membrane blebbing (21). These events were shown to be orchestrated by a family of proteases known as caspases which cleave many of the cell's proteins (21). However, now it appears that a number of related but caspase independent pathways also contribute to this form of cell death (22, 23). Apoptosis is primarily induced via one of two pathways, an extrinsically triggered sequence and an intrinsic response actuated by the mitochondria (24, 25)(See Figure 3). The caspase proteins can be subdivided into two groups commonly referred to as response

initiators and effectors. They are synthesized as the procaspase form and possess little catalytic ability, cleavage of the inactive zymogen detaches the inhibitory pro domain and activates the enzymatic properties (25). Caspase-8 initiates the extrinsic apoptosis pathway. It is activated following ligand binding to death receptors which then induces the downstream apoptotic signalling (25). The intrinsic stimuli converge on mitochondrial outer membrane permeabilization (MOMP). Mitochondrial proteins cytochrome c and Apaf-1, once released into the cytoplasm, form a complex together with procaspase-9. This complex, often referred to as the apoptosome, helps cleave caspase-9 and initiate the execution of apoptosis (25). Both caspase-8 and -9 are initiator caspases and once they are activated by the upstream stimuli they initiate the caspase cascade and cleave the apoptotic effectors. The effector caspases in turn are responsible for mediating the apoptotic events, this group includes caspase-3, -6 and -7. Among their targets are caspase activated DNases which fragment DNA, nuclear laminins and other cytoskeletal proteins (21).

The mitochondrion is an organelle responsible for the majority of cellular ATP production in oxygen rich environments. Disruption of this mitochondrial function could lead to cell death independent of apoptotic signalling (26, 27). However, mitochondria are also central to the integration of intrinsic apoptotic stimuli (26, 27). MOMP and the consequent release of certain mitochondrial contents into the cytoplasm is considered a point of no return in programmed cell death. While many events have the capacity to contribute leading up to this stage, the opening of the mitochondrial permeability transition pore (mtPTP) is an event that will lead to organelle swelling, loss of membrane potential and eventually rupture of the outer membrane. mtPTP opening could be triggered by stimuli such as excessive amount of intracellular calcium or reactive oxygen species (ROS) (26, 27). The Bcl-2 family of proteins are

also crucial regulators of MOMP. These proteins are divided into two groups as either pro or anti-apoptotic. The Bax and Bak proteins oligomerize to form a pore in the outer mitochondrial membrane which would cause permeabilization (26, 27). This interaction is prevented via competitive binding with the anti-apoptotic members of the Bcl-2 family such as Bcl-2, Bcl-XL, and MCL1. Other apoptosis promoting proteins, known as Bcl-2 homology 3 – only (BH3-only) proteins, are unable to form channels in the mitochondrial outer membrane, however they bind and sequester the anti-apoptotic Bcl-2 factors thus preventing them from inhibiting Bax or Bak (26, 27). This sort of interrelation between the various types of Bcl-2 proteins allows for the integration of multiple stimuli to help regulate tissue homeostasis through apoptosis. DNA damage and errors during the cell cycle progression will lead to p53 activation (see Cell Cycle section), which will in turn increase gene transcription of the pro-apoptotic Bax as well as the BH3-only PUMA and NOXA (28). This response is critical for the maintenance of DNA integrity or the prevention of oncogenic transformations as damaged cells are removed by undergoing cell death. Growth factor abundance is a strong survival signal, it negatively regulates programmed cell death trough Bcl-2 proteins. Downstream of growth factors binding to their respective receptors the Akt pathway is activated. Akt phosphorylates and inhibits BAD, a BH3-only factor, such post translational modification prevents the sequestration of the anti-apoptotic Bcl-2 proteins and promotes survival (29, 30). Malfunctions of the p53 mediated DNA damage response and growth factor influenced PI3K/Akt pathways often contribute to tumorigenesis, as the organism loses the capability to remove these unwanted damaged cells which promotes the spread of the disease.

As mentioned earlier a number of contributing factors also participate in programmed cell death, some interact with the elements of the caspase cascade while others act independently. A

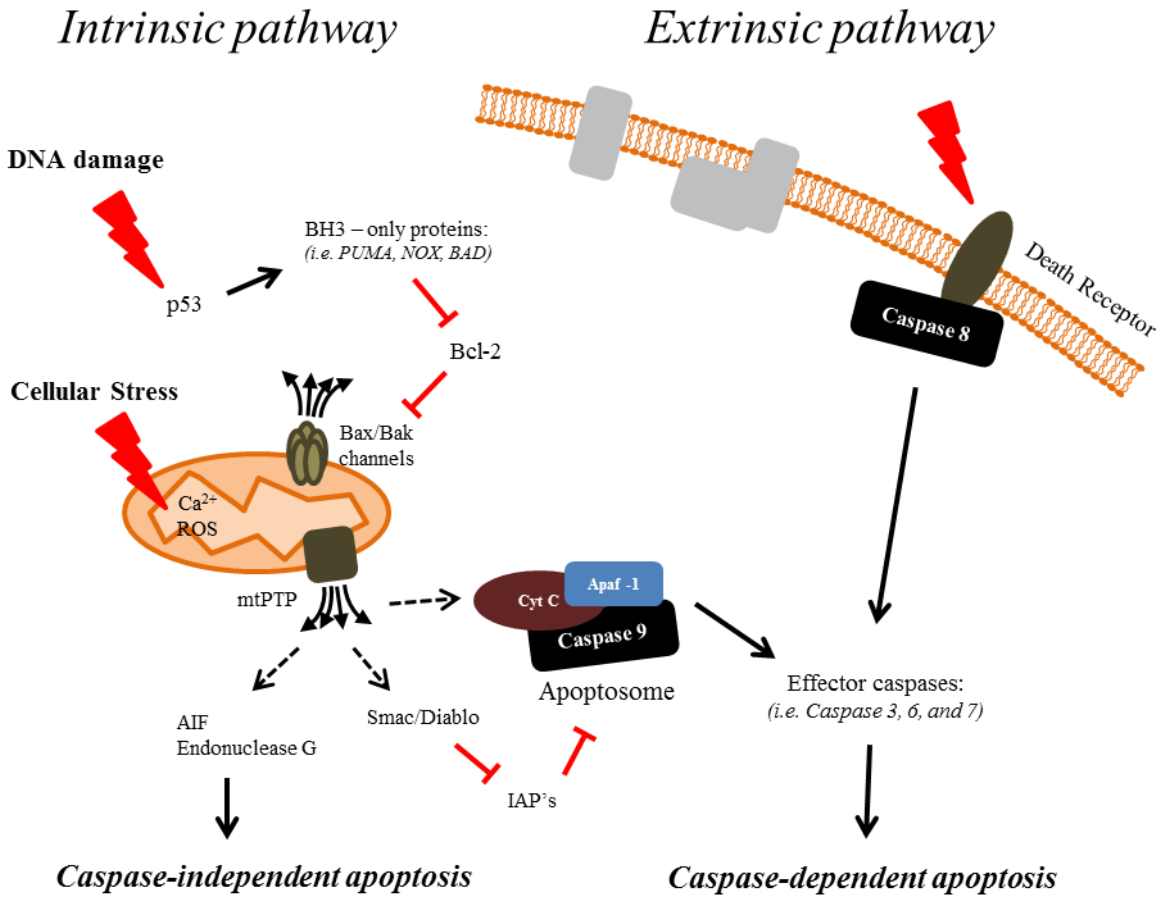

Figure 3. The molecular regulation of caspase-dependent and independent apoptosis. The intrinsic apoptosis pathway influences the permeabilization of the outer mitochondrial membrane. The consequent release of mitochondrial contents into the cytoplasm mediates both caspase-dependent and independent apoptotic responses. Ligand binding to the death receptor initiates the extrinsic pathway. Recruitment and cleavage of caspase-8 causes the downstream activation of effector caspases.

family of proteins known together as inhibitors of apoptosis (IAPs) prevent caspase activation. Smac/Diablo factors are released into the cytoplasm following MOMP and inhibit IAP activity, thus enhancing the apoptotic program (22). AIF (apoptosis inducing factor) and endonuclease G also translocate from the mitochondria into the nucleus where they help degrade DNA (22). The relative importance of each individual component is subject to debate. It has been demonstrated that apoptotic cell death is possible in the absence of caspase actions, even though these event are morphologically different (22, 23). Interestingly while deactivation of apoptotic effectors

leads to a shift in the phenotype of apoptotic cell death, knock-out of both Bax and Bak renders cells apoptosis deficient and results in a switch to autophagy (20).

2.2 Autophagy

Autophagy is another form of programmed cell death that is generally initiated in response to nutrient starvation and involves the lysosomal degradation of cytoplasmic contents (31). Unlike apoptotic cell death autophagy at first is an attempt by the cell to recycle its contents when facing an energy deficit or cytotoxic stress. However, when excessive it results in cell death (32). Autophagy is a multistep pathway which involves a series of complex interactions between proteins encoded by the Atg genes (31, 33)(See Figure 4). These proteins guide the nucleation and maturation of the autophagosome, a double membrane vacuole which will enclose cellular contents destined to be degraded. Energy sensing pathways play a central role in the regulation of autophagy, with the antagonistic actions of AMPK and mTOR central in the control of the pathway induction (31, 33). Once upstream stimuli are activated the Atg6 (Beclin-1 in mammalian cells) complex initiates vesicle nucleation and autophagosome formation (33). LC3 (also known as Atg8 in yeast) is activated through two protein conjugation systems and it completes autophagosome formation (33). This is followed by the fusion of the autophagosome with the lysosome and the degradation of the cargo it contains (31, 33). Autophagic cell death is primarily characterized by profuse engulfment of cytoplasmic portions (31, 33).

The dynamic molecular mechanism of autophagy is increasingly more often implicated in states of health and disease. Upstream signals converge on the complex ULK1/2 the mammalian ortholog of Atg1 forms with multiple cofactors. This complex then phosphorylates components of the Beclin-1 complex to initiate the formation of the autophagosome (33). ULK1/2 and its

cofactors interact with mTOR which inhibits the initiation of autophagy. AMPK is activated in response to a high AMP/ATP ratio, it both inhibits mTOR and independently activates ULK1/2 (33). The initial stages of autophagosome formation are mediated by the Beclin-1 complex. This step is influenced through numerous mechanisms which include transcriptional control, post translational modifications, and protein-protein interactions (33). Interestingly, Beclin-1 interacts with Bcl-2 as it has a BH3 binding domain, which serves as a point of cross-talk with the apoptotic pathways. This interaction is inhibitory and is alleviated during cellular stress conditions (33). The activation of LC3 is critical during the vesicle elongation and autophagosome maturation (33, 34). It is achieved through two interrelated systems that resemble ubiquitin-like conjugation. Briefly, Atg12 is covalently linked to Atg5, they then form a complex with Atg16. In turn, this complex will catalyze the conjugation of LC3 with phophotidylethanolamine (PE). This lipidation step converts the soluble LC3-I form into a membrane associated LC3-II state. Leading up to this step LC3 has to first be cleaved by Atg4 then processed by the E1-like enzyme Atg7 and E2-like Atg3. Once the autophagosome is formed it will fuse with a lysosome to degrade its contents (33). The progress or flow of the autophagic pathway can be influenced in a number of different ways. Decreased upstream initiation will downregulate the flow through pathways, however reduced lysosome fusion will lead to accumulation of autophagosome intermediates. Deficiencies related to Atg genes also can impair the removal of unwanted cellular contents (31). Thus, unlike apoptosis where MOMP is a critical turning point, autophagy flux is somewhat difficult to discern.

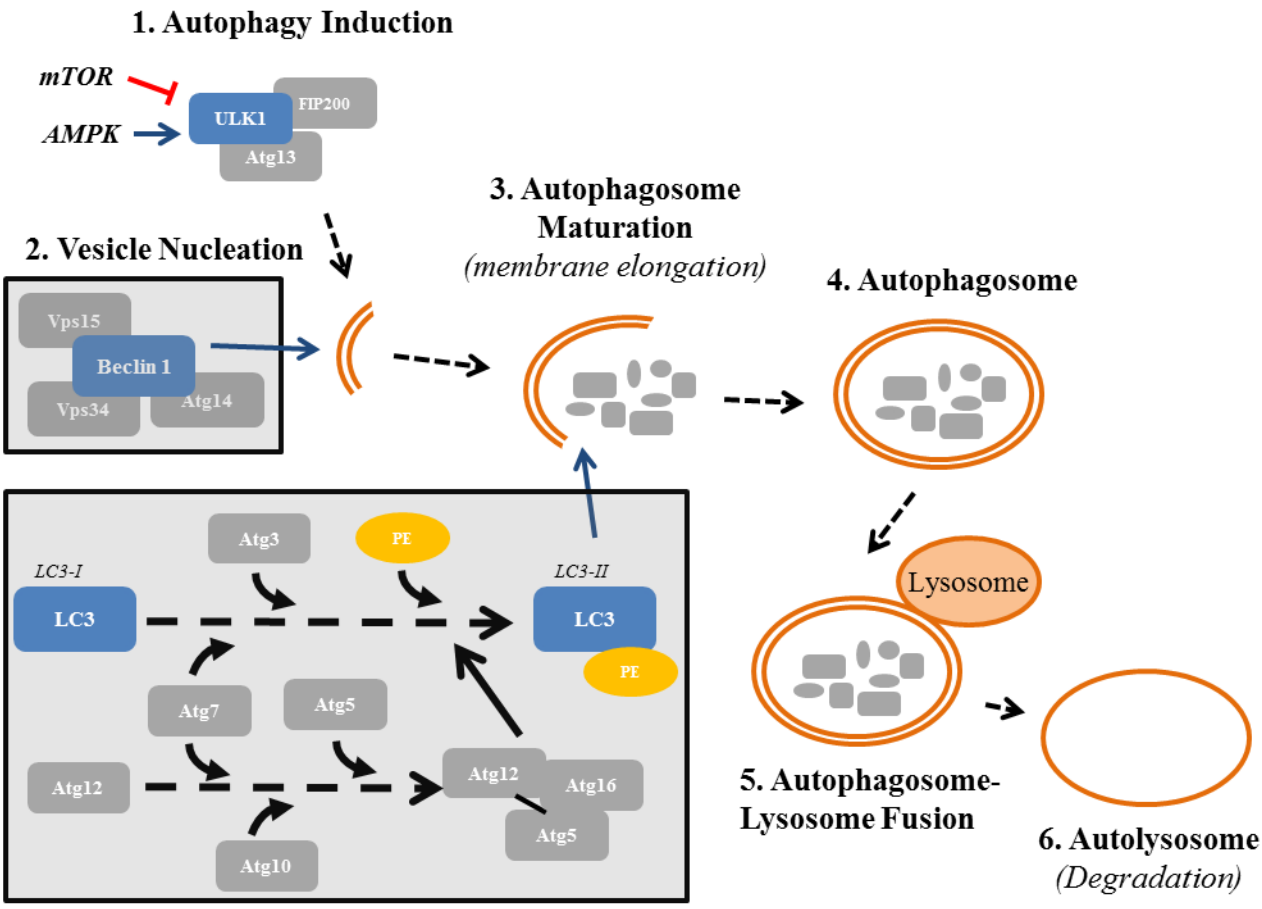

Figure 4. The regulation of autophagy. The autophagy pathways consist of a number of distinct stages. The progress from one stage to the next (black dashed arrows) is regulated by the Atg proteins. AMPK and mTOR are cellular energy and nutrient sensing kinases that regulate the induction of autophagy through the ULK1 complexes. Beclin 1 complexes contribute to vesicle nucleation, while LC3 regulates the autophagosome formation step.

3. Myogenesis

The skeletal musculature is a dynamic and highly regulated tissue that plays a pivotal role in mammalian functions. Muscle is generated by a series of complex events during embryonic development, and it also has the capacity to regenerate and restructure itself postnatally. Muscle cells originate in the somites, which are epithelial spheres formed from the paraxial mesoderm during the early stages of embryonic development (35). The dorsal region of the somites will then develop and form the dermomyotome which will be the source for most cells of the myogenic lineage (35). The dermomyotome is further subdivided into epaxial and hypaxial regions, which eventually form the myotome that will develop into trunk musculature (36). Other muscle progenitor cells will delaminate from the hypaxial dermomyotome and migrate to form the muscle of the limbs and the rest of the body core (36).

Muscle development at the molecular level is regulated by a family of proteins known as the myogenic regulatory factors (MRFs) (reviewed in (37, 38)). The MRFs in turn are regulated by early myogenic determinants such as PAX 3 and 7 (37). Numerous studies outline distinct roles for the different MRFs and early myogenic factors as well as the hierarchy within the group. The first identified member of the MRF family, MyoD, was reported to have the capacity to convert fibroblasts and some other cell lineages into myoblasts (39). Myf5, another MRF, acts in a similar fashion (40). Mouse knock-out models with either of the genes missing appear to have relatively normal muscle development, with the other MRF compensating for the deficiency (41). Animals with both genes knocked out did not develop any skeletal muscle (41). The roles of Myf5 and MyoD within the developing embryo differ in a region specific pattern. While MyoD is the more prominent factor in muscles derived from the hypaxial region of the developing somite, Myf5 is important in the epaxial region (42). Myogenin is also capable of

converting a variety of cell types into myogenic cells (43). Moreover, downregulation of Myogenin prevents the late stages of myoblast maturation and fusion into multinucleated myotubes (35). Embryos with a deleted Myogenin gene die immediately after birth and they display severe defects such as reduced muscle size and lack of multinucleated muscle fibres (43). The role of MRF4 is somewhat disputed. Initially the protein was thought to regulate the later stages of muscle development, however some evidence argues that it might participate in earlier events as well (38).

The actions of MRFs, or myogenesis in general, is further complicated by a number of additional levels of regulation. Those include transcriptional control of the myogenic regulators, post-translational modifications, chromatin remodelling, as well as protein-protein interaction (reviewed in (44)). The 4 MRFs are transcription factors that regulate the expression of genes required for myogenic development. They all contain a basic-helix-loop-helix (bHLH) domain which is essential for their function (45). The MRF proteins heterodimerize with ubiquitous non-myogenic bHLH containing proteins such as E12 and E47, both products of the E2A gene, as well as a number of others, collectively termed E proteins. Once the heterodimer complex is active it binds to specific sites in the promoter regions of myogenic genes known as the E box (45). Proteins such as Id possess a HLH domain and are capable of binding to either MRFs or E proteins, however they do not activate gene transcription. Thus, the presence of such proteins dilutes the amount of MRFs and E proteins that would be available to associate with each other and activate muscle gene expression (45). The MRF/E protein heterodimer will further form a complex with other coactivators such as the histone acetyltransferaces p300 and PCAF. These complexes will then recruit the transcriptional machinery in addition to affecting chromatin remodelling (45). The Myocyte Enhancer Factor-2 (MEF2) family of MADS-box transcription

factors is another class of proteins that activate the expression of myogenic genes (reviewed in (46)). The MADS-box motif is required for DNA binding, while the MEF2 domain increases the DNA binding affinity and participates in protein-protein interactions (46). Although it is questionable if MEF2 proteins are capable of evoking myogenic determination on their own, their cooperation with the MRF proteins is well established. In fact, often the MEF2 and E box sites on the promoters of muscle specific genes are located in close proximity. MEF2 proteins physically interact with the bHLH regions of the MRFs and synergize in the activation of the myogenic program (46). MyoD and the other MRFs are also regulated at the post translational level through both phosphorylation and acetylation (45). Ultimately, this complex network of molecular mechanisms converges on MRF controlled gene expression to help shape the myogenic program which guides the development and maintenance of skeletal muscle tissue.

PAX3, and to some degree PAX7, are believed to act as key early myogenic regulators. It was demonstrated that PAX3 is able to activate the expression of both Myf5 and MyoD (47). Moreover, cells expressing PAX3 and/or PAX7 are a key contributor to all skeletal muscle. Knock out of both these genes results in arrest of muscle development (48). While promoting the early events of the myogenic program, PAX3 supresses the late stages, and it must be downregulated to allow myogenic cells to undergo terminal differentiation (49). PAX3 cooperates with and also regulates a number of factors that help promote the myogenic program. SIX transcription factors family and their cofactors EYA proteins participate in the activation of the MRFs (37). In addition, Msx1 helps ensure adequate expansion of the myogenic progenitors by inhibiting differentiation (37). The c-Met receptor and its ligand HGF (hepatocyte growth factor), as well as Lbx1 are required for proper migration of the myogenic cells to the limb regions of the developing embryo (37).

Throughout the embryogenesis a number of secreted factors control the spatio-temporal patterns of skeletal muscle development. Shh, a factor released from the notochord, together with the Gli transcription factor promotes myogenesis through upregulating Myf5 expression (38). BMP (bone morphogenic protein) is antagonistic to the actions of Shh (35). Furthermore, Wnt factors are released from the neural tube and surface ectoderm and are also important regulators of myogenesis (38). Notch and its ligand Delta inhibit MyoD, while fibroblast growth factors (FGF) are also implicated in the regulation of MRFs (36). These morphogens collectively influence the myogenic program to control orderly tissue patterning in the developing organism.

Once functionally mature, muscle tissue still has the capability to regenerate and restructure following certain event such as injury or training (44). By and large, this response is mediated by a population of myogenic progenitor cells known as satellite cells (reviewed in (50)). Other muscle residing stem cells as well as bone-marrow derived stem cells also contribute to the regeneration process, albeit to a lesser degree (50). The satellite cells reside within the basal lamina which surrounds muscle fibres, and are mitotically quiescent during normal conditions (50). During the process of regeneration satellite cells re-enter the cell cycle, undergo multiple rounds of cell division, they then proceed to the differentiation step and fuse with the existing muscle fibres (44)(See Figure 5). Some of the daughter cells created during the proliferation stage do not differentiate and fuse, but rather revert back into the quiescent state to replenish the progenitor pool (50). Irradiation or treatment with drugs that prevent cell division debilitates muscle regeneration following injury (50). Once activated, satellite cells start expressing MRFs which control the myogenic program (44). Out of the four MRF family members, MyoD is reported as essential for proper myogenic stem cell function in adult skeletal muscle (51). PAX7 appears to play an important role in those cells, since animals with this gene

mutated display lack of satellite cells and reduced muscle size (52). A number of growth and inflammatory factors secreted in the muscle tissue appear to promote myogenesis, those include HGF, FGF, IGF and LIF (50). The TGF-β family of proteins, which includes myostatin, inhibit the proliferation and differentiation of satellite cells (50). Similar to muscle formation in embryogenesis the execution of the separate events must be properly timed. The satellite cells must proliferate to adequately expand their population, while excessive or insufficient cell number will compromise tissue healing. Furthermore, following the initial proliferative stage the myogenic progenitor cells switch to differentiation then they must fuse with the damaged muscle fibres. This stage is also critical to skeletal muscle maintenance, while deficiencies often lead to degenerative conditions such as muscular dystrophy (50).

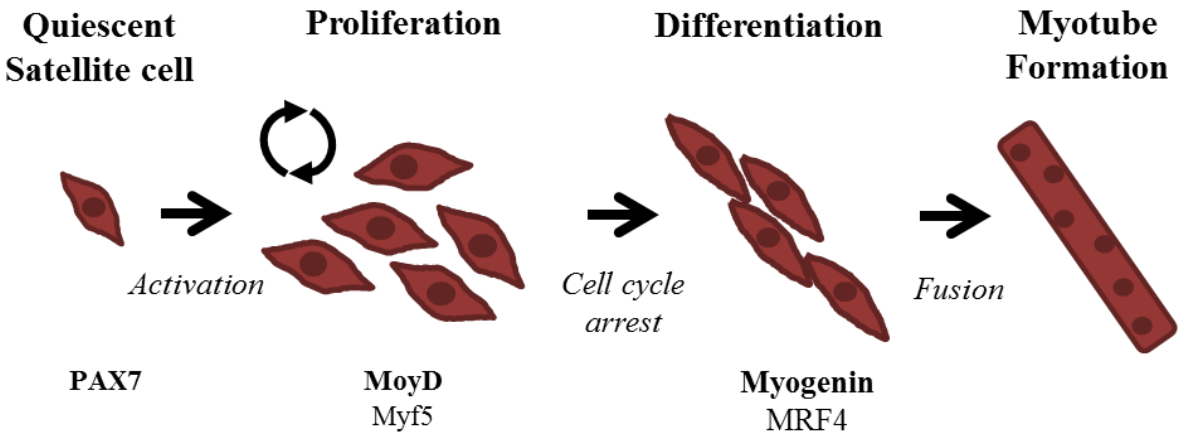

Figure 5. Stages of satellite cell recruitment and myogenic regulators. Quiescent satellite cells once activated begin to proliferate in order to expand the cell population. Differentiating satellite cells must first exit the cell cycle, once terminally differentiated these cells fuse into multinucleated myotubes or with existing muscle fibres. Satellite cell recruitment is regulated by PAX7 and the MRFs.

3.1 Cell cycle in Myogenesis

Regulation of the cell cycle is an essential part of the myogenic program (reviewed in (53)). Muscle precursor cells must halt cellular replication to allow differentiation. During the

final stages of muscle cell maturation they will irreversibly exit the cell cycle and reach a state of terminal differentiation. Following that, myoblasts fuse with muscle fibres or form syncytial myotubes (53). These events are controlled by a complex regulatory network that involves MRFs and cell cycle regulators. Myf-5 and MyoD, the MRFs implicated in myogenic determination, undergo distinct cell cycle dependent regulation (54). Myf-5 protein levels are notable in G_0, the quiescent state, and are downregulated as cells proceed through G_1. Conversely, the MyoD protein is highly expressed during G_1 and its levels diminish as cells near the G_1/S transition (54). Knock-out studies revealed that Myf-5 deficient cells undergo premature differentiation, which is accompanied by higher than normal myogenin gene expression together with other late muscle specific markers (55). Furthermore, MyoD-null cells fail to proceed in their myogenic program (55). Induction of MyoD expression is known to act as a potent inhibitor of cell proliferation (56). In fact, myogenic stem cells lacking MyoD continue proliferating but fail to undergo further development (51). Inefficient MyoD activity is an underlining characteristic of rhabdomyosarcomas which are characterized by over-proliferation (57).

Since cell cycle arrest is necessary for muscle differentiation, conditions that boost replication also prevent muscle gene expression. Either overexpression of E2F1 (58), or knock-out of its inhibitor Rb (59) abolish the differentiation of myoblasts. Moreover, as myogenic cells progress towards terminal differentiation E2F expression is downregulated (58). Also, Rb protein levels are increased through direct transcriptional activation by MyoD (60). Hence, regulators of the Rb/E2F axis, such as CDKs and CKIs, inevitably have effects on the differentiation program in muscle progenitors. Most of the interphase CDKs and cyclins are reported to be downregulated as cells stop proliferating and begin expressing muscle specific genes (53). p21 is a well characterized MyoD target gene and its expression is increased to allow terminal

differentiation independently of p53 signalling (61). Animals with p21 and p57 genes both knocked-out display severe muscle deficiencies (62). p27 is also expressed in the developing musculature and it enhances MyoD activity (63).

Besides regulating myogenic differentiation through the Rb/E2F pathway at the G_1/S transition point, many of the cell cycle regulators are reported to also participate in regulation of MRF activities through protein-protein interactions and post-translational modifications. The Rb protein directly binds to MyoD and this interaction is reported to promote muscle cell commitment and differentiation (64). CDK4 binds to MyoD and prevents its DNA binding ability (65). Cyclin D is required for nuclear import of CDK4 where the interaction between the two proteins takes place (65). Conversely, CDK4 kinase activity is also inhibited when it is bound to MyoD (66). CDK1 and CDK2 where shown to phosphorylate MyoD on serine 200 and this post-translational modification inhibits its activity and promotes degradation (67). p57 acts to inhibit the CDK2 and CDK1 complexes and it is thought to also directly bind and stabilize MyoD (68). The reciprocal interaction of MyoD with positive cell cycle regulators and its cooperation with cell cycle inhibitors highlights the vital role this MRF plays in the transition from proliferation to myogenic differentiation. These interactions also help elucidate why muscle gene expression is normally absent during the proliferative stages.

3.2 Regulation of Myogenesis

The effectors of myogenic and cell cycle processes are regulated through a number of signal transduction pathways. These signalling sequences integrate the various external stimuli and internal cues and comprise of a series of post-translation modifications often carried out by protein kinases. Amongst the most researched and established pathways implicated in the

myogenic process are the mitogen activated protein kinase (MAPK) family (69) and the PI-3 Kinase (PI3K) (70) signalling cascades. Both of these pathways play a role in the proliferation and differentiation stages. Extracellular signal regulated kinase (ERK), a member of the MAPK family, is a well characterized regulator of cell proliferation (reviewed in (71)). Following the binding of mitogenic factors to their respective receptors on the cell membrane Ras activation is triggered. Raf, a downstream target of Ras, is a kinase that activates MEK which consequentially phosphorylates ERK1 and ERK2 (71). ERK1/2 in turn promotes activation of Fos and Jun, both being components of AP-1 transcription factor, and induces Cyclin D gene expression (71). ERKs also stabilize the c-Myc factor which plays an important role in regulating cell cycle progression (71). Active MEK1 was shown to block the activity of the nuclear MyoD transcriptional complex, potentially through direct protein-protein interaction (72). Fos and Jun are reported as inhibitors of transcriptional activation of myogenic genes by MyoD and Myogenin (73). In addition, c-Myc possesses a bHLH domain and was shown to repress MyoD initiated differentiation as well (74). The protein kinase Akt is thought to influence both proliferation and myogenic differentiation. The numerous targets downstream of Akt participate in various essential cellular functions, some of which were shown to have an impact on the state of myoblast cells. The PI3K/Akt pathway is activated following mitogenic stimulation, and it is thought to enhance the G_1/S cell cycle progression (75). Akt regulates p27 protein localization within the cell. Following phosphorylation of the T157 residue the CKI is then trapped in the cytoplasm, and thus unable to inhibit the nuclear CDK complexes (76). In addition, Akt is also a negative regulator of FOXO activity. The FOXO transcription factors are responsible for upregulating p27 gene expression (77), while reducing Cyclin D levels (78). GSK3β is inactivated by Akt which makes it unable to tag Cyclin D for degradation (79). The mTOR

complex, a well characterized Akt target is also a positive cell cycle regulator (80). Altogether, both ERK and Akt signalling pathways cooperate to promote G_1/S stage transition, hence promoting the proliferation and inhibiting the differentiation of myogenic progenitors. This step is responsible for the proper expansion of the muscle progenitor pool prior to differentiation and fusion.

Upon initiation of differentiation myoblasts exit the cell cycle and induce the activity of myogenic regulators. Those events are coordinated through the p38 MAPK and PI3K/Akt signal transduction pathways. Inhibition of either one results in abrogation of myogenic differentiation (81). p38 kinase activity increases over the course of differentiation and it promotes cell cycle exit and expression of late myogenic genes (82). PI3K/Akt signalling is thought to downregulate the MAPK/ERK pathways as myoblasts switch from proliferation to differentiation (83). Inhibition of ERK signalling normally accompanies muscle differentiation, while deactivation of this pathway promotes expression of myogenic genes (84). GSK3β is target of Akt directed inhibitory phosphorylation and it is reported that deactivation of GSK3β induces myogenic differentiation (85). Furthermore, Akt is an important mediator of cell survival. Inhibition of this enzyme in differentiating myoblasts is reported to impair survival (86). Calcium regulated signalling is another emerging regulator of myogenesis. Reduction of intracellular calcium levels attenuates the differentiation of myocytes (87). The Calcineurin/NFATc pathway is a potential mediator of calcium signalling with its upregulation promoting the expression of myogenic genes (88). Calcineurin is a calcium/calmodulin regulated phosphatase that removes a phosphate group from the NFATc transcription factors, which is essential for their nuclear localization (88). Interestingly, the actions of Calcineurin upon NFATc3 are antagonized by GSK3β, with inhibition of GSK3β overcoming the blockade of myogenic differentiation caused by Calcineurin

suppression (89). Furthermore another calcium regulated enzyme, Calcium/calmodulin dependent kinase (CaMK), is a major positive regulator of MEF2 DNA binding ability (90). The enzymes involved in the regulation of myogenesis could potentially be targeted by therapeutic interventions to alter the course of the muscle tissue maintenance.

3.3 Electrical Stimulation of Muscle Cells

The electrical stimulation model was originally developed to mimic the innervation of muscle tissue, to help study the effects of depolarization induced contractile activity (reviewed in (91)). It is known to evoke a wide range of cellular responses within the muscle fibres. However, it also promotes the activation of satellite cells and their fusion with those muscle fibres (91). *In-vitro* use of electrical stimulation on myoblast cells, as well as differentiated myotubes, is a helpful tool in identifying specific signalling events involved in the various phenotypical changes observed with the use of this model (91, 92). Cell cycle arrest is one of the changes elicited by electrical stimulation in myoblasts (93). It is accompanied by increased protein levels of MyoD and markers of mitochondrial biogenesis, which points to the induction of myogenic differentiation (93). Those changes appear to be mediated through AMPK activity downstream of calcium signalling (93). AMPK is a well-known regulator of cellular energy balance and it is thought to inhibit proliferation during energy deficient states (94). Activated AMPK is reported to phosphorylate p27 on T198 and increase its protein stability (95). In fact, electrically stimulated myoblasts displayed higher $p27^{T198}$ and total p27 protein levels, as well as an increased amount of cyclin E bound to p27. Moreover, these changes were attenuated following removal of intracellular calcium or AMPK inhibition (93).

4. Cancer

Cancer is a disease characterized by progressive transformation of normal cells within an organism into malignant derivatives (96). Tumorigenesis in its essential form is fuelled by dynamic genomic changes that produce continuous and uncontrollable growth and expansion of malignant cancer cells (96). The majority of tumors are genetically unstable. As a result they accumulate mutations in oncogenes and tumor suppressor genes, which promote the clonal expansion of those cells (97). Cancer cells acquire a number of capabilities that are essential to pathogenesis of the disease including self-sufficiency in growth signals, insensitivity to anti-growth cues, ability to evade apoptosis, limitless replicative capacity, sustained angiogenesis, and the ability to metastasize and invade other tissues (96). Furthermore, the tumor mass can be characterized as an organ that contains a heterogeneous population of cells, which all contribute to the creation of a microenvironment that reinforces cancer development (98). Uncontrolled cell proliferation seen in cancer cells is caused through continuous deregulated entry and inability to exit the cell cycle (99). Many mechanisms that drive cancer progression converge on Rb/E2F axis and its regulation (100). Given the importance of Rb mediated repression of the cell cycle in normal cellular functions, it is not surprising that many of the regulators of this pathway are implicated in cancer. p27 deregulation and Cyclin D over-activation are common culprits of uncontrolled proliferation seen in a large number of cancers (101). In the big picture, the G_1 stage of cell cycle is a period when a wide range of signals determine the fate of cell growth and proliferation. Deregulation within this stage and its many components plays a crucial role in the onset and progression of malignant growth (101). Underlying this deregulation are proliferation promoting pathways such as Ras/ERK and PI3K/Akt which are often hyper activated in cancerous cells (101). Healthy cells have built-in mechanisms that ensure the integrity of the

DNA before proceeding to further cell cycles phases. Progress will be halted and DNA repair initiated as a consequence of genomic damage and cell death pathways are often activated if those repairs fail (see above). Cancer cells are often defective in the control of these checkpoints leading to the inability to prevent progressive DNA damage and the resultant genomic instability characteristic to cancers (15). The dynamic nature of the disease in turn contributes to the progressive malignant transformation, making the identification of specific molecular culprits difficult.

5. Rhabdomyosarcoma

Rhabdomyosarcoma (RMS) is a form of cancer where tumor cells have characteristics of myogenic heritage (reviewed in (102)). These cells initiate the myogenic program but fail to exit the cell cycle and do not undergo terminal differentiation (57). Unlike most tumors, rhabdomyosarcoma affects mostly children and adolescents. Pathologically, this cancer can be subdivided into 3 distinct variants. The embryonal form (eRMS) most often affects young children, it commonly arises in the head and neck, paraspinal region and the genitourinary system (103). The alveolar form (aRMS) mostly localizes to the extremities and axial musculature (102, 103). The third pleomorphic form affects the musculature of adults and is a rare event (102). A portion of the alveolar rhabdomyosarcoma tumors carry a well-defined genetic mutation, a chimeric gene is formed through fusion of 2 regions from separate chromosomes and it is thought to drive the malignancy (104, 105). A portion of a gene for the Forkhead (FKHR) family member FOXO1 located on the 13th chromosome translocates into the region encoding either PAX 3 on chromosome 2, or PAX7 on chromosome 1 (104). aRMS tumors negative for the fusion gene are clinically and molecularly indistinguishable from the eRMS forms (106). Furthermore, recently published outcomes of whole genome analyses of a large set of RMS samples demonstrate that based on molecular signatures all RMS tumors can be categorized into two groups, defined by the either presence or absence of the fusion gene (107). The variants of rhabdomyosarcoma have different prognostic fates. The PAX3/FOXO1 fusion positive tumors are associated with the least favourable outcome followed by PAX7/FOXO, while fusion negative aRMS and eRMS are statistically linked with a lesser risk of mortality (108). This risk stratification is important to identify and prioritize treatment strategies and research efforts. Despite continuous advances in our understanding of this disease severe cases

are still linked to high death rates and future research is essential for improvements to these outcomes.

The characterization of RMS cells as both cancerous and myogenic has led to extensive study of the molecular properties that produce such unique state. At the genomic level, there are identifiable abnormalities that contribute to the pathology of rhabdomyosarcoma. Even though aRMS is often associated with the distinct PAX/FOXO1 chromosomal translocation, eRMS tumors exhibit a common underlying genetic signature as well. Loss of heterozygosity on a specific locus on the 11th chromosome is a frequent characteristic of the rhabdomyosarcoma tumor genotype (109). This region contains a number of imprinted genes that could potentially act as drivers of the malignancy, those include H19, p57Kip2, IGF2 and a number of others (110). A familial germline mutation in the p53 gene, called Li-Fraumeni Syndrome (LFS), is often associated with increased incidence of pediatric cancers (111). A number of young RMS patients are reported to have this familial disorder, while some others exhibit de-novo p53 mutations (111). MyoD, an important regulator of muscle formation, is expressed in rhabdomyosarcoma cells but does not initiate terminal differentiation. It is reported that the MyoD protein is capable of binding to DNA, however its transcriptional activation abilities are deficient in both eRMS and aRMS (112). This evidence suggests that a factor required for the activation of MyoD activity is deficient in those cells (112). Indeed, forced dimerization of MyoD with E proteins promotes differentiation of RMS cells (113). Furthermore, MEF2D expression is reported to be downregulated in some RMS cases and exogenous reinstatement of this protein enhances myogenic differentiation and inhibits tumorigenicity (114). aRMS associated PAX/FOXO1 fusion proteins prevent MyoD transcriptional activity, though it appears that this mechanism is independent of the MyoD-E protein interaction (115). As discussed earlier

MyoD, is crucial for cell cycle exit and induction of differentiation in myogenic progenitors (see Myogenesis section). Hence, it is not surprising that RMS cell continue to proliferate uncontrollably and that it coincides with impaired MyoD mediated transcriptional activity. These deficiencies pose a challenge for therapeutic approaches that would target myogenic differentiation signalling to help induce cell cycle arrest in RMS cells.

Examination of the PAX/FOXO1 fusion gene properties has yielded a better understanding of the contribution of this chromosomal translocation to tumor development. The ability to activate transcription of target genes due to PAX DNA-binding domain interaction with specific promoters is more potent when fused with the transactivation domain of FOXO, compared to wild type (116). The expression of the PAX/FOXO gene itself is amplified in rhabdomyosarcoma which leads to accumulation of the protein (117). Moreover, the chimeric protein interferes with the activity of wild type PAX3 in developing muscle progenitors (118). When PAX3/FOXO is expressed ectopically in fibroblasts the cells acquire a number of tumorigenic characteristics (119), underlining the enhancement of proliferation and invasiveness of cancer cells (120). In contrast, downregulation of PAX3/FOXO protein expression promotes cell cycle arrest, induction of myogenic differentiation and also cell death in RMS cells (121, 122). Targeting the fusion gene product directly, through altering either the upstream regulators or downstream effectors could serve as a strategy for possible therapeutic interventions in this subtype of RMS tumors.

Despite the obvious muscle resemblance, research has yet to clearly establish which cells in the body give rise to RMS (reviewed in (123)). A portion of RMS tumors are located in and around the skeletal muscle, however some also develop where muscle tissue is generally absent. To accentuate the variability in the origin of RMS cells, tumors were shown to develop from

both committed myogenic progenitors as well as uncommitted mesodermal cells (123). These inconsistencies raise the question of whether RMS are in fact muscle cells that fail to differentiate or if they are cancer cells which acquire some myogenic properties. Primary tumor samples, *in-vitro* cell culture and *in-vivo* animal models of RMS help researches address this question and uncover the underlying molecular properties of these tumors. Commonly used cell lines are the eRMS RD line and the aRMS Rh30. The RD line was established from a biopsy from a refractory tumor in the pelvic region of a 7 year old female patient who has previously undergone chemotherapy and radiation therapy. This cell line is reported to carry a MYC amplification, as well as Ras and p53 mutations (124). The Rh30 line contains the PAX3/FOXO fusion gene and was established from a bone marrow metastasis sample from a previously untreated 16 year old male. These cells carry a heterozygous p53 mutation and amplification on the 12^{th} chromosome in a region containing CDK4 (124). Neither cell line originated from a sample taken in a body area where skeletal muscle in generally found, however both clearly exhibit the myogenic signatures characteristic to RMS. The heterogeneous origins of RMS call for categorization that is based on the molecular properties rather than the localization of those tumors.

Interestingly, ectopic insertion of the PAX3/FOXO fusion gene under the wild type PAX3 promoter does not lead to tumorigenesis *in-vivo*. Rather, abnormalities in embryonic development similar to a PAX3 deficiency arise, which suggests that the PAX3/FOXO fusion gene negatively affects normal PAX3 function (125). However, as demonstrated by Keller et al., conditional PAX3/FOXO knock-in into differentiating muscle cells aided by INK4a/ARF and p53 loss of function led to aRMS development *in-vivo* (126). Hence, the fusion protein likely cooperates with a number of secondary mutations to produce a malignant *in-vivo* tumor (126,

127). According to whole genome scans, a portion of aRMS tumors carry common chromosomal amplifications and deletions that possibly alter the expression of cell cycle regulators. Also, fusion gene positive aRMS samples appear to undergo genetic mutagenesis to a lesser extent than the fusion gene negative or the eRMS subtype (107). Therefore, the molecular signature of the fusion gene positive aRMS tumors outside of presence of PAX/FOXO is not as clearly identifiable as in eRMS.

Sharp et al. in 2002 created an *in-vivo* eRMS model by overexpressing the HGF/SF (hepatocyte growth factor/scatter factor) in INK4a/ARF knockout mice (128). INK4a is an inhibitor of CDK4 and is implicated in Rb regulation while ARF represses MDM2, a negative regulator of p53. HGF/SF is a ligand of c-Met, a receptor tyrosine kinase (RTK) which upon binding activates Ras signalling downstream (128). Amongst eRMS tumors cell cycle checkpoint regulation is often deficient and Rb mutations are a common example of that (57, 105, 129). A number of other studies show that some form of Ras pathway deregulation together with p53 deficiency will lead to RMS development *in-vivo* (130-133). Numerous RTK are implicated in eRMS tumorigenesis, these include FGFR, IGFR, PDGFR (134, 135). Mutations in Ras genes are also common in this malignancy (107, 133). In a separate study, Shh (sonic hedgehog) pathway deregulation in mice was shown to lead to eRMS development in a non-myogenic cell lineage (136). Ptch1 is a negative regulator of Shh and heterozygous deletion of this gene contributed to the transformation of myogenic progenitors into eRMS cells (129). Furthermore, in human samples hyper-activation of the Shh pathways is associated with a less favourable prognosis (137). Overall, examination of animal models as well as human samples identifies common patters in eRMS pathogenesis. Deregulation of the RTK/Ras pathway, deficiency in p53 signalling and hyper-activation of the Shh are drivers of fusion negative RMS development

and those are in turn aided by deregulation of Rb and possibly other cell cycle checkpoint pathways.

The Akt pathway is an intriguing target in RMS research as it plays a role in both cell proliferation and survival, as well as myogenic differentiation. Increasing Akt activity may promote pro myogenic events, which in turn could potentially lead to reduced tumorigenicity. However, inhibition of this pathway might help reduce proliferation and induce cell death in RMS cells. Dissecting the specific roles played by the targets of this kinase in RMS oncogenesis will likely help determine which option is best for therapeutic intervention. As discussed earlier, the PI3K/Akt pathway is a potent pro-survival mediator (see Programmed Cell Death Pathways section). Also, it was demonstrated that the mTOR complex inhibitor, rapamycin, prevents proliferation of RMS cells and induces p53-independent cell death (138). mTOR activity is positively regulated by Akt mediated phosphorylation (139) and recently emerging evidence shows GSK3 inhibition elicits favourable consequences in both the embryonal and alveolar RMS forms (140-142). Since Akt phosphorylates and inhibits GSK3 (139), increased Akt activity may result in desirable tumor inhibiting effects. The most captivating target of Akt in the RMS context is perhaps FOXO. FOXO1 is known to be mislocalized out of the nucleus following phosphorylation by Akt on 3 separate residues (143). However, despite the fact that 2 of those residues are present in the chimeric protein product of the PAX/FOXO1 gene, it is not responsive to Akt mediated regulation (143). This finding was challenged by Jothi et al. who showed that Akt does in fact modulate the activity of the fusion gene (144). Activation of Akt is reported to promote arrest of proliferation and cell death in aRMS cells (140, 145). Evidence helps argue that inhibition of Akt is in some aspects beneficial in lessening the oncogenic properties of RMS. In contrast, activation of this pathway is also shown to weaken the RMS phenotype. The relative

importance of Akt targets is hard to specifically identify as their activity encompasses such a broad range of cellular functions. Ultimately, in light of the conflicting outcomes to varying Akt activity in RMS cells, the roles of the individual Akt targets could be further investigated as opposed to the comprehensive function of the pathway.

The regulation of myogenesis, as previously discussed, is complex and involves a number of signalling pathways. Targeting some of those molecular mechanisms could potentially be beneficial in offsetting the RMS phenotype. Since muscle differentiation is coupled with cell cycle arrest, inhibition of signalling sequences involved in proliferation could potentially induce differentiation in RMS. For instance, Cyclin D/CDK4 complexes promote G_1 phase progression and inhibit MyoD activity, while pharmacological inhibition of the complex arrests proliferation in RMS cells (146). Also, both ERK and Akt signalling are well known mediators of myoblast proliferation (see Myogenic Signalling section). Studies show that concurrent inhibition of both MAPK/ERK and Akt/mTOR in RMS cells yields a synergetic interaction detrimental to the growth of these tumors, in comparison the inhibition of either pathway individually is rather inefficient (147, 148). Conversely, p38 signalling is critical to the myogenic differentiation process since it contributes to cell cycle arrest. Forced activation of p38 in rhabdomyosarcoma cells resulted in an increased expression of late myogenic genes that are usually absent in the malignant state (149). In support of this line of evidence, inhibition of Notch is reported to result in p38 activation and the consequent differentiation events (150), while Notch hyper-activation in RMS samples is shown to yield a more aggressive tumor phenotype (151). Despite the potential of pharmacologically targeting these pathways in experimental settings, the incorporation of such drugs into comprehensive patient treatment strategies is complicated. Issues such as toxicity, bioavailability, and targeted delivery all limit the possible applicability of

such therapies. Furthermore, RMS patients are often children who must still biologically develop and mature, drugs could also potentially interfere with this crucial aspect.

6. Hypotheses

My research will center around the use of the *in-vitro* electrical stimulation model on rhabdomyosarcoma cells. The ultimate goal of the project is to investigate whether the myogenic properties of those cancerous cells could be manipulated in a way similar to healthy myoblasts. The central tenet of my thesis is that electrical stimulation of rhabdomyosarcoma cells will elicit growth arrest and potentially promote myogenic differentiation. This could be further broken down into the following specific hypotheses:

1. Electrical stimulation of RMS cells will activate AMPK and induce cell cycle arrest, a response analogous to that seen in myoblasts.

2. Electrical stimulation will induce myogenic differentiation in RMS cells.

3. Electrical stimulation will affect Akt signalling in RMS cells as part of the induction of myogenic differentiation.

7. Manuscript

Electrical Stimulation of Rhabdomyosarcoma Cells Induces Cell Cycle Arrest and Autophagy

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7.1 Abstract

Rhabdomyosarcoma (RMS) is a soft tissue cancer that manifests in young patients and demonstrates muscle-like properties. Unlike muscle cells, RMS cells fail to undergo cell cycle arrest and terminal differentiation despite the presence of the necessary molecular regulators to do so. Electrical stimulation (ES) elicits a variety of phenotypical changes in skeletal muscle, and we set out to determine whether ES induces any effects in RMS cells. Both alveolar (Rh-30) and embryonal (RD) RMS cells were subjected to ES (5Hz, 5V) for 4 hours/day followed by a 20 hour rest period for 1-5 days. ES of RD and Rh-30 cells led to a reduction in the number of cells and an altered phenotype when compared to non-stimulated control (CTL) cells. FACS analyses revealed that ES induced an accumulation of RD and Rh-30 cells in the G_2/M phase of the cell cycle. ES also increased the conversion of LC3-I into LC3-II which suggests an increase in autophagy. Furthermore, ES induced the phosphorylation of Akt/PKB on T308, important for cell survival and myogenic differentiation, while protein levels of MyoD and myogenin were also altered by ES in RMS cells. Thus, it appears that electrical stimulation of rhabdomyosarcoma cells leads to cell cycle arrest and cell death, which potentially provides the basis for the development of a novel disease therapy.

7.2. Introduction

Rhabdomyosarcomas (RMS) are a family of juvenile soft-tissue cancers that express many of the molecular factors that regulate muscle maturation (103, 152). RMS tumors are subcategorized with two main forms being embryonal (eRMS) and alveolar (aRMS)(152). RMS origins are highly heterogeneous, eRMS often affects young children and presents in the head and neck, the paraspinal region and the genitourinary system. In contrast, aRMS tumors mostly localize to the extremities and axial musculature and generally develop in adolescents (103, 152). A portion of aRMS tumors carry a chromosomal fusion between genes encoding either PAX3 or PAX7 and the Forkhead family member FOXO1. The resultant chimeric PAX/FOXO protein is a potent transcription factor which is thought to induce the aRMS malignant phenotype (57, 104, 152). Fusion gene positive aRMS are associated with the high mortality rates, while fusion gene negative aRMS are indistinguishable from eRMS (106, 108, 152). Activating alterations in receptor tyrosine kinases and ras signalling pathways as well as dysfunctions in developmental patterning pathways and cell cycle regulation are all evident in RMS patients (57, 107). This vast range of oncogenic properties makes identification of a singular molecular therapeutic intervention in RMS patients extremely difficult.

Maintenance of multinucleated skeletal muscle mass is assisted by muscle derived stem cells, termed satellite cells (50). Following nuclear degeneration or muscle damage, quiescent satellite cells first proliferate to increase their numbers and subsequently undergo differentiation and fusion by inducing the expression of myogenic regulatory factors (MRFs) (37, 50, 153). MRFs heterodimerize with E proteins to activate the transcription of myogenic genes (45). One of these MRF, MyoD, is indispensable for proper myogenic stem cell function (51). MyoD increases p21 expression to promote cell cycle exit in myoblasts (53, 61). The importance of

MyoD in muscle regeneration is illustrated by rendering satellite cells differentiation incompetent in MyoD$^{-/-}$ mice or *in-vitro* (51, 55). Unlike other cancers, RMS cells express MRFs and many other genes unique to myogenic progenitor cells, however unlike myoblasts they fail terminally differentiate due to lack of MyoD transcriptional activity (112, 113). What remains unclear is whether RMS originates from myogenic cells with a disrupted differentiation program, or if they are primarily tumor cells that somehow acquire muscle-like characteristics. Regardless of the reason, these myogenic properties may allow for the induction of cell cycle exit and terminal differentiation in RMS cells.

Electrical stimulation (ES) is an effective method whereby to induce a wide range of phenotypical changes in excitable cells including skeletal muscle and neuronal cells *in-vivo* and in cell culture *(91, 92).* Earlier findings from our lab demonstrate that when electrically stimulated myoblasts exit the cell cycle due to the activation of AMPK and the subsequent direct phosphorylation of the CDK2 inhibitor p27^{KIP1} on T198, leading to its accumulation in the cell. This increase in p27 protein levels caused cell cycle arrest and differentiation of the myoblasts in culture (93). Based on these results we electrically stimulated eRMS and aRMS cells to evaluate whether ES of those cells will activate the myogenic program and induce cell cycle exit and differentiation. We show that stimulation of eRMS and aRMS cell leads to a drastic reduction in cell numbers. In response to ES there appears to be no activation of pathways that induce G_1 arrest in these cell types, suggesting an involvement of alternative mechanism. In agreement with this FACS analyses revealed an accumulation of RMS cells in G_2/M and an enhancement in the activation of LC3 a critical regulator of autophagy. These effects are accompanied by an increase in Akt activity as protein levels of the T308 phosphorylated form are elevated in both RMS cell lines in response to ES. Overall, our findings suggest that electrical stimulation reduces

proliferation and induces autophagy in RMS cells and may provide a novel therapeutic avenue for disease intervention.

7.3. Materials and Methods

Cell Culture. RD (eRMS) and Rh-30 (aRMS) cells were a generous gift from Dr. John McDermott (York University, Toronto, Ontario) and were maintained at 37^{o}C and 5% CO_2, in high glucose Dulbecco's Modified Eagle's Medium (DMEM, Wisent, St.Bruno, Quebec), supplemented with 10% fetal bovine serum and 2% antibiotic-antimycotic solution (Wisent). The media was replenished every 48 hours.

Electrical stimulation. Cells were plated onto 0.1% gelatin coated 6 well plates. Cells that were stimulated for a longer duration were plated at a higher density to account for cell loss. The lids of the plates were fitted with two parallel platinum wire electrodes, placed at the opposite ends of each well and extending into the media. The wires from all wells were arranged in parallel and connected to an electrical stimulator (Harvard Apparatus Canada, Saint-Laurent, Quebec, Canada). Cells were stimulated at 5V and a frequency of 5Hz for 4 hours/day with alternating potential followed by a 20 hour recovery period for 1 to 5 days. Cells were harvested following the recovery period throughout the protocol in 0.2% TENT buffer (TRIS, EDTA, NaCl and 0.2% Triton x-100) supplemented with phosphatase inhibitor (phosSTOP, Roche Diagnostics, Laval, Quebec) and protease inhibitor (Sigma, Oakville, Ontario). Samples were then sonicated and centrifuged (16,100x g), the supernatants were collected and stored at -84^{o}C for subsequent analyses.

Western Blot. Protein concentrations in the samples were determined using the Bradford assay (Bio-rad, Mississauga, Ontario). 25µg of each sample was loaded onto a 12% polyacrylamide-SDS gel, separated via electrophoresis and transferred onto a PVDF (Bio-rad). Following blocking in 10% skim milk, membranes were incubated overnight with primary antibodies for pAkt^{T308} (1:500), Akt (1:2000), pAMPK^{T172} (1:1000), AMPK (1:1000), ,

pGSK3β^{S9} (1:1000), GSK3β (1:1000)(Cell Signaling, Whitby, Ontario); p27^{T198} (1:1000) (R&D Systems, Minneapolis, MN); p27(1:2000)(BD Biosciences, Mississauga, Ontario); Cyclin E (1:2000), COX IV (1:5000), Myogenin (1:500), β-Actin (1:50000)(Abcam, Cambridge, MA); MyoD (1:500)(Santa Cruz, Dallas, TX). Subsequently, membranes were probed with appropriate HRP linked secondary antibodies (1:5000) (Promega, Madison, WI). Protein expression was measured using enhanced chemiluminescence reagent (Millipore, Whitby, Ontario) the emitted signal was detected using a Kodak In vivo FX Pro and quantified with Carestream molecular imaging software (Marketlink Scientific, Burlington, Ontario).

Cell Cycle Analyses. To determine the cell cycle profiles of RD and Rh30 cells they were collected by trypsination, washed twice in cold phosphate buffered saline (PBS), then fixed by dropwise addition of ice cold 70% ethanol and stored at -20^{o}C for further analyses. Prior to sorting, cells were incubated overnight in a staining solution containing PBS, 1 µg/mL propidium iodide (PI, Bioshop, Burlington, Ontario) and 0.5µg/mL RNase (Sigma). DNA content was measured using Gallios™ flow cytometer (Beckman Instruments, Mississauga, Ontario) and cell cycle profiles were determined using ModFit LT 4.0 software (Verity Software House, Topsham, ME).

Statistical Analysis. All statistical analyses were performed using GraphPad Prism 5 software. Two-way ANOVAs were used to assess the effects of ES and time on protein content. Following the correction of protein levels in stimulated cells to reflect variation in control conditions one-way ANOVAs followed by Tukey's post-hoc tests were used to identify differences between individual time points. Changes were considered statistically significant when $p < 0.05$ (n=3-5/group).

7.4. Results

ES of RD eRMS cells over 5 days resulted in a drastic decrease in cell numbers when compared to non-stimulated same day control (CTL) cells. This was accompanied by substantially altered cell morphology. ES cells appear larger in size and exhibited a more elongated shape after 5 days (Fig. 1*A*). Cell cycle analysis revealed that ES of RD cells causes a statistically significant decrease in the proportion of cells in the G_0/G_1 population and an accumulation during the G_2/M when compared to CTL cells. While the amount of cells in S phase remained unchanged. After 5 days of ES the G_2/M population of RD cells increased by 30.6% ± 8.25, correspondingly the percentage of cells in G_0/G_1 decreased by 11.5% ± 4.7 (Fig 1*B, C*). This alteration in the cell cycle profiles of ES RD cells is observed as early as day 1, the first time point, and last throughout the protocol.

Despite featuring a different molecular etiology than RD cells, aRMS Rh-30 cells exhibit a similar response to ES. On day 4 there are clear distinctions evident with respect to cell numbers in ES cells compared to CTL, these Rh-30 cells appear enlarged or detached despite the use of gelatin coated culture plates which enhance adhesion (Fig 2*A*). FACS of Rh-30 cell identifies possibly a presence of two distinct populations of cells (Fig 2*B*). aRMS tumors have been shown to often harbour recurrent amplifications or deletion of chromosomal regions (107), this could potentially yield the observed shift in the cell cycle profile in a portion of Rh-30 cells. Nevertheless, this unusual appearance of FACS outcomes prevented precise cell cycle profile determination. Yet, when the number of cells in regions corresponding to the G_0/G_1 and G_2/M in each of the populations was estimated a shift from G_0/G_1 to G_2/M following ES in clearly identifiable (Fig 2*B, C*). An observation consistent with the results obtained for RD cells. After 4

days of ES one population of cells displays an 80.3% ± 40.05 ($p = 0.1$) increase in G_2/M proportion, while the second changed by 64.0% ± 15.23 ($p<0.05$).

RMS cells do not arrest in the G_1 cell cycle stage following ES. Electrical stimulation of C2C12 myoblasts was previously shown to increase the expression of CDK inhibitor protein p27 and p21 in a AMPK dependent mechanism (93). p27 restricts the activity of Cyclin E/CDK2 complexes at the G_1/S transition which leads to cell cycle arrest and induction of the myogenic differentiation program. Unlike the results observed in C2C12 myoblasts, ES does not activate AMPK in RD cells as indicated by the phosphorylation levels at T172. pAMPKT^{172} levels are decreased in stimulated cells as early as day 1 and remain reduced throughout the protocol (Fig 3*A*). Phosphorylation of p27 on T198 increases protein stability and contributes to growth arrest and autophagy (95). Moreover, the energy sensor AMPK was shown to directly phosphorylate p27 on T198 (95). Electrical stimulation of skeletal myoblasts was previously shown to activate AMPK and increase the levels of both p27^{T198} and consequentially total p27 protein (93). In RD cells p27^{T198} protein levels on day 1, 2 and 3 while not significantly lower compared to day 0 are decreased relative to the value observed on day 5 (Fig S1). Total p27 protein expression is decreased in ES RD cells on day 1, however there is variations observed in the levels of this protein in the following days possibly implicating other regulatory mechanism. This demonstrated that pAMPK, p27, and p27^{T198} are affected differently by electrical stimulation in RD cells when compared to C2C12. Cyclin E was used as another indicator of the G_1/S transition. ES induced a statistical change in Cyclin E levels over time yet no differences were observed between individual days (Fig 3*A*). Unlike in RD cells, ES activates AMPK in Rh-30 cells with pAMPK^{T172} levels were determined to be 1.92 ± 0.51 fold above control after 4 days

(Fig 3*B*). The protein levels of p27 were not altered in Rh-30 cells. Similarly, there were no ES-induced changed in Cyclin E levels in these aRMS cells (Fig 3*B*).

Given the decrease in the number of RMS cells after ES we wished to evaluate whether this reduction is a result of programmed cell death pathways. FACS analyses showed no indication of cells undergoing DNA fragmentation, as evidenced by the lack of a sub-G_1 population (Fig 1*A*, 2*A*). To assess the effects of ES on autophagy we measured protein levels of two members of the pathways. LC3 is an important regulator of autophagosome maturation. The activation of LC3 is critical to the autophagic pathway and it is achieved via the conversion of the cytosolic LC3-I into the lipidated, membrane bound LC3-II form. Five days of ES of RD cells induced a 2.72 ± 0.37 fold increase in the levels of LC3-II as a fraction of total protein content above those in non-stimulated controls (Fig 4*A*), while in Rh-30 cells the value on day 4 is 2.29 ± 0.18 relative to day 0 (Fig 4*B*). Total LC3 protein levels were also measured following electrical stimulation. Similar to the outcomes observed for the fraction of activated LC3-II, total protein levels increase significantly by day 4 in Rh-30 cells (Fig S2*B*) and appear to accumulate in RD cells (n=2) (Fig S2*A*). Beclin-1 protein content did not statistically vary between the conditions in either cell line.

Akt activation is enhanced in electrically stimulated RMS cells. In RD cells, ES elucidated a 2.21 ± 0.25 fold increase in the abundance of active $pAkt^{T308}$ (Fig 5*A*). Similarly, ES of Rh-30 cells induced a 3.00 ± 1.60 fold increase in $pAkt^{T308}$ above those levels in CTL cells (Fig. 5*B*). Akt plays a role on proliferation and survival but also in myogenic differentiation, and the significance of the observed activation of this kinase in ES cells is yet to be fully clarified. To further investigate the influence of Akt in RMS cells we used a pharmacological inhibitor of Akt activation in conjunction with electrical stimulation. It is evident that Akt inhibition further

enhanced cell loss following electrical stimulation in Rh-30 cells (Fig S3). However, it is not yet clear what the molecular implications of that are. This result puts forth a notion that despite the positive outcomes of enhancing the ability of RMS cells to undergo myogenic differentiation, it might instead be more advantageous to strengthen the cell death response induced by electrical stimulation in the Rh-30 cells.

Since RMS tumor cells express MRFs, we measured MyoD and myogenin protein levels in RD and Rh-30 cells in order to evaluate the myogenic changes in these cells following ES. Myogenin protein levels were drastically decreased in both RD and Rh-30 cells as early as day 1 and remained supressed for the duration of the protocol. By the final day myogenin content decreased 38% ± 12 and 71% ± 7 in RD and Rh-30 cells respectively relative to the value observed on day 0 (Fig 5*A, B*). The expression of the MyoD protein is increased 1.67 ± 0.28 fold at day 5 of ES in RD and 2.0 ± 0.25 fold on day 4 in Rh-30 cells (Fig 5*A, B*).

The mitochondrion is an organelle implicated in the maintenance of the cellular energy status and homeostasis, it is also an important mediator of apoptotic and autophagic pathways (27). Increased mitochondrial content in myoblasts is shown to parallel the differentiation progress (93). In tumorigenic conditions increasing the mitochondrial content potentially increases the likelihood these cancers cells would initiate apoptosis. The major function of this organelle network is to produce ATP within the cell it relies on aerobic mechanisms to do so. Aggressive tumors are often highly hypoxic, they are reliant on anaerobic glycolysis for ATP generation (154). In this light, increased mitochondrial content in RMS cells could indicate a shift from cancer promoting conditions to detrimental ones. COXIV (cytochrome c oxidase subunit IV) is an often used marker of overall mitochondrial content within a cell. COXIV protein levels were shown to increase in electrically stimulated myoblasts (93). In RMS cells

besides imposing cell cycle arrest, enhancing myogenic differentiation may also contribute to increasing the mitochondrial content as in myoblasts. However, since RMS cells are also cancerous increased mitochondrial content might contribute to cell death induction. Yet, electrical stimulation of RMS cells did not induce any changes in COXIV protein levels (Fig S4). This evidence further corroborates the notion that RMS cells do not behave as myoblasts when subjected to electrical stimulation.

Based on the results obtained following electrical stimulation of the myogenic rhabdomyosarcoma cells, which indicate that these cells respond differently from myoblasts, we tested the application of this protocol towards non-myogenic PC3 prostate cancer cells. After 5 days of electrical stimulation there is a drastic decrease in the number of prostate cancer cells (Fig S5). This result demonstrates that electrical stimulation does not necessarily relies on myogenic properties to induce phenotypical changes. Furthermore, this potentially opens an avenue to future applications of this intervention in other cancer types, not limited to the myogenic RMS cells.

7.5. Discussion

Electrical stimulation induces rapid phenotypical changes in skeletal muscle that mimic those seen during adaptation to chronic contractile activity. While myoblasts undergo enhanced cell cycle arrest and differentiation in response to ES, we show that RMS cells respond differently. This challenges the notions that RMS is truly myogenic in nature. Despite this, ES of RMS cells did drastically reduce the number of these cells which is a positive outcome. These cells respond in a way distinct from myoblasts but do show signs of arrested proliferation and enhanced cell death. This raises the possibility that other non-myogenic cancer cells may respond likewise to electrical stimulation.

ES induces distinct and rapid cell cycle responses in RMS cells with an apparent shift of the population from G_0/G_1 to G_2/M. This could be due to a specific arrest of cells in G_2/M prior to undergoing autophagy and consistent with the lack of G_1/S arrest. The observed arrest could be due to a number of factors including lack of Cyclin B/CDK1 induction, insufficient Anaphase Promoting Complex activity, or deficiencies in the separation of the sister chromatids in mitosis (13). Further experiments are required to evaluate the exact nature of this ES-dependent effect. Nonetheless, ES is likely to restrict the progress through the cell cycle and help inhibit proliferation and induce autophagy, both of these processes contribute to the observed reduction in cell numbers. It was previously demonstrated that electromagnetic forces are able to disrupt the separation of the sister chromatids during mitosis and cause cell cycle arrest and cell death in various cancer cells (155, 156). We show similar effects of ES on RMS cells with further evidence of autophagy activation which leads to cell death. Akt is necessary for cell cycle progression through the G_2/M into the following G_1 stage, also kinase activity is enhanced during this step and when it was inhibited cell death was prevalent (139, 157, 158). We show that ES

promotes Akt activation, as indicated by measuring $pAkt^{T308}$ levels. In contrast, the G_2/M progression is inhibited. The reasons for this are unclear, but Akt kinase activity may not be actually increased in despite the raised levels of $pAkt^{T308}$ in ES RMS cells. Ultimately, the increase in $pAkt^{T308}$ may in fact be a response of the cell to prevent the ES-dependent effects towards autophagy induction, as Akt mediates the activation of mTOR complex which in turn inhibits autophagy (33, 139). Further work needs to be conducted to establish the specific effects of Akt activation in RMS cells following ES.

Deficiencies in the apoptotic pathway and DNA damage response are common in tumors (15, 96). p53 is a critical mediator of DNA damage response and is mutated in both RD and Rh-30 cells (124). This may explain the observed lack of apoptosis in electrically stimulated RMS. We show that despite p53 deficiencies ES activated autophagy, giving RMS cells an alternative mechanism to induce cell death which represents an attractive target for therapeutic intervention (20, 159). Lipidation of LC3 is often used to evaluate the autophagy pathway, as it is an event critical to the maturation of the autophagosome (31, 34). The autophagosome in turn fuses with a lysosome to degrade the cytoplasmic cargo it contains, while excessive degradation of cellular components leads to cell death (31, 33). Leading up to the conversion of LC3-I into LC3-II, the Beclin 1 complexes must be activated (33). Beclin 1 insufficiency contributes to the development of some cancers (160), yet it is also extensively regulated at the protein level through post-transcriptional modification as well as protein-protein interactions (33). Also, Beclin 1 contains a BH3-only domain and the activity of this protein is inhibited through the binding of the anti-apoptotic Bcl-2 factors (33). This mechanism may permit Akt mediated intervention in Beclin 1 activation. Akt phosphorylates and inhibits the pro-apoptotic BAD factor which binds and sequesters the anti-apoptotic Bcl-2 protein, thus preventing it's the downstream effects (29, 139).

This may explain why we observed ES-induced changes in LC3 yet no effects on Beclin 1. With Akt being activated in response to ES in RMS cells, this may have prevented the induction of Beclin 1 with no inhibitory effects on LC3. This allowed cell death to proceed as indicated by the observed decrease in RMS cell numbers following ES. This result may also suggest the LC3 plays a more critical role in autophagy activation than Beclin 1.

The expression of factors essential for myogenesis is a well-documented feature of RMS, yet the contribution of the myogenic regulators to the manifestation of this cancer type remains undefined. The MRF myogenin regulates the expression of genes necessary for the late stages of differentiation and myoblast fusion (44). RMS tumors express myogenin, however its transcriptional activity was shown to be inhibited in PAX3/FOXO positive cells as a results of a direct phosphorylation by GSK3β (140). ES-induced activation of Akt inhibits GSK3β, alleviating its suppressive effects on myogenin and consequentially increasing its transcriptional activity, and decreasing proliferation in aRMS Rh-30 cells (140). Furthermore, PAX3 transcriptional activity is known to inhibit differentiation in myoblast (49, 57). Given that aRMS cells express a constitutively active chimeric protein this may also be occurring in aRMS cells. The FOXO protein is phosphorylated by Akt which leads to its inactivation by translocation out of the nucleus (143). The regulatory domain of FOXO is part of the PAX/FOXO chimeric protein and was initially thought to be unresponsive to Akt-dependent regulation because it only possesses 2 out of the 3 phosphorylation sites (143). However, it was recently demonstrated that Akt does in fact modulate the activity of PAX/FOXO (144, 145), since ES promotes Akt activation in Rh-30 cells it might serve as a potential therapeutic intervention (144, 145). As opposed to Akt mediated inhibition of cell death pathways which would enhance survival, ES

mediated effects towards myogenin and PAX/FOXO in aRMS cells further prevent proliferation and invasiveness.

Given our results it appears that ES of RMS represents a potential novel therapy. ES induces G_2/M arrest which is accompanied by cell death via autophagy. Concomitant activation of Akt further reduces the proliferation of Rh-30 cells by inhibiting GSK3β which represses myogenin transcriptional activity. Moreover, ES may only affect proliferating cells as shown with the application of electric fields (155, 156), making unwanted side effects minimal.

7.6. **Figure Legends**

Figure 1. Electrical stimulation of RD cells induces cell cycle changes. Stimulation (ES) of RD cells over 5 days progressively decreased cell numbers, altered cell morphology **(*A*)** and caused an accumulation of cells in the G_2/M phase of the cell cycle **(*B*)** compared to non-stimulated control (CTL) RD cells. **(*C*)** Graphical summary of the percentages of cells in each phase of the cell cycle in response to 1, 3 and 5 days of ES. All values are mean ± S.E.M.; *p<0.05 compared to same day CTL (n=4).

Figure 2. Electrical stimulation of Rh-30 cells induces cell cycle changes. Following 2 or 4 days of electrical stimulation (ES) Rh-30 cells display decreased cell numbers and altered cell morphology compared to non-stimulated control (CTL) cells ***(A)***. FACS profiles of Rh-30 cells indicate a shift towards G_2/M in response to ES ***(B, C)***. All values are mean ± S.E.M.; *p<0.05 compared to same day CTL (n=3-5).

Figure 3. Electrical stimulation affects AMPK, p27 and Cyclin E in RMS cells. Electrical stimulation **(**ES) decreases $pAMPK^{T172}$ protein levels in RD cells while concomitantly decreasing p27 and increasing Cyclin E protein levels ***(A)***. ES increased $pAMPK^{T172}$ protein levels in Rh-30 cells but no ES induced changes in protein levels of p27 and Cyclin E were observed ***(B)***. β-actin was used to correct for protein loading. Proteins in stimulated cells (S) were quantified relative to those in non-stimulated control (C) cells. All values are mean ± S.E.M.; *p<0.05 compared to Day 0; **p<0.05 main effect of one-way AVOVA (n=5).

Figure 4. Electrical stimulation activates autophagy in RMS cells. ES leads to conversion of LC3-I (upper arrow) into the active LC3-II (lower arrow) in both RD ***(A)*** and Rh-30 ***(B)*** cells. Protein levels of Beclin 1, another autophagy mediator, remained unchanged by ES ***(A, B)***. β-actin was used to correct for protein loading. Proteins in stimulated cells (S) were quantified

relative to those in non-stimulated control (C) cells. All values are expressed as mean ± S.E.M. *p<0.05 compared to Day 0 (n=3-5).

Figure 5. Electrical stimulation of RMS cells activates Akt and affects myogenic regulatory factors. ES activates Akt ($pAkt^{T308}$) in RD cells ***(A)***. In addition ES decreased myogenin and increased MyoD in RD cells ***(A)***. Similar responses to ES were observed in Rh-30 cells ***(B)***. β-actin was used to correct for protein loading. Proteins in stimulated cells (S) were quantified relative to those in non-stimulated control (C) cells. All values are expressed as mean ± S.E.M. *p<0.05 compared to Day 0 (n=3-5).

7.7. **Figures**

Figure 1

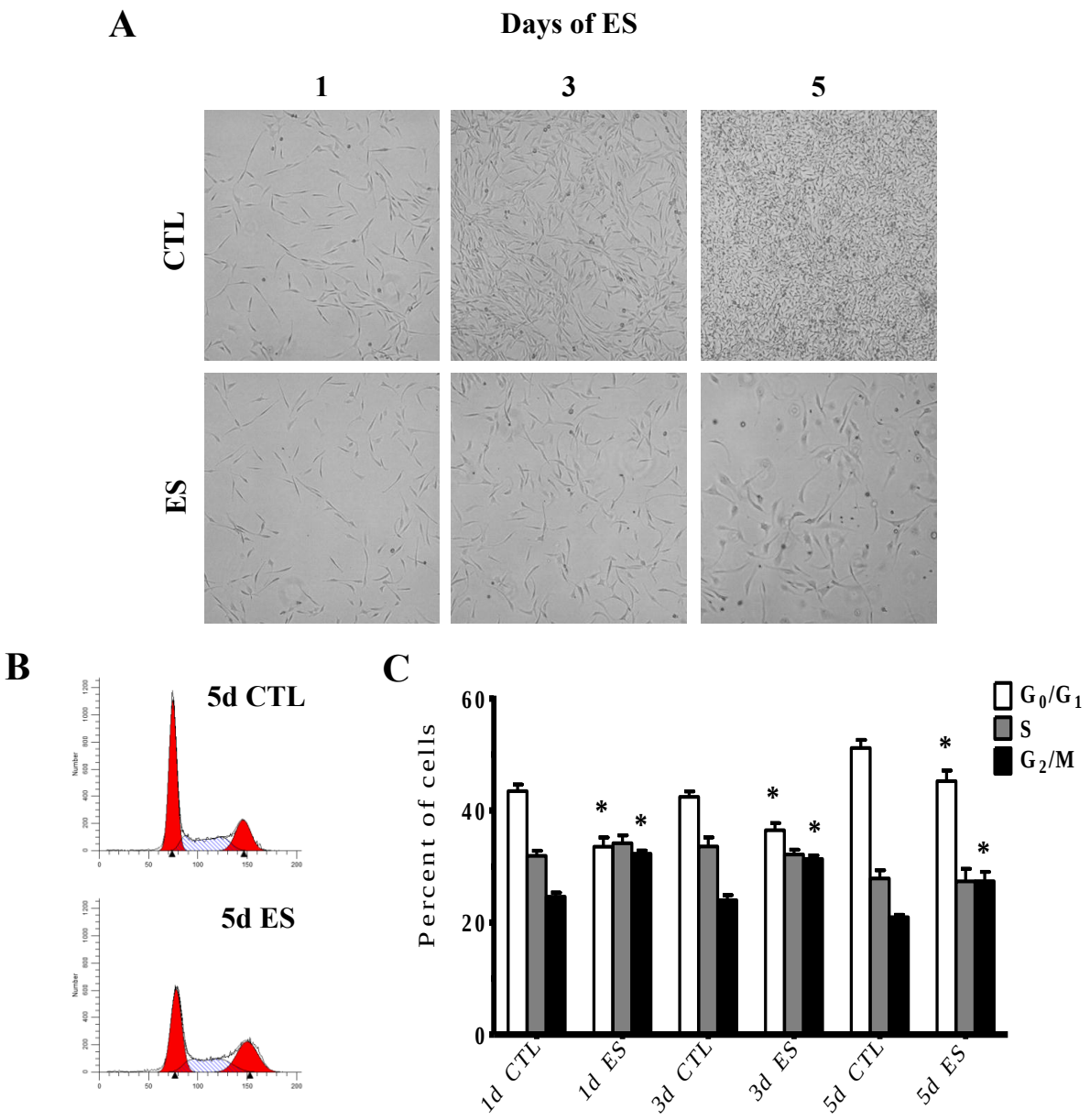
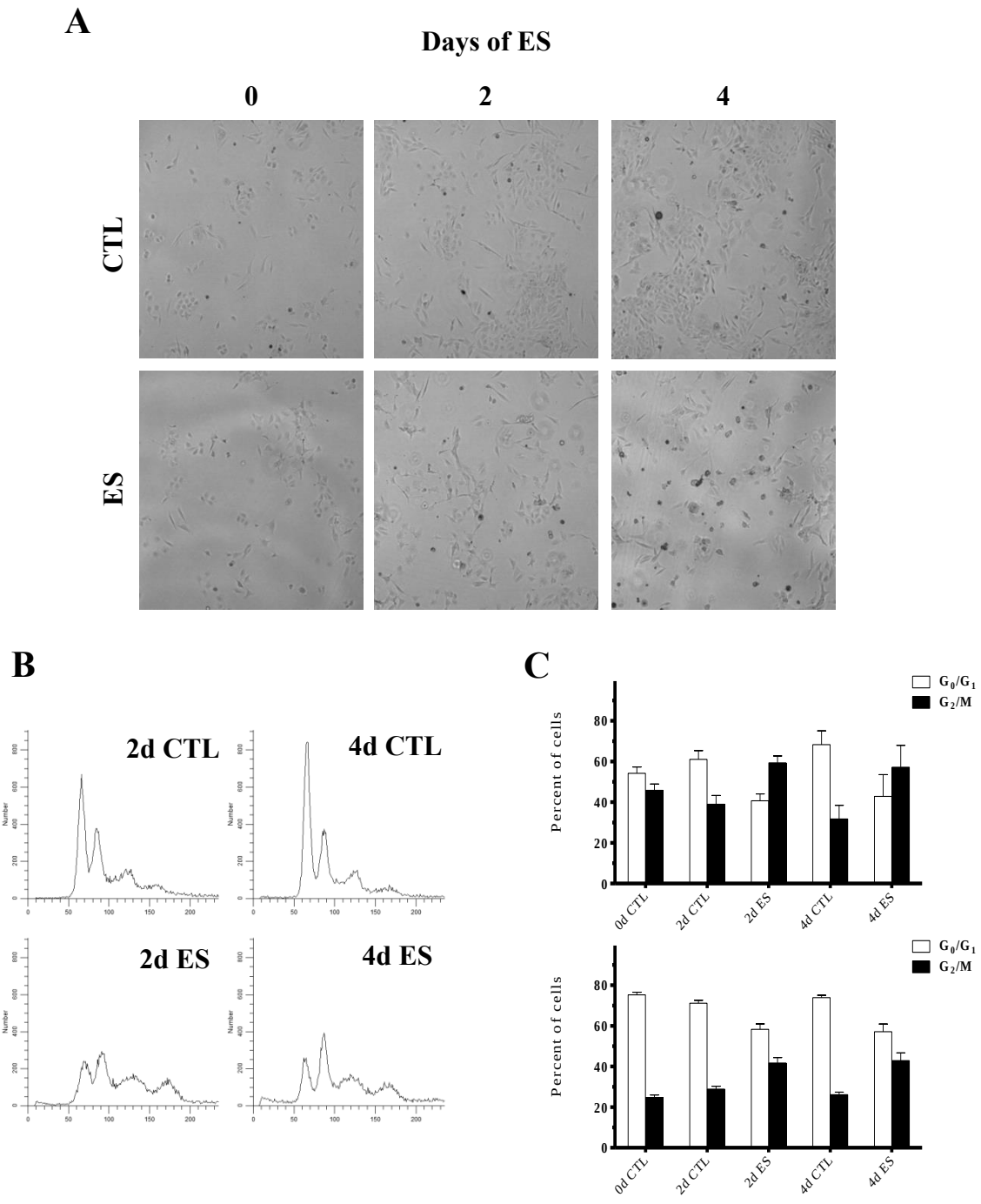


Figure 2


A
Days of ES
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2
4
CTL
ES
B
2d CTL
4d CTL
2d ES
4d ES
Number
C
Percent of cells
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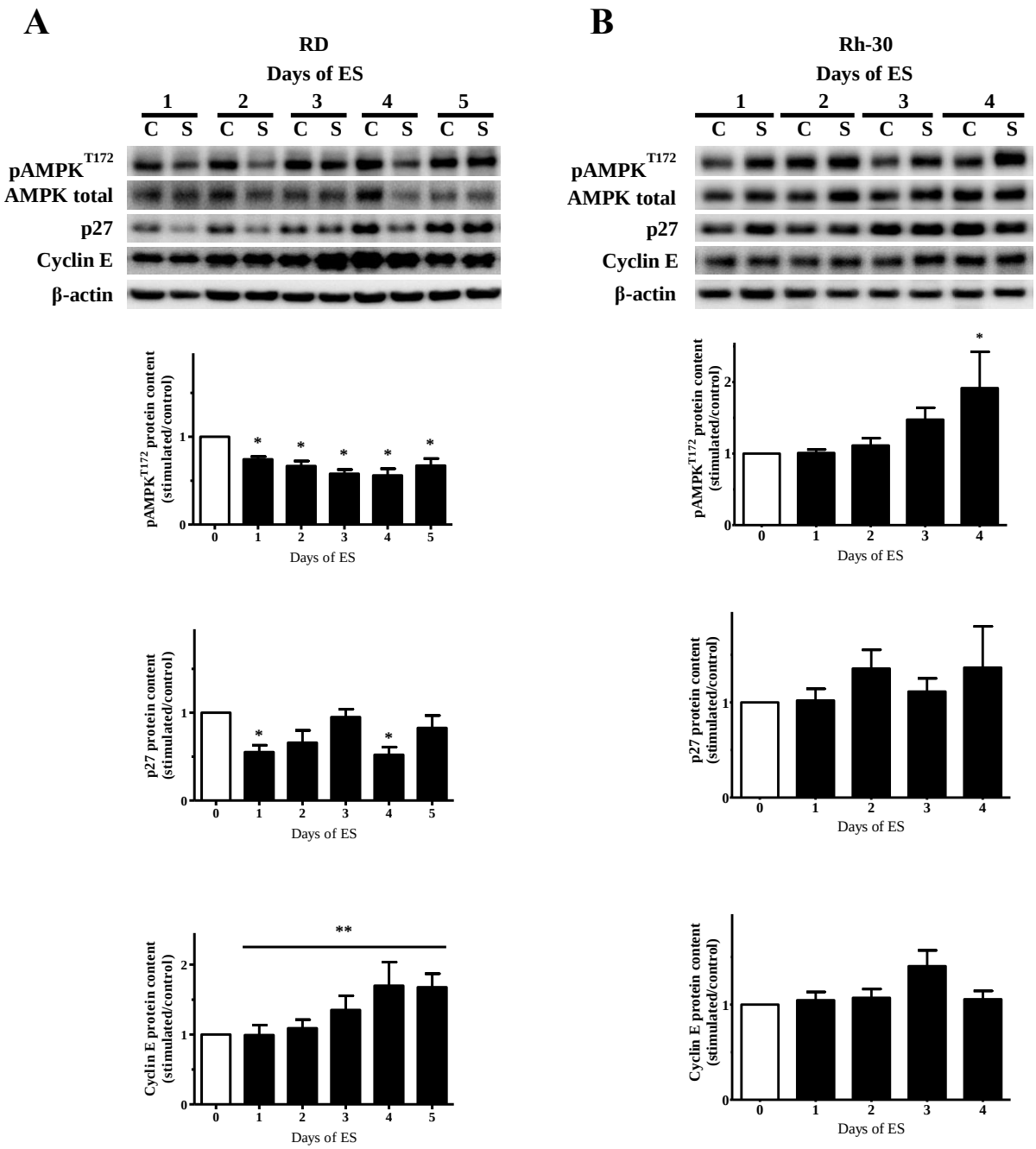
4d ES

Figure 3


A
RD
Days of ES
1 2 3 4 5
C S C S C S C S C S
pAMPK T172
AMPK total
p27
Cyclin E
β-actin
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p27 protein content (stimulated/control)
Cyclin E protein content (stimulated/control)
Days of ES
B
Rh-30
Days of ES
1 2 3 4
C S C S C S C S
pAMPK T172
AMPK total
p27
Cyclin E
β-actin
pAMPK T172 protein content (stimulated/control)
p27 protein content (stimulated/control)
Cyclin E protein content (stimulated/control)
Days of ES

Figure 4

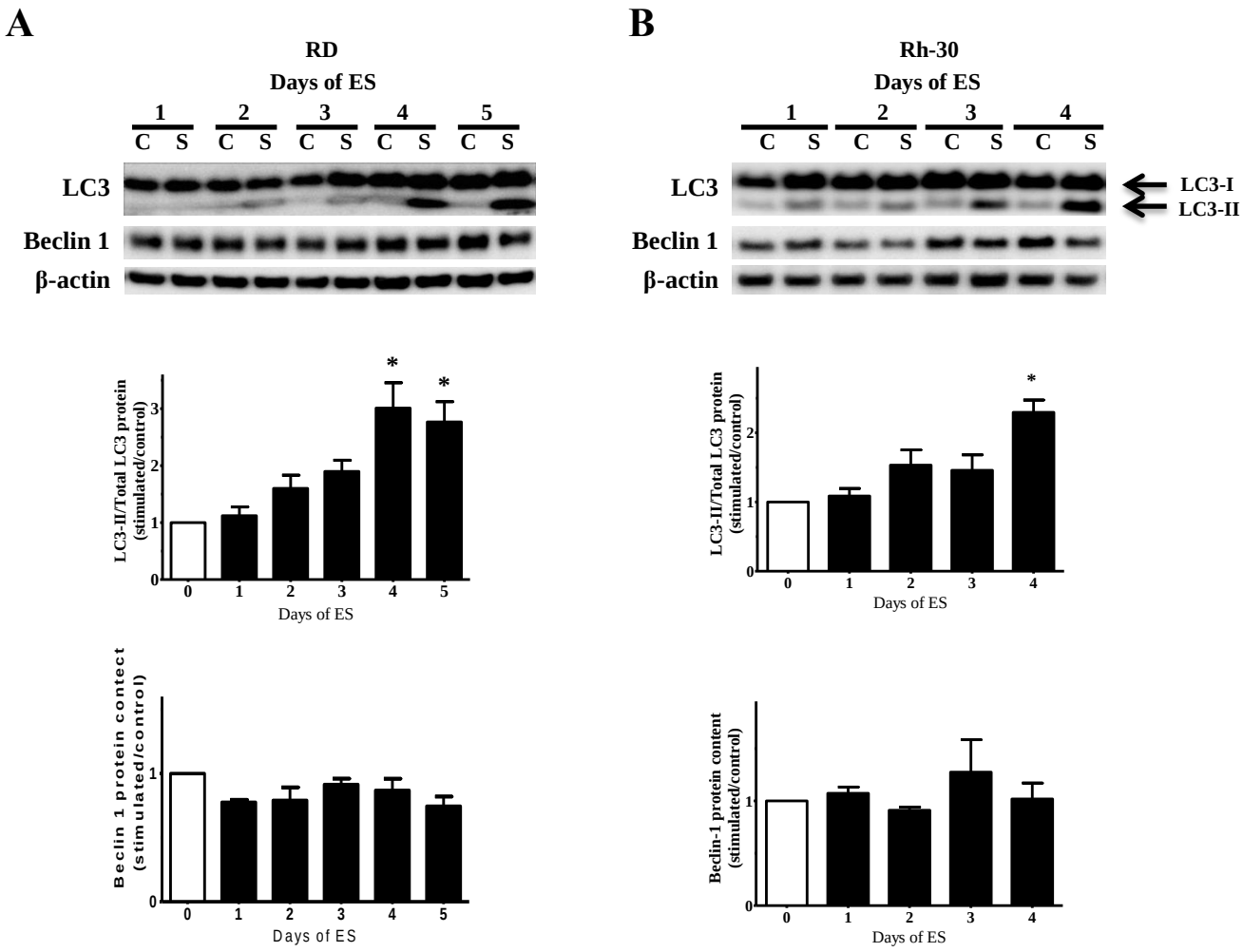
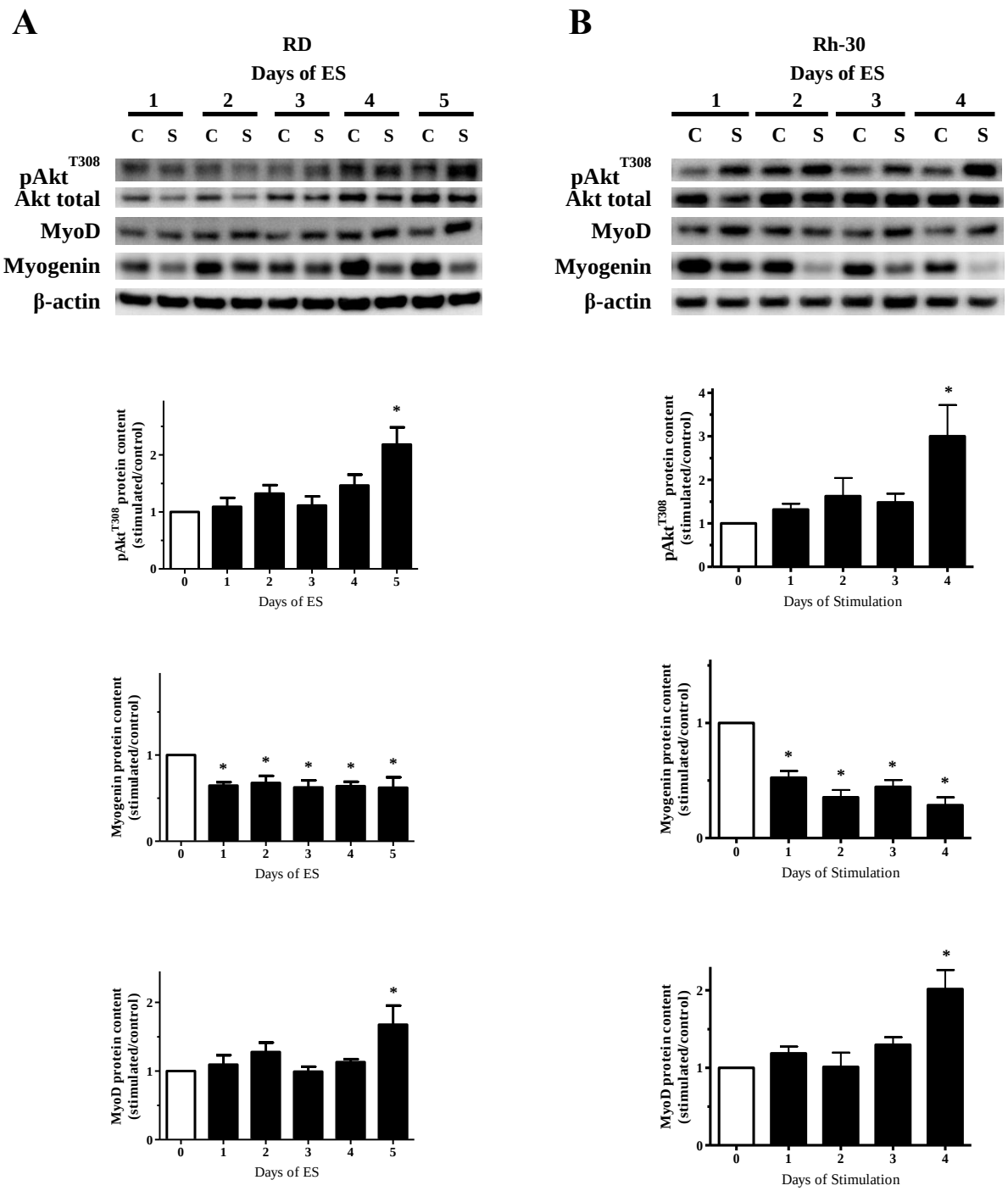

A
RD
Days of ES
1 2 3 4 5
C S C S C S C S C S
LC3
Beclin 1
β-actin
LC3-II/Total LC3 protein (stimulated/control)
Days of ES
Beclin 1 protein contect (stimulated/control)
Days of ES
B
Rh-30
Days of ES
1 2 3 4
C S C S C S C S
LC3
LC3-I
LC3-II
Beclin 1
β-actin
LC3-II/Total LC3 protein (stimulated/control)
Days of ES
Beclin-1 protein content (stimulated/control)
Days of ES

Figure 5


A
RD
Days of ES
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Akt total
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Myogenin
β-actin
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Days of ES
Myogenin protein content (stimulated/control)
Days of ES
MyoD protein content (stimulated/control)
Days of ES
B
Rh-30
Days of ES
1 2 3 4
C S C S C S C S
pAkt T308
Akt total
MyoD
Myogenin
β-actin
pAkt T308 protein content (stimulated/control)
Days of Stimulation
Myogenin protein content (stimulated/control)
Days of Stimulation
MyoD protein content (stimulated/control)
Days of Stimulation

7.8. **Supplemental Figures**

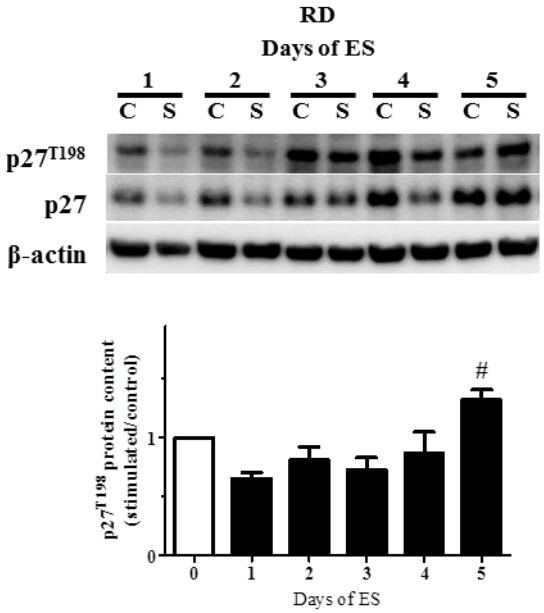

Figure S1. $p27^{T198}$ is a direct phosphorylation target of pAMPK, following this post-transcriptional modification protein stability is increased. β-actin was used to correct for protein loading. Proteins in stimulated cells (S) were quantified relative to those in non-stimulated control (C) cells. All values are expressed as mean ± S.E.M. *$p<0.05$ compared to Day 0, #$p<0.05$ compared to days 1, 2, and 3, n=5.

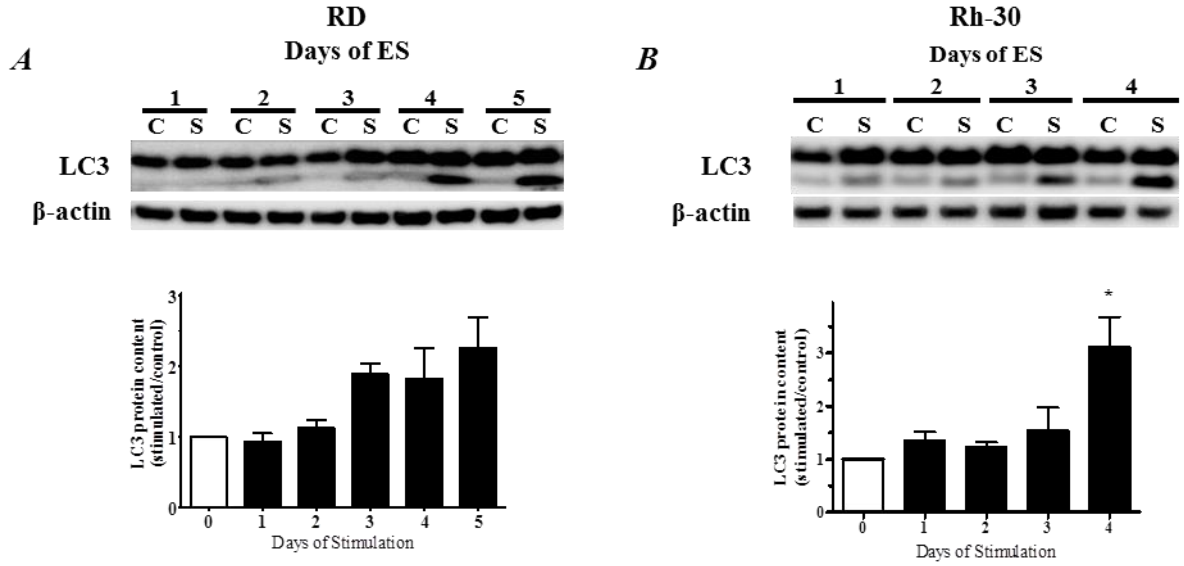

Figure S2. Total LC3 protein levels in electrically stimulated RD **(A)** and Rh 30 **(B)** cells. β-actin was used to correct for protein loading. Proteins in stimulated cells (S) were quantified relative to those in non-stimulated control (C) cells. All values are expressed as mean ± S.E.M. *p<0.05 compared to Day 0, n=2 in RD and n=4 in Rh-30 cells.

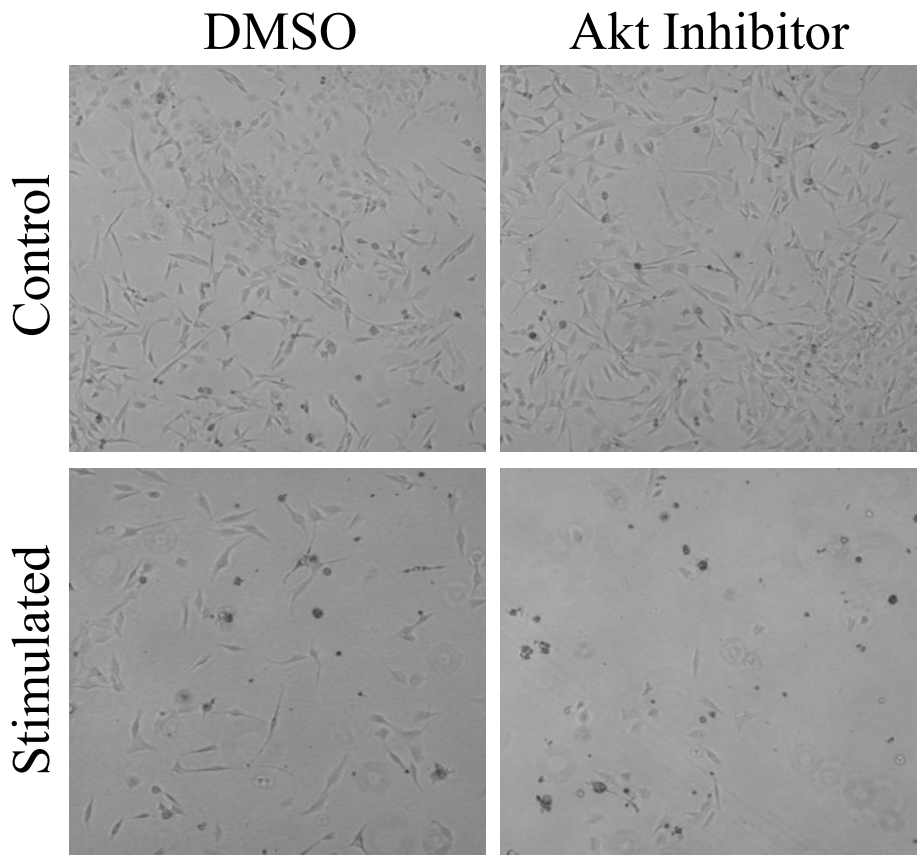

Figure S3. Rh-30 cells following 4 days of electrical stimulation or control condition with and without pharmacological Akt inhibition. Cells were treated with 10 μM of 1L6-Hydroxymethyl-chiro-inositol-2-(R)-2-O-methyl-3-O-octadecyl-*sn*-glycerocarbonate a commercially available, specific inhibitor of Akt activation. The drug prevents binding of the Akt protein to its upstream activator via competitive interaction. Dimethyl sulfoxide (DMSO) was used as vehicle control. The drugs were replenished every 48 hours, prior to the stimulation.

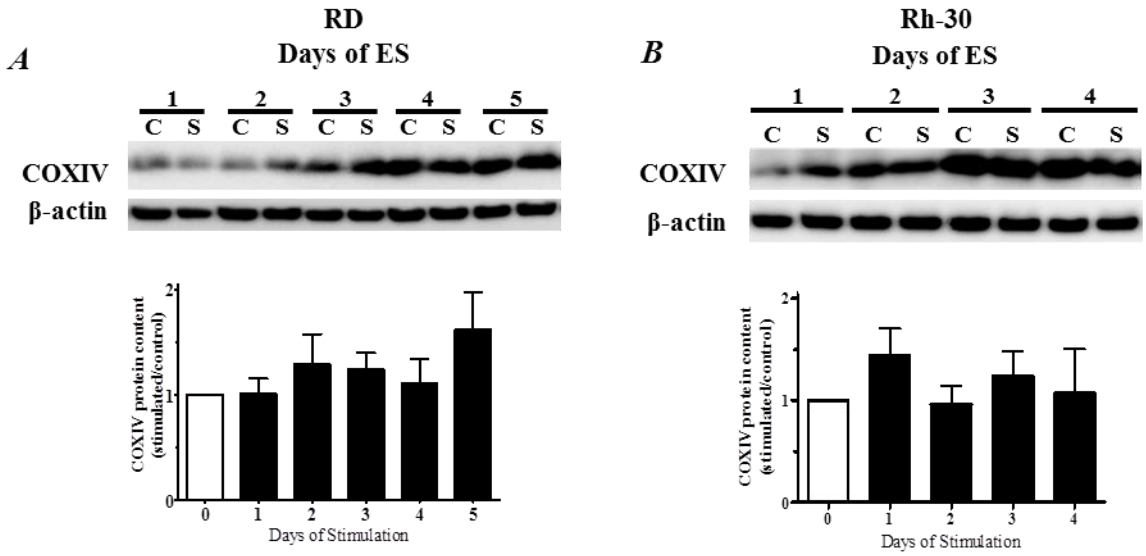

Figure S4. COXIV was measured to help reflect on the changes in the mitochondrial content in electrically stimulated cells. β-actin was used to correct for protein loading. Proteins in stimulated cells (S) were quantified relative to those in non-stimulated control (C) cells. All values are expressed as mean ± S.E.M. *$p<0.05$ compared to Day 0, n=5 in RD **(A)** and n=3 in Rh-30 **(B)** cells.

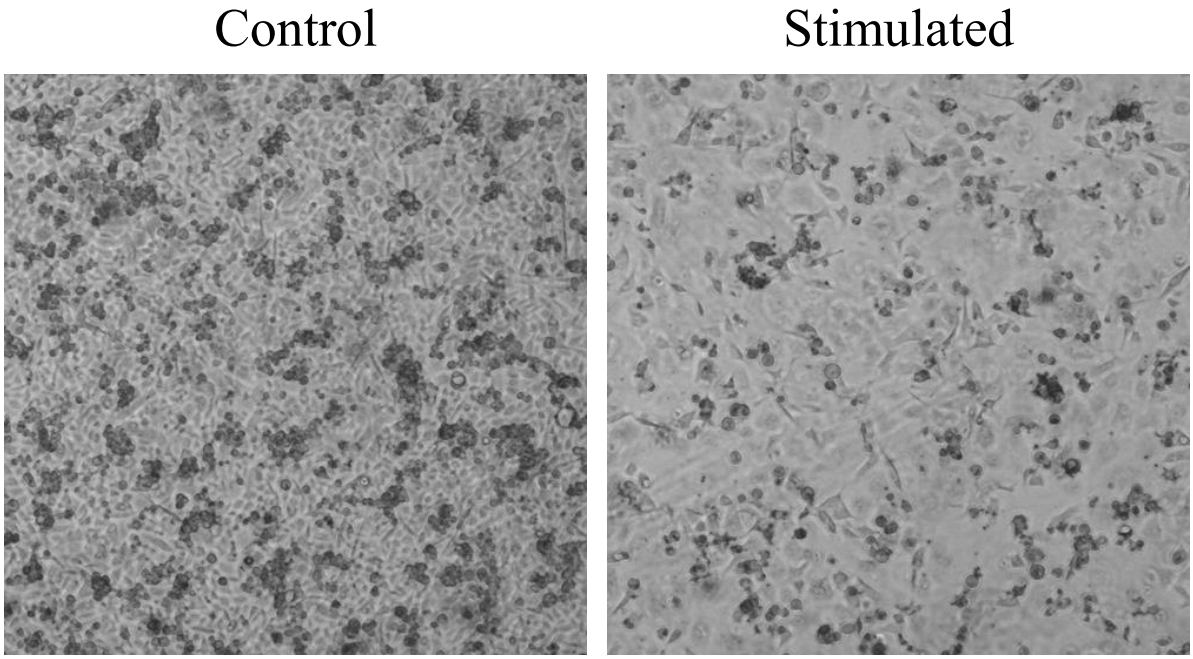

Figure S5. PC3 cells, a non-myogenic prostate cancer cell line, following 5 days of electrical stimulation or control conditions.

8. Conclusions

The nature of RMS tumors as both oncogenic and myogenic offers a unique opportunity to capitalize on the knowledge of muscle biology and apply it in a cancer context. Cancer is a disease that most often presents itself in the aged population and is frequently classified on the basis of the tissue it originates from. RMS tumors are statistically rare and they manifest themselves during childhood and adolescence and in a variety of locations. However, at the molecular level most cancers, including RMS, exhibit some common set of molecular determinants which shape the malignancy. Unlike other cancer forms RMS tumors express a range of factors implicated in the myogenic differentiation program. While, myogenesis is a popular research field and numerous approaches exist to intervene in the molecular mechanisms which regulate this process, few are applied to RMS. The differentiation program is deficient in RMS cells and the potential of therapeutic strategies aimed towards myogenic regulators is questionable. In this thesis the use of the electrical stimulation on RMS cells was evaluated, as previously it was shown to induce cell cycle arrest and differentiation in myoblasts.

Electrical stimulation induces arrest of proliferation and cell death in both subtypes of RMS, it also leads to obvious morphological changes. However, the alterations observed in RMS cells following electrical stimulation are unlike the ones observed in myoblasts. In myoblasts electrical stimulation induces cell cycle arrest likely at the G_1/S transition mediated by an increase in p27 protein levels and decrease in Cyclin E. In RMS cells such changes were not induced by electrical stimulation, and in agreement cell cycle profiling indicated no build up in G_1/S but instead there was an accumulation of cells in the G_2/M phase. It is unclear at this point which molecular mechanisms mediate this cell cycle alteration, nor whether the cells arrest at the G_2/M transition or during M phase.

Programmed cell death pathways can be initiated in response to cell damage and repair must take place in a timely manner to ensure survival. This fact is utilized in many cancer therapies as the induction of massive DNA damage in tumors can lead to induction of cell death pathways and destruction of tumor cells. However, the regulators of cell death pathways are often deficient in cancers. p53 is an important regulators of cellular responses to stress and it is often mutated in RMS, including RD and Rh-30 cell lines. Both apoptosis and autophagy were evaluated in electrically stimulated RMS cells as these cell death modes could potentially contribute to the cell loss elicited by electrical stimulation. At this point it is unclear if cell cycle precedes cell death in electrically stimulated RMS cells. The G_2/M accumulation was observed at the earliest time point of electrical stimulation in both cell lines studied, yet signs of apoptotic cell death were not detected. However, the autophagy pathways protein LC3 was activated by day 4 in both RD and Rh-30 cells. Nevertheless, both cell cycle arrest and cell death are implicated in the apparent decrease in RMS cell numbers following electrical stimulation.

The Akt pathway fulfills a diverse range of roles important for cellular functions with some individual effects having conflicting implications in RMS cells. In cancer cells excessive Akt activation is often observed and it contributes to proliferation and evasion of cell death. Conversely, in myogenesis Akt is required for differentiation to take place. In an earlier study we report that myogenin transcriptional activity is prevented through the influence of GSK3β in PAX3/FOXO positive aRMS cells. Inhibition of GSK3β alleviated the suppression of myogenic gene expression and decreased both proliferation and survival of Rh-30 cells (140). We demonstrated that electrical stimulation induces Akt activity, inhibits GSK3β, and activates the expression of myogenin targets (140). Separately, Akt activity towards the PAX/FOXO fusion gene was shown to inhibit growth and invasiveness while also inducing cell death in aRMS cells

(144, 145), a process likely rooted in the myogenic traits of these cells. In conventional tumor cells Akt signalling promotes proliferation and survival, however since RMS possess myogenic properties, Akt also mediates effects with contribute to enhanced differentiation, growth arrest and cell death. Thus, Akt actions in this myogenic context are in direct conflict with its pro survival function, and it is not clear which option is more advantageous in preventing the growth of RMS tumors if this kinase was to be targeted through a pharmacological approach. Electrical stimulation on the other hand likely acts through numerous mechanisms as changes in cell cycle, survival and myogenic status all take place in conjunction with Akt activation. The relative effectiveness of the contribution of Akt mediated changes in myogenic status to the overall phenotype observed in electrically stimulated Rh-30 cells is yet to be determined. However, preliminary evidence suggests that pharmacological inhibition of Akt coupled with electrical stimulation might further enhance the degree of cell loss. Inhibition of GSK3 was recently shown to lead to cell death in eRMS cells as well (141). However, neither GSK3 analyses nor Akt inhibition are have been completed in RD cells following electrical stimulation.

The fact the RD and Rh-30 cell do not exhibit a response comparable to the one observed previously in C2C12 myoblasts questions the merit of treating RMS cells as myogenic. However, the nature of the response and the fact it is unlike the one elicited in myoblasts puts forth an interesting possibility that other non-myogenic cancer cells could respond in a manner similar to RMS cells when subjected to electrical stimulation. To test this possibility we electrically stimulated a non-myogenic prostate cancer PC3 cell line and the preliminary evidence points to a decrease in the number of cells after 5 days of this treatment. Further experiments are required to elaborate on this line of evidence as it might carry significant implications on cancer therapeutic approaches.

9. Limitations and Future Directions

Evidence obtained so far, taken together with the main hypotheses of this thesis, help identify a number of prospective areas that need to be further investigated. Other areas could also be addressed to help strengthen the validity of the collected data. *In-vitro* application of the electrical stimulation model proved promising but not without its out challenges. Ultimately while the outcomes observed *in-vitro* provide an exciting opportunity to investigate the molecular properties of RMS cells, the potential of electrical stimulation as a therapeutic intervention will depend of whether or not it can be successfully and effectively applied *in-vivo*. Moreover, in light of the results obtained to date it is important to test whether the effects electrical stimulation on RMS cells could also be consistently replicated in other non-myogenic cancer cell lines or if they are unique to RMS. It is also imperative to establish a baseline through the use of non-cancerous yet dividing cells, besides the aforementioned myoblasts.

Even though the loss of RMS cells following electrical stimulation is clear and both cell cycle arrest and cell death appear to be implicated, further experiments are required to strengthen our grasp of these events. Originally it was hypothesized that the G_1/S transition will be prevented following stimulation if the myogenic RMS cells behave similar to myoblasts, this was not the case. An immunoprecipitation of Cyclin E bound p27 in combination with p27 cellular localization assays would add to the strength of the conclusion that could be made about this cell cycle stage. FACS results strongly suggest a G_2/M accumulation of cells following stimulation, yet it is not clear what molecular mechanisms are responsible for that. Cyclin B/CDK1 and APC activity, as well as the mitotic spindle assembly process could all be assessed to further evaluate the nature of the observed G_2/M arrest. Also, it might be important to distinguish if the cells arrest at the G_2/M transition or during Mitosis. Nevertheless, p27 does also inhibit the Cyclin

B/CDK1 complexes which regulate the transition stage. The inability to detect the $p27^{T198}$ in Rh-30 cells, and the other important CDK inhibitor p21 in both RMS cell lines, leave gaps in our understanding of the mechanism responsible for the cell cycle effects of electrical stimulation.

Electrical stimulation of RMS cells causes the activation of Akt signalling. While the activity of this kinase is believed to prevent cell death and contribute to cell cycle progression, it may also serve a different role in myogenic and Rh-30 cells by promoting differentiation. Pharmacological inhibition of Akt may further decrease the number of cells remaining following electrical stimulation. The full consequences of Akt inhibition in Rh-30 cells, as well as in the RD line, are yet to be determined. It is important to further establish that by combining stimulation and pharmacological inhibition of Akt activation. Furthermore, recent evidence points to the contributions of calcium signalling to Akt activity and apoptosis in aRMS cells (145). Chelation of calcium out of electrically stimulated myoblasts attenuated cells cycle arrest (93). Together, these observations put forward a notion that calcium signalling could act as the mediator of the responses RMS cells exhibit following electrical stimulation. However, further research is required to establish the possibility of such connection. At this stage it is not clear what mechanisms contribute to the activation of the Akt pathway. Often this kinase is activated downstream of the growth factor mediated receptors, yet this is not the case in electrically stimulated RMS cells.

GSK3β activity is important to the manifestation of the tumorigenic state in PAX/FOXO positive cells. As we demonstrate Akt mediated inhibition of GSK3β in Rh-30 cells helps alleviate the suppression of myogenin transcriptional activity. Changes in E box driven promoter reported activity was used to help assess myogenic differentiation status. Yet, these experiments could be revisited to offer a more comprehensive outlook on the myogenic changes occurring in

these cells. While after 2 days of electrical stimulation E box driven luciferase expression was statistically increased, the full length MCK promoter activity did not show any changes. To expand our understanding measurements of myogenic gene expression in electrically stimulated aRMS cells at additional time points including day 0 and day 4 should be conducted. Moreover, the enhanced activation of the E box but not the full length MCK promoter driven reporter gene expression implicates the participation of other transcriptional regulators that impose the differentiation block in aRMS. Finally, E box activation is not exclusively targeted by myogenin, it is also mediated by other MRFs such as MyoD. MyoD mediated transcriptional activity was not addressed in this project, yet taking into account the significant increase in the protein levels of this factor in electrically stimulated Rh-30 cells, it could serve as a potential topic for further research. Moreover, recently GSK3 was also shown to support the oncogenic state in eRMS cells, yet it is unclear how electrically stimulated RD cells respond to changes in this kinase. It is possible the myogenic program in eRMS cells is influenced similarly to Rh-30 cells in terms of GSK3 effects towards myogenic differentiation. Thus, it could provide an exciting opportunity to expand our understanding of the myogenic state in eRMS cells as well.

Appendix A

Glycogen synthase kinase 3β represses MYOGENIN function in alveolar rhabdomyosarcoma

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Glycogen synthase kinase 3β represses MYOGENIN function in alveolar rhabdomyosarcoma

MG Dionyssiou[1], S Ehyai[1], E Avrutin[2], MK Connor[2] and JC McDermott*[,1,3,4,5]

MYOGENIN is a member of the muscle regulatory factor family that orchestrates an obligatory step in myogenesis, the terminal differentiation of skeletal muscle cells. A paradoxical feature of alveolar rhabdomyosarcoma (ARMS), a prevalent soft tissue sarcoma in children arising from cells with a myogenic phenotype, is the inability of these cells to undergo terminal differentiation despite the expression of MYOGENIN. The chimeric PAX3-FOXO1 fusion protein which results from a chromosomal translocation in ARMS has been implicated in blocking cell cycle arrest, preventing myogenesis from occurring. We report here that PAX3-FOXO1 enhances glycogen synthase kinase 3β (GSK3β) activity which in turn represses MYOGENIN activity. MYOGENIN is a GSK3β substrate *in vitro* on the basis of *in vitro kinase* assays and MYOGENIN is phosphorylated in ARMS-derived RH30 cells. Constitutively active GSK3β(S9A) increased the level of a phosphorylated form of MYOGENIN on the basis of western blot analysis and this effect was reversed by neutralization of the single consensus GSK3β phosphoacceptor site by mutation (S160/164A). Congruently, GSK3β inhibited the *trans*-activation of an E-box reporter gene by wild-type MYOGENIN, but not MYOGENIN with the S160/164A mutations. Functionally, GSK3β repressed muscle creatine kinase (MCK) promoter activity, an effect which was reversed by the S160/164A mutated MYOGENIN. Importantly, GSK3β inhibition or exogenous expression of the S160/164A mutated MYOGENIN in ARMS reduced the anchorage independent growth of RH30 cells in colony-formation assays. Thus, sustained GSK3β activity represses a critical regulatory step in the myogenic cascade, contributing to the undifferentiated, proliferative phenotype in alveolar rhabdomyosarcoma (ARMS).

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Subject Category: Cancer

Rhabdomyosarcoma (RMS) is the most common pediatric soft tissue sarcoma, accounting for 5% of all childhood cancers and approximately 50% of soft tissue sarcomas.[1–3] There are two main subtypes: embryonal and alveolar RMS and although embryonal RMS is more common, alveolar RMS is considered to carry a worse prognosis. A gene fusion resulting in the *t*(2;13)(q35;q14) somatic cell chromosomal translocation fuses *PAX3* and *Foxo1* to create a potent transcription factor (PAX3-FOXO1) which is a predominant causative genetic lesion for the development of alveolar rhabdomyosarcoma (ARMS).[1] ARMS is a highly malignant mesenchymal tumor that has properties of immature striated muscle tissue resulting in dense aggregates of poorly differentiated cells that are separated by fibrous membranes resulting in a loss in cellular cohesion.[2,3] PAX3 is a key determinant of somatic myogenesis and, is involved in the migration of progenitor cells to the dermomyotome region of the somite where they grow and divide in the presence of growth factors.[4] PAX3 is also required to activate the myogenic determination gene, *MYOD*.[5] MYOD is one of four myogenic regulatory factors (MRFs, which include MYF-5, MRF4 and MYOGENIN) from the basic helix-loop-helix superfamily of transcription factors which interact with myocyte enhancer factor-2 (MEF2) proteins in the hierarchical control of muscle-specific gene expression.[6] Two kinases that potently exert effects on this myogenic regulatory cascade are p38 mitogen activated protein kinase (MAPK) and glycogen synthase kinase 3β (GSK3β). p38 MAPK is a key regulator of skeletal myogenesis that critically interacts with and activates MEF2 in the somite myotome during development.[7–9] Conversely, GSK3β activation leads to a repression in skeletal and cardiac muscle differentiation, in part by antagonizing p38 MAPK-mediated activation of MEF2.[10,11] GSK3β usually targets proteins that have already been phosphorylated by another kinase at a 'priming' serine or threonine residue located four amino acids C-terminal to a consensus (S/T)XXX(S/T)-PO_4 motif.[12,13] Regulation of MEF2 and the MRFs leads to morphological changes including epithelial to mesenchymal transition, cell alignment and fusion to form multinucleated myotubes that eventually develop into

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Keywords: alveolar rhabdomyosarcoma; PAX3-FOXO1; MYOGENIN; GSK3β; cell proliferation; tumorigenicity
Abbreviations: GFP, green fluorescent protein; GSK3β, glycogen synthase kinase 3β; Luc, luciferase; MAPK, mitogen activated protein kinase; MCK, muscle creatine kinase; MEF2, myocyte enhancer factor-2; PI3K, phosphoinositide 3-kinase; PKB, protein kinase B
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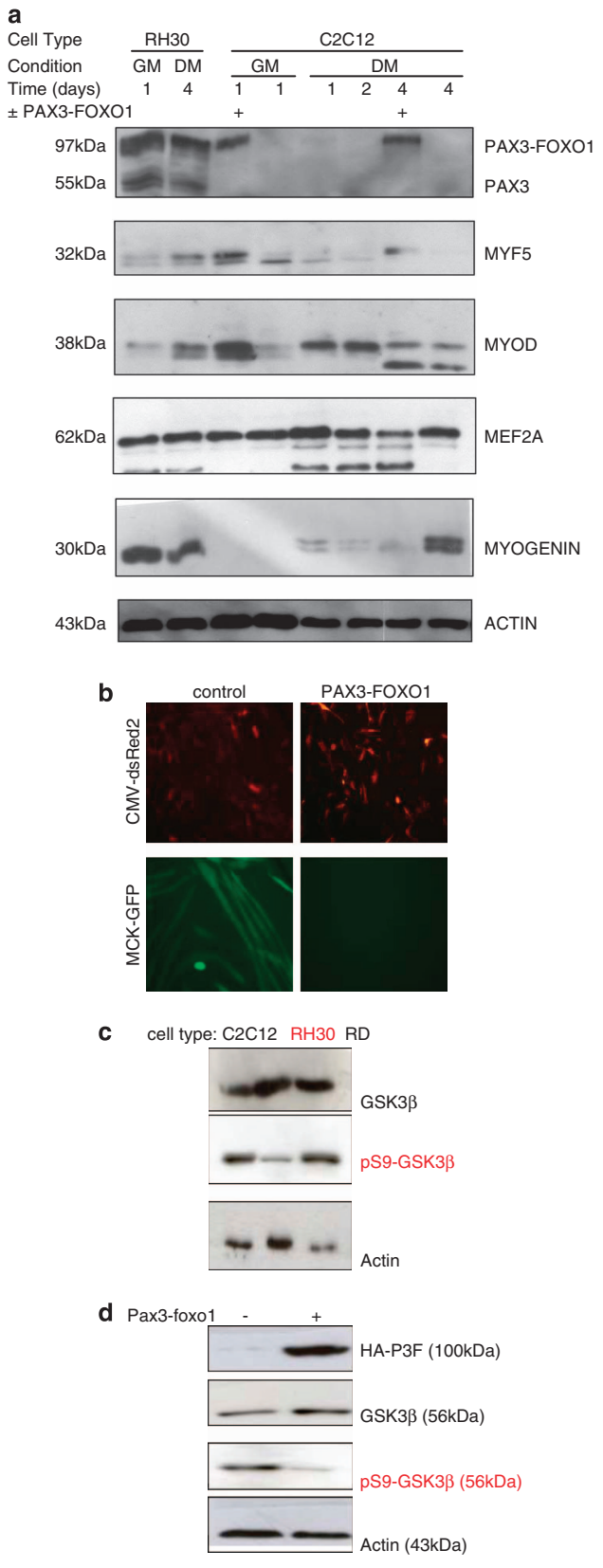

functional, contractile muscle fibers. In particular, cells that express MYOD and MYOGENIN are typically fusion competent[14,15] with the exception of ARMS cell types. To date, lack of myogenic differentiation of PAX3-FOXO1 expressing ARMS cells has been attributed to their inability to upregulate $p57^{Kip2}$ activity, hence destabilizing the DNA binding affinity of MYOD transcription complexes.[16] Dysfunctional MYOD/E-protein complex association and transcriptional control is a common feature between ARMS and the non-PAX3-FOXO1 expressing embryonal rhabdomyosarcoma (ERMS). Subsequent restoration of the MYOD/E12 complex has been shown to switch ERMS cells from an arrested myofibroblast phase to a more differentiated state.[17] Similarly p38 MAPK activity can potentiate myogenic differentiation in ERMS cells by enhancing MYOD *trans*-activation properties.[18] Therefore, it is fairly clear that in both rhabdomyosarcoma subtypes the ability of MYOD to potentiate transcription is compromised. However, the role of MYOGENIN in RMS is more equivocal. For normal myogenesis to occur, *both in vitro and in vivo,* an absolute requirement for MYOGENIN is evident. Thus, MYOGENIN activity constitutes a pivot point for irreversible commitment to terminal differentiation.[19,20] The combination of data from gene targeting studies of the MRFs[21,22] supports the prevailing consensus that while the other three MRFs can compensate each other's functional roles,[23–26] MYOGENIN is absolutely essential for skeletal muscle fiber formation.[20] Despite its expression in RMS, the paradox as to why MYOGENIN cannot mediate competence for differentiation is unknown.

Here, we examined the posttranslational regulation of MYOGENIN in ARMS. On the basis of the *in silico* prediction of a single consensus phosphorylation site for GSK3β on the MYOGENIN protein and also high levels of GSK3β activity in these cells, we determined that MYOGENIN function is potently repressed by GSK3β activity in ARMS. Moreover, pharmacological inhibition of GSK3β results in a profound decrease in size and, to a certain extent, number of RMS colonies in a colony-formation assay. This effect is mimicked by introduction of MYOGENIN bearing neutralizing mutations in the GSK3β consensus site. In combination, these data reveal MYOGENIN as a key target of GSK3β activity in

Figure 1 MYOGENIN protein expression and GSK3β activity are both maintained in ARMS: (**a**) C2C12 myoblasts were transfected with HA-PAX3-FOXO1 or pcDNA3.1 control plasmid for 1 day before extraction or serum withdrawal and then extraction at 1 day increments for up to 4 days as indicated. Protein levels were compared with protein extracts from PAX3-FOXO1 expressing RH30 cells 1 day in growth media (GM) and 4 days in differentiation media (DM). The results show that despite the expression of PAX3-FOXO1, RH30 cells also express MYOGENIN. On the other hand, HA-PAX3-FOXO1 overexpression in C2C12 inhibits MYOGENIN expression and subsequent myogenic differentiation. (**b**) C2C12 myoblasts were transfected with CMV-dsRed2, MCK-eGFP and, either HA-PAX3-FOXO1 or pcDNA3.1 control plasmid. HA-PAX3-FOXO1 overexpression repressed the formation of multinucleated myotubes. (**c**) Endogenous GSK3β protein levels and phosphorylation at serine 9 were compared in C2C12 myoblasts, RH30 and ERMS RD cells. Although GSK3β is expressed in all three cell types, it is predominantly phosphorylated and hence inactive in C2C12 myoblasts and RD cells but not PAX3-FOXO1 expressing RH30 cells. (**d**) C2C12 myoblasts were transfected with HA-PAX3-FOXO1 or pcDNA3.1 control plasmid for 1 day before extraction. Overexpression of HA-PAX3-FOXO1 resulted in decreased phosphorylation of GSK3β at serine 9 indicating its activation

ARMS, indicating that pharmacologic manipulation of this signaling axis may provide an opportunity for therapeutic intervention.

Results

MYOGENIN is expressed in PAX3-FOXO1 expressing RH30 cells. Serum (10% FBS) contains growth factors that repress the transcriptional activity of MRFs and also stimulate cell cycle progression hence rendering C2C12 myoblasts proliferative. In tissue culture, serum withdrawal (2% HS) results in activation of MEF2 and MRFs causing cell alignment and fusion to form multinucleated myotubes. Initially, in order to investigate the effect of PAX3-FOXO1 on this differentiation program, proliferating C2C12 myoblasts were transiently transfected with CMV-dsRed2, MCK-eGFP, and either HA-PAX3-FOXO1 or pcDNA3.1 control vector. Growth media (GM) was replaced with differentiation media (DM) 19 h after transfection and cells were allowed to differentiate for 96 h. SDS-PAGE samples were prepared from populations of myoblasts that either expressed or did not express PAX3-FOXO1, (a) before serum withdrawal (time = 0; GM = 10% FBS) and (b) at 24 h increments upon serum withdrawal (days 1–4; DM = 2% HS). Protein expression levels of these samples were then compared with protein samples from PAX3-FOXO1 expressing RH30 cells in GM and DM, by western blotting. These data indicate that despite the expression of PAX3-FOXO1, MYOGENIN protein expression is maintained in human ARMS-derived RH30 cells (Figure 1a). In addition, PAX3-FOXO1 repressed myotube formation in C2C12 myoblasts (Figures 1a and b). Detection of myogenic differentiation using an MCK promoter driving GFP expression[27] revealed GFP expressing, multinucleated myotubes in the controls but not in cells expressing PAX3-FOXO1 (Figure 1b).

It is well documented that MRFs and MEF2 proteins are highly sensitive to pro-myogenic kinases such as p38 MAPK[9,28–30] and also kinases such as GSK3β which are

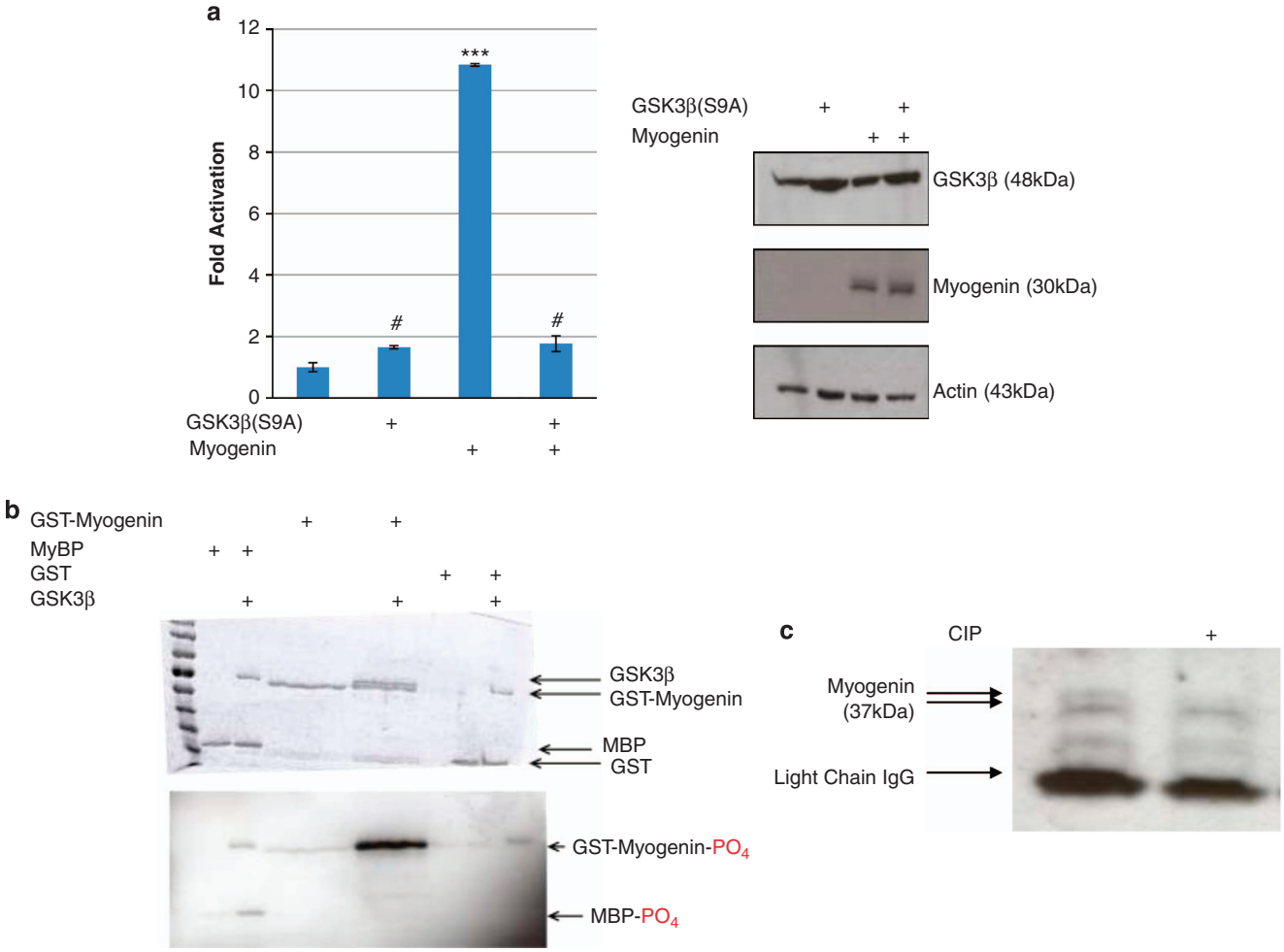

Figure 2 Overexpressed, constitutively active GSK3β (S9A) represses MYOGENIN *trans*-activation of E-box. (**a**) C2C12 myoblasts were transfected with 4x E-box Luc reporter and different combinations of HA-GSK3β(S9A) and MYOGENIN or pcDNA3.1 control plasmid as indicated. Overexpressed HA-GSK3β(S9A) repressed MYOGENIN transcriptional activity ($P<0.001$). (**b**) GSK3β directly phosphorylates MYOGENIN *in vitro*: Purified GST-MYOGENIN was incubated *in vitro* with GST-GSK3β and (γ-^{32}P) ATP. GST and MBP proteins were used as negative and positive control respectively as indicated. Bands were resolved using SDS-PAGE and visualized by Coomassie Blue staining. Gels were dried and exposed to X-ray film for 21 h after the assay. (**c**) Calf-intestinal phosphatase (CIP) treatment of immunoprecipitated MYOGENIN that was obtained from 1000 μg of RH30 protein extract. The data shows that CIP treatment causes a loss of a high-molecular weight, phosphorylated form of MYOGENIN. $^{\#}$ns, $^{***}P<0.001$

repressive to myogenesis.[10,31] Therefore we tested for GSK3β activity under conditions when myogenesis is supressed. As GSK3β is constitutively active until it is repressed by phosphorylation at serine 9 (by PKB), we assessed both total GSK3β protein expression levels and S9 phosphorylation levels using appropriate antibodies as indicated. We document that GSK3β is expressed in proliferative C2C12 myoblasts, PAX3-FOXO1 expressing ARMS cells (RH30) and, non-PAX3-FOXO1 ERMS cells (RD). However only in PAX3-FOXO1 expressing RH30 cells, is GSK3β predominantly in its unphosphorylated form (at serine 9) and, hence fully active state (Figure 1c). In addition, ectopic expression of PAX3-FOXO1 resulted in reduced phosphorylation of GSK3β at serine 9 in C2C12 myoblasts (Figure 1d).

MYOGENIN *trans*-activation function is repressed by GSK3β. To assess the effect of GSK3β activity on MYOGENIN function, *trans*-activation of a 4x E-box Luciferase construct was measured in proliferating C2C12 myoblasts that were transfected with different combinations of constitutively active GSK3β(S9A) and MYOGENIN as indicated in Figure 2a. The data indicate that MYOGENIN potentiates the 4x E-box Luc reporter gene and that GSK3β(S9A) abrogates this effect ($P<0.001$) indicating repression of MYOGENIN by active GSK3β (Figure 2a, left panel) without affecting the MYOGENIN protein expression levels (Figure 2a, right panel).

GSK3β directly phosphorylates MYOGENIN *in vitro*. In order to determine whether MYOGENIN is a substrate for GSK3β, an *in vitro* kinase assay was performed using GST-MYOGENIN (1–225), purified GST-GSK3β and γ-^{32}P ATP. Bands were resolved using SDS-PAGE and subsequent autoradiography showed ^{32}P labeled bands for MYOGENIN, autophosphorylated GSK3β and MyBP (positive control, Figure 2b). In addition, Coomassie Blue staining revealed a lower mobility band indicative of phosphorylation (Figure 2b). To further test the idea that the lower mobility band is hyperphosphorylated, we used calf-intestinal phosphatases on RH30 cell lysates and found that the low mobility band was eradicated (Figure 2c). Collectively these data suggest that MYOGENIN is a GSK3β substrate *in vitro*.

Pharmacologic manipulation of GSK3β activity alters MYOGENIN properties. To further investigate the effect of GSK3β on MYOGENIN, COS7 cells were co-transfected with MYOGENIN and GSK3β(S9A) and, then treated with or without 10 μM GSK3β inhibitor, AR-A014418, as indicated in Figures 3a and b. Western blot analysis revealed two predominant forms of MYOGENIN, a low mobility hyperphosphorylated isoform and a high mobility, hypophosphorylated isoform (Figure 3a, lane 2). The lower mobility, hyperphosphorylated band is reduced upon pharmacological treatment with AR-A014418 as indicated (Figure 3a, lane 3). This corresponded with a significant increase in *trans*-activation of an E-box *cis* element driven reporter gene ($P<0.001$, Figure 3b). In contrast, constitutively active GSK3β(S9A) without pharmacological inhibition resulted in an increase in the low mobility, hyperphosphorylated band (Figure 3a, lane 4) which corresponded to a decrease in E-box luciferase activity in reporter gene assays ($P<0.05$, Figure 3b).

Mutation of a consensus GSK3β phosphoacceptor site on MYOGENIN (S160/164A) prevents GSK3β-mediated repression. By *in silico* analysis, MYOGENIN contains a highly conserved putative GSK3β consensus phosphoacceptor site (Table 1), which we targeted by neutralizing site-directed mutagenesis. We observed that although wild-type MYOGENIN is sensitive to the repressive effects of constitutively active GSK3β(S9A), MYOGENIN

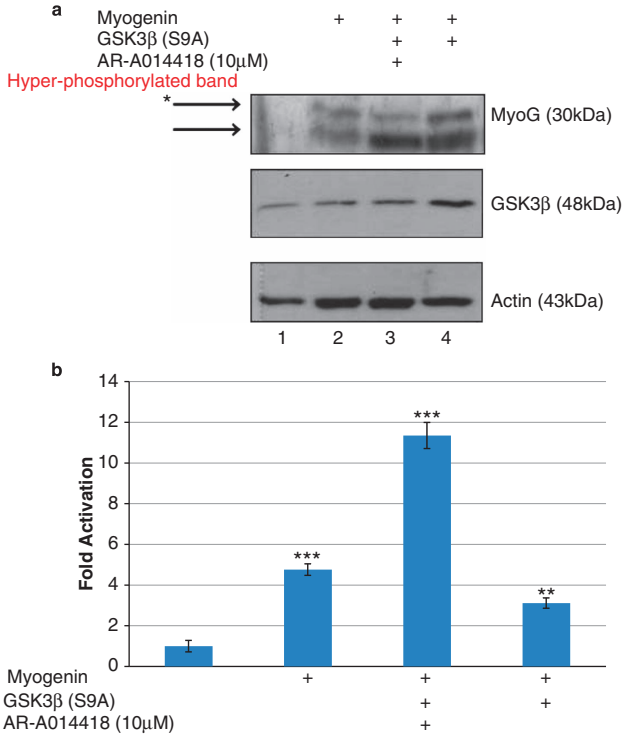

Figure 3 GSK3β increases MYOGENIN protein, possibly through phosphorylation and this corresponds with decreased transcriptional activity. (**a**) Cos7 cells were transiently transfected with or without MYOGENIN and/or GSK3β(S9A) and then treated for 19 h with either 10 μM GSK3β inhibitor or DMSO 24 h after transfection as indicated. Protein samples were extracted and western blot analysis revealed an increase in a slower migrating, hyperphosphorylated MYOGENIN band (lane 4) in the presence of overexpressed HA-GSK3β(S9A), which was reduced in the presence of GSK3β inhibitor (lane 3). (**b**) E-box Luc reporter gene was co-transfected in Cos7 cells using the same conditions that were described above. Overexpressed MYOGENIN significantly enhanced transcriptional activity of the E-box promoter (***$P<0.001$) and, this effect was further increased in the presence of GSK3β inhibitor despite overexpression of GSK3β(S9A) ($P<0.001$). Overexpression of GSK3β(S9A) repressed MYOGENIN transcriptional activity (**$P<0.05$)

Table 1 GSK3β consensus sequence within Myogenin

Myogenin sequence:	Species:
158 VP**S**ECS**S**HSA**S**CSP 171	Human
158 VP**S**ECN**S**HSA**S**CSP 171	Mouse
158 VP**S**ECN**S**HSA**S**CSP 171	Rat

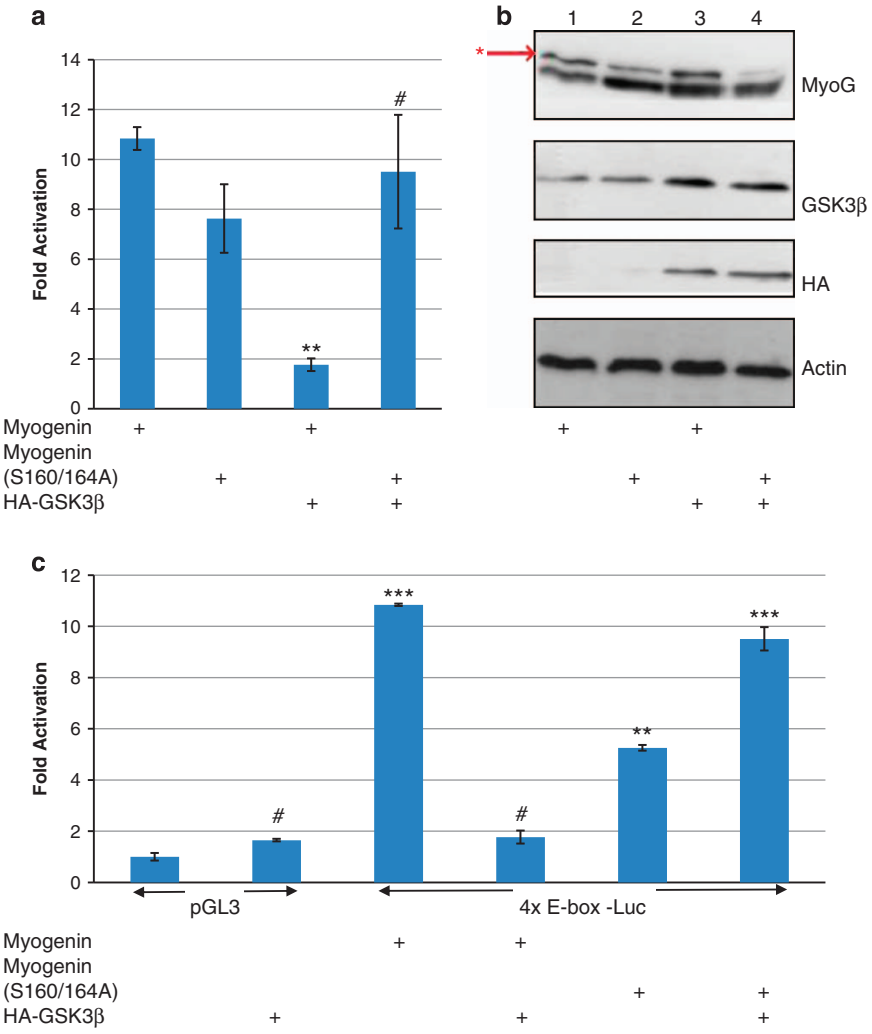

Figure 4 MYOGENIN neutralizing phosphomutant (S160/164A) is resistant to GSK3β repression of transcription activity as well as an increased slower migrating, hyperphosphorylated MYOGENIN band. (**a**) 4x E-box Luc activity was assessed in C2C12 myoblasts that were transfected with either wild-type MYOGENIN or MYOGENIN (S160/164A) and, co-transfected with HA-GSK3β(S9A) or pcDNA3.1 control plasmid as indicated. HA-GSK3β(S9A) repressed MYOGENIN *trans*-activation of the 4x E-box promoter region ($P<0.001$) but had no effect on mutated MYOGENIN (S160/164A) transcriptional activity. (**b**) Western blot analysis of the same samples revealed a decrease in a slower migrating, hyperphosphorylated band for overexpressed MYOGENIN (S160/164A, lane 2) with respect to overexpressed wild-type MYOGENIN (lane 1). Co-transfected HA-GSK3β(S9A) caused an increase in the slow migrating, hyperphosphorylated MYOGENIN band (lane 3) but not with overexpressed mutated MYOGENIN (S160/164A, lane 4). (**c**) Independent analysis of E-box Luc activity in C2C12 myoblasts with different combinations of overexpressed MYOGENIN, mutated MYOGENIN (S160/164A), HA-GSK3β(S9A) or pcDNA3.1 control plasmid as indicated. ***$P<0.001$, **$P<0.05$, $^{\#}$ns

(S160/164A) was not (Figure 4a). Western blot analysis revealed that MYOGENIN (S160/164A) mutations correspond with a decrease in the low mobility, hyperphosphorylated upper band (Figure 4b, lane 2) and that this effect was not altered by ectopically expressed HA-GSK3β(S9A). Together these data indicate that S160/164A mutations in MYOGENIN render it insensitive to the repressive effect of GSK3β. GSK3β(S9A) expression resulted in an increase in the low mobility, hyperphosphorylated form of wild-type MYOGENIN (Figure 4b, lane 3) and this corresponded with decreased E-box luciferase activity ($P<0.001$, Figure 4a). Although *trans*-activation of the skeletal muscle gene E-box *cis*-element by mutated MYOGENIN (S160/164A) is marginally less potent than wild-type MYOGENIN ($P<0.05$, Figure 4c); it is resistant to inhibition by activated GSK3β ($P<0.001$, Figure 4c).

PAX3-FOXO1 activation of GSK3β antagonizes muscle creatine kinase promoter activation. To further examine the functional significance of our findings, we used MCK promoter activity, as a key indicator of the activation of myogenic differentiation, in C2C12 myoblasts that were transfected with or without the PAX3-FOXO1 oncogene (Figure 5a). These data depict that PAX3-FOXO1 represses MCK promoter activation in myoblasts that have been co-transfected with MYOGENIN ($P<0.01$) and this effect is not only abrogated by pharmacological inhibition of GSK3β, but further activated ($P<0.001$, Figure 5a). Interestingly, in PAX3-FOXO1 expressing, human ARMS-derived RH30 cells, ectopically expressed MYOGENIN had no effect on MCK promoter activity unless it was coupled with pharmacological inhibition of GSK3β using AR-A014418 ($P<0.001$, Figure 5b). Conversely, mutated MYOGENIN (S160/164A)

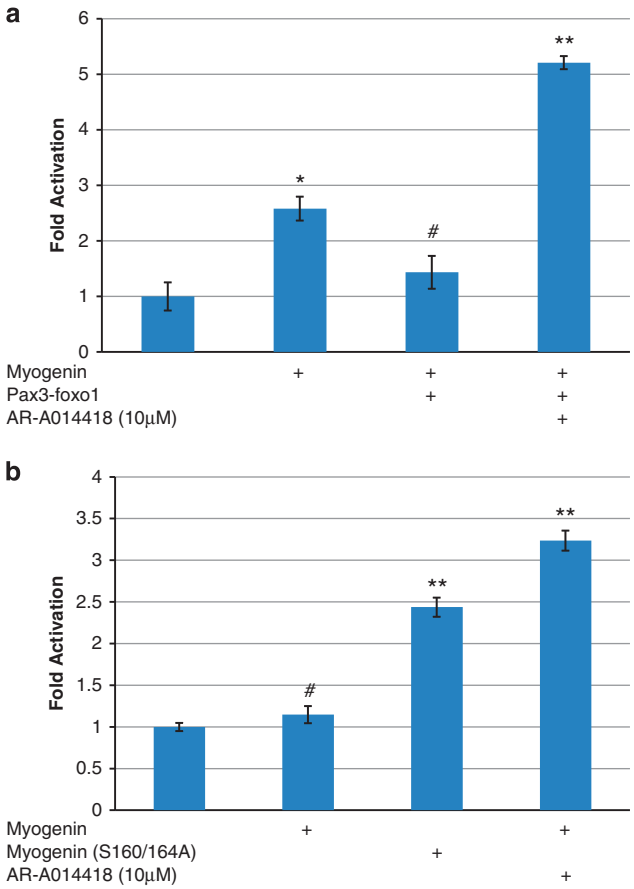

Figure 5 Pharmacological inhibition of GSK3β rescues PAX3-FOXO1 repression of MYOGENIN's transcriptional activation of MCK promoter in both C2C12 myoblasts and RH30 human ARMS cells. (**a**) MCK-Luc promoter activity was assessed in C2C12 myoblasts that were transfected with different combinations of MYOGENIN, PAX3-FOXO1 and pcDNA3.1 control plasmid as indicated and then treated with either 10 μM AR-A014418 or DMSO solvent. MYOGENIN enhanced MCK-Luc activity as expected ($P<0.001$) and this effect was repressed by co-expression of PAX3-FOXO1 ($P<0.01$). Pharmacological inhibition of GSK3β not only reversed the effect of PAX3-FOXO1 but resulted in a super-activation ($P<0.001$). (**b**) To assess the importance of these findings in human-derived ARMS, RH30 cells were transfected with either MYOGENIN or mutated MYOGENIN(S160/164A) and MCK-Luc promoter activity was assessed. The data shows that wild-type MYOGENIN could not *trans*-activate the MCK promoter region unless it was coupled with pharmacological inhibition of GSK3β ($P<0.001$). This was in contrast to mutated MYOGENIN (S160/164A), which could potentiate MCK promoter activity ($P<0.001$) regardless of GSK3β inhibition. (**c**) Summary of our findings: GSK3β activity in ARMS represses the activation of muscle-specific genes by repressing the transcriptional activity of MYOGENIN. $^{\#}$ns, $^{*}P<0.01$, $^{**}P<0.001$

was able to potentiate MCK promoter activity regardless of GSK3β inhibition ($P<0.05$, Figure 5b). Taken together, these data provide evidence that S160/164 on MYOGENIN are likely key targets of GSK3β signaling in alveolar rhabdomyosarcoma resulting in a diminution of the critical E-box dependent gene activation that is necessary and sufficient for differentiation.

Manipulation of GSK3β and MYOGENIN activity reduces tumorigenic properties of ARMS-derived RH30 cells. Colony-formation assays were performed as previously described using RH30 cells[32] which can grow in an anchorage independent manner. Equal numbers of RH30 cells that have been transiently transfected with or without MYOGENIN containing the S160/164A mutations were seeded in growth media with or without 10 μM AR-A014418 (GSK3β inhibitor) and allowed to form colonies for 21 days (Figure 6). The addition of 10 μM AR-A014418 significantly impaired the ability of RH30 cells to form colonies ($P<0.05$) and remarkably reduced the size of the colonies ($P<0.0001$). A similar reduction in colony numbers and size were also evident in RH30 cells that were transfected with MYOGENIN (S160/164A) mutations (Figures 6a and b). In addition, we confirmed that pharmacological inhibition of GSK3β significantly reduced cell proliferation of PAX3-FOXO1 expressing cells (Figure 6c). Collectively these findings strongly indicate that GSK3β activity promotes the tumorigenicity of RH30 cells and that this effect is neutralized by expression of MYOGENIN bearing mutations that render it insensitive to GSK3β.

Electrical stimulation of ARMS-derived RH30 cells reduces GSK3β activity through Akt (PKB). Electrical stimulation of skeletal muscle cells in cell culture has been shown to induce phenotype alterations and differentiation.[33] Given that rhabdomyosarcoma shares properties of the skeletal muscle lineage, we electrically stimulated cultured RH30 cells for 4 h/day (5 Hz) for up to 4 days with the idea that it might promote differentiation by affecting the Akt/GSK3β signaling pathway.[34] Stimulation of these cells resulted in an increase in pAktT308 to levels that were 3.00 ± 0.72-fold higher than those in non-stimulated cells after 4 days of stimulation (Figures 7a and b). Concomitantly, pGSKβS9 was also increased 2.25 ± 0.37 fold following 4 days of stimulation (Figures 7a and c). These increases in pAktT308 and pGSKβS9 were not a result of increases in total protein (Figure 7a) as indicated by the 3.76 ± 1.32 and 2.05 ± 0.55 increases in relative phosphorylation, respectively (Figures 7d and e). These changes in kinase activity corresponded with increased E-box promoter activity in stimulated cells compared with controls (Figure 7f). Collectively, these data indicate that electrical stimulation suppresses GSK3β activity and correspondingly activates MRF activity supporting our previous findings and also highlighting the possibility of using electrical stimulation as a therapeutic intervention in ARMS patients.

Discussion

ARMS, unlike ERMS, has a well-characterized cytogenetic basis in the majority of patients resulting from chromosomal translocations between chromosomes 1 and 13 and also 2 and 13 that result in fusion of the DNA binding domains of either Pax7 or PAX3 with the *trans*-activation domain of the Forkhead (FKHR) transcription factor family member Foxo1.[1,2,35] In view of the well-substantiated crucial role of PAX3 and 7 in the development of skeletal muscle[4,5] it is therefore not surprising that the signature of ARMS tumor cells is a muscle-like phenotype and the expression of a variety of structural muscle marker genes such as myosin heavy chain and desmin.[36] What is surprising is the sustained expression of MYOD and MYOGENIN in ARMS,[37,38]

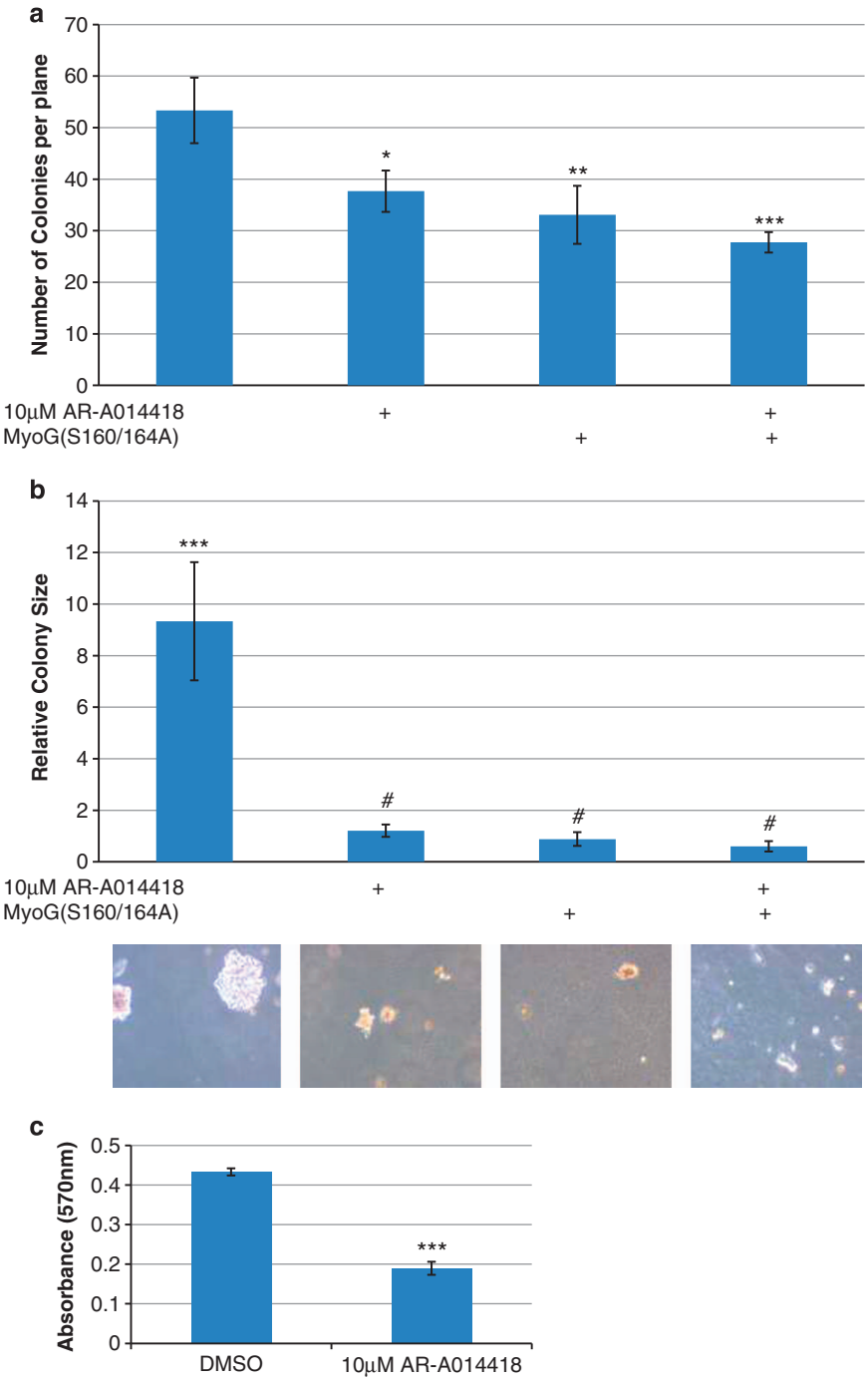

Figure 6 Soft agarose colony formation and MTT cell proliferation assays: (**a**) Equal numbers of RH30 cells were seeded under different conditions as depicted, and allowed to form colonies for 21 days. On the 22nd day the colonies were stained with 0.005% Crystal Violet overnight. Colonies were counted at different planes ($n = 10$) in four independent experiments done in triplicate. The total number of colonies was reduced by (i) 10 μM AR-A014418, $P < 0.05$ (ii) Transient transfection of MYOGENIN containing the S160/164A neutralizing mutations, $P < 0.01$ and (iii) both, $P < 0.001$. Also see Supplementary Figure 1 for visual representation of the data. (**b**) We searched for the three largest colonies in each of the 12 plates for each condition and calculated the area using ImageJ software. The data revealed that the control colonies could grow up to 9 $\times$ bigger than any of the experimental conditions, $P < 0.001$. (**c**) MTT cell proliferation assays were performed in PAX3/FOXO1A-expressing cells with and without 10 μM AR-A014418 treatment. The experiment revealed that GSK3β inhibition reduces cell proliferation by $\sim$2-fold, $P < 0.001$. $^{\#}$ns, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$

which are transcription factors that are intimately associated with the terminally differentiated, non- proliferative phenotype of normal myogenic cells, begging the question as to why they cannot exert this effect in ARMS. In particular, the function of MYOGENIN in the myogenic regulatory hierarchy places it at a pivotal and required step in the terminal commitment of myogenic progenitors to the differentiation program.[19,20,34] Thus, our observations reported here, that MYOGENIN function in ARMS is repressed by inappropriate sustained signaling by the kinase GSK3β, may be of considerable

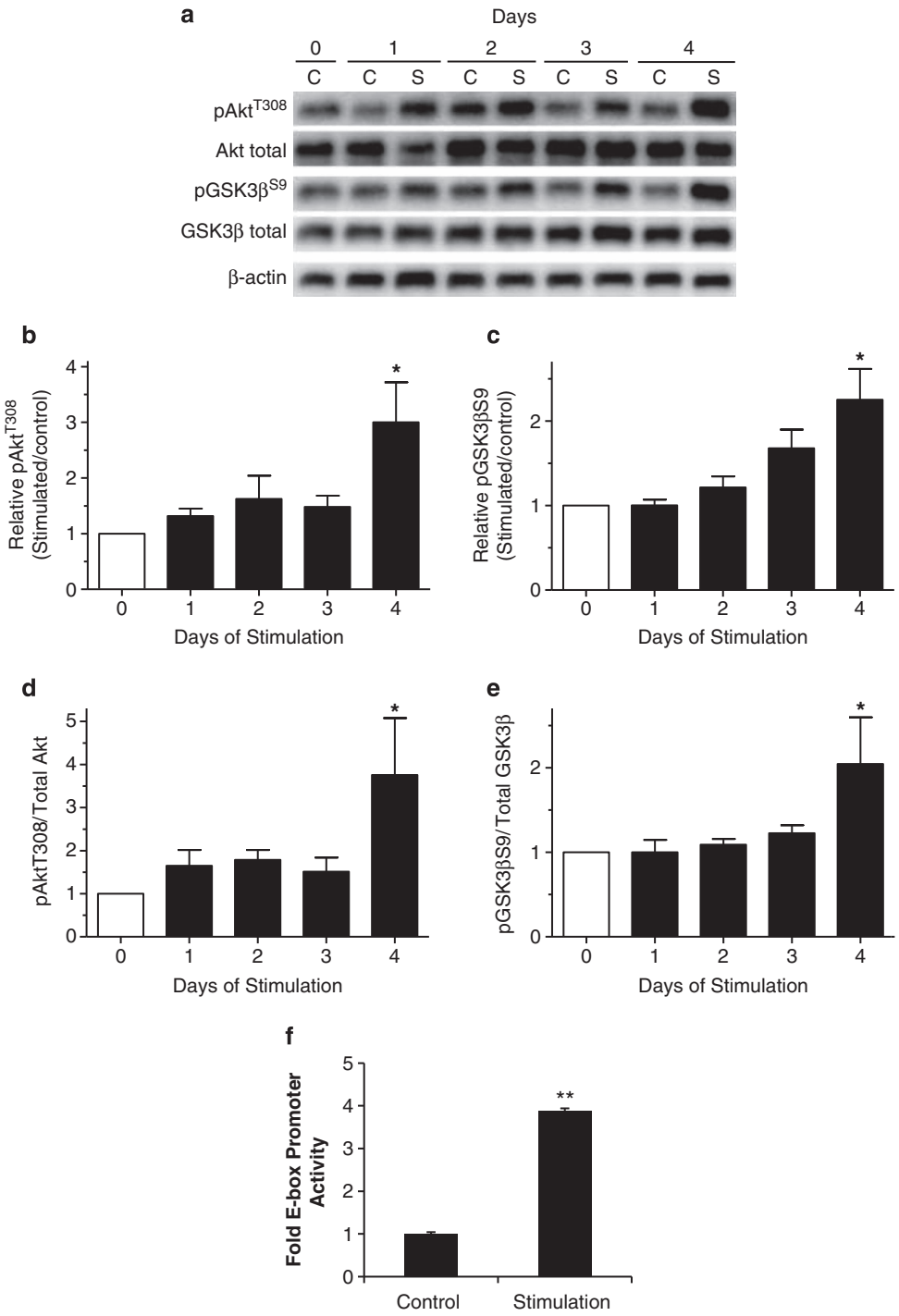

Figure 7 *In vitro* electrical stimulation of RH30 cells. (**a**) Western blot analysis revealed that electrical stimulation increased PKB/Akt activity and that this corresponded with increased phosphorylation of GSK3β at S9. Relative increase of phosphorylation at: (**b**) Akt at T308 and (**c**) GSK3β at S9, over time. Graphical representation of phosphorylated to total amounts of: (**d**) Akt and (**e**) GSK3β. (**f**) E-box promoter activity decreased with electrical stimulation and this also corresponded with inhibition of GSK3β at S9. $^{*}P<0.01$, $^{**}P<0.001$

significance for understanding the etiology of this disease. Moreover, as repression of kinase activity is, in many cases, a tractable pharmacologic approach, we now propose targeting GSK3β activity as a tangible therapeutic strategy for ARMS.

In support of the above, a recent study showed that ARMS-associated PAX3/7-Foxo1 fusion proteins inhibit MYOD target genes.[39] It was also reported that forced MYOD/E-protein dimer expression could not rescue PAX3/7-Foxo1 repression of myogenic factors.[39] Here, we also report that ectopically expressed PAX3-FOXO1 represses the induction of muscle genes, even when MRFs are expressed. We propose that the posttranslational repression of MYOGENIN

activity is due to sustained GSK3β activity and, through a cross-talk mechanism, subsequent repression of p38 MAPK (Supplementary Figure 1) as we have previously described.[10] p38 MAPK and PKB/Akt are both required for activation of MEF2/MYOD transcriptional control and chromatin remodeling events at crucial myogenic loci for the differentiation program.[11,40]

In other systems, GSK3β phosphorylation of its protein substrates results in subsequent targeting for proteasomal degradation.[12,13] However, GSK3β does not appear to affect MYOGENIN protein stability in our experiments as we observe an increase in a slow migrating, hyperphosphorylated form of MYOGENIN in response to GSK3β signaling that is not reduced in terms of its level of expression suggesting that proteasomal degradation of MYOGENIN is not enhanced by GSK3β. Conversely, neutralizing mutations of the GSK3β consensus enhanced MYOGENIN *trans*-activation of the muscle creatine kinase promoter, and also reduced the tumorigenic properties of ARMS cells (RH30) in a colony-formation assay. These findings suggest that GSK3β-mediated inhibition of MYOGENIN *trans*-activation properties impairs MYOGENIN's ability to promote terminal differentiation in tumorigenic RH30 cells.

Cell cycle control is an essential component of normal growth control and development which goes awry in tumorigenesis. To date several growth-promoting PAX3-FOXO1 target genes have been implicated in RMS such as the IGF-R and c-Met although, while their contribution to proliferation is likely, the extent of their precise involvement in ARMS is still not clear.[41] During normal skeletal myogenesis, upregulation of a cyclin-dependent kinase inhibitor, p21, stalls myoblasts in the G2/M phase of the cell cycle thus priming them for differentiation by promoting cell cycle exit, which is a requirement for subsequent muscle-specific gene expression.[42] Consistent with the idea that GSK3β activation may contribute to the oncogenic properties resulting from PAX3-FOXO1 expression in ARMS, we observed that the number of proliferative RH30 cells is approximately halved by pharmacological inhibition of GSK3β. So far, the exact mechanism by which GSK3β regulates cell proliferation in ARMS is unknown. However, GSK3β has recently been shown to activate KLF6[43] and we recently identified that KLF6 enhances cell proliferation in myogenic cells through a TGFβ/Smad3 dependent pathway.[44] We therefore speculate that PAX3-FOXO1/GSK3β enhancement of cell proliferation may involve KLF6 as a downstream effector as it is also highly expressed in various RMS cell types.

In summary, MYOGENIN normally activates genes that regulate cell fusion and terminal differentiation of skeletal muscle. In PAX3-FOXO1 expressing ARMS cells, our data indicate that sustained GSK3β activity represses MYOGENIN function, contributing to the transformed, proliferative phenotype of these cells. On the basis of this evidence, we propose that pharmacologic targeting of GSK3β kinase activity may constitute a tractable therapeutic strategy for ARMS.

Materials and Methods

Plasmids. E-box, MYOGENIN and MCK reporter constructs in pGL3 and expression vectors for MYOGENIN in EMSV were used in reporter gene assays. HA-tagged PAX3-FOXO1 was cloned into pcDNA3.1 and kindly donated by Dr. Malkin at MaRS, Toronto. HA-tagged GSK3β(S9A) was cloned in pcDNA3 ORF 995–2305.

Antibodies. Anti-MYOGENIN and anti-HA mouse monoclonal antibodies as well as anti-MEF2A rabbit polyclonal antibody were produced with the assistance of the York University Animal Care Facility; anti-PAX3 (1 : 250; Cell Signaling, Whitby, ON, Canada) GSK3β, phospho-GSK3β (1 : 1000; Cell Signaling); actin, MYOD, Myf-5, GFP, dsRed2 (1 : 2000; SantaCruz, Santa Cruz, CA, USA) were used for immunoblotting experiments.

Cell culture and transfection. C2C12, Cos7 and RH30 cells were maintained in DMEM supplemented with 10% fetal bovine serum (HyClone, Burlington, ON, Canada), 1% L-glutamine and 1% penicillin-streptomycin. Cells were maintained in a humidified, 37 °C incubator with a 5% CO_2 atmosphere. For transfections, cells were seeded 1 day before transfection and transfected according to the standard calcium phosphate method previously described. A mixture of 50 μl 2.5 M $CaCl_2$ per 25 μg DNA with an equal volume of 2x HeBS (2.8 M NaCl, 15 mM Na_2HPO_4, 50 mM HEPES, pH = 7.15) was used and the cells were incubated overnight followed by washing and addition of fresh media. The cells were counted and transferred to pre-gelatin-coated plates.

Protein extractions, immunoblotting and reporter gene assays. Cells were collected using an NP-40 lysis buffer (0.5% NP-40, 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 10 mM sodium pyrophosphate, 1 mM EDTA (pH 8.0), 0.1 M NaF) containing 10 μg/ml leupetin and aprotinin, 5 μg/ml pepstatin A, 0.2 mM phenylmethylsulfonyl fluoride and 0.5 mM sodium orthovanadate. Protein concentrations were determined using the Bradford method (Bio-Rad, Mississauga, ON, Canada) with bovine serum albumin (BSA) as a standard. An amount 20 μg of total protein extracts were used for immunoblotting, diluted in sample buffer containing 5% β-mercaptoethanol and boiled.

Transcriptional assays were done using luciferase reporter plasmids. The cells were collected for these assays using 20 mM Tris, (pH 7.4) and 0.1% Triton-X 100 and the values obtained were normalized to β-galactosidase activity expressed from a constitutive SV40 driven expression vector and represented as relative light units (RLU) or in some cases corrected Luciferase values for control, reporter alone transfections were arbitrarily set to 1.0, and fold activation values were calculated. Bars represent the mean ($n = 3$) and error bars represent the standard error of the mean ($n = 3$). Independent two sample *t*-tests of all quantitative data were conducted using R software. *P*-values are indicated with respect to controls where appropriate.

***In vitro* kinase assay.** A total of 3 μg of purified recombinant GST-MYOGENIN was mixed with either 0.5 μg purified recombinant GST-GSK3β (1–433; Cell Signaling) and with (γ-^{32}P) ATP and incubated for 30 min at 30 °C. Samples were denatured for 5 min at 95 °C in SDS sample buffer. Protein samples were then separated by 10% SDS-PAGE and exposed on X-ray film (Kodak X-Omat, Toronto, ON, Canada) for 21 h to detect ^{32}P incorporation. The lanes containing GST-MYOGENIN are elongated because two lanes were pooled to fit a higher total reaction volume to accommodate for the low concentration of purified GST-MYOGENIN (0.06 μg/μl). All lanes contain equal total amounts of proteins (3 μg).

Electrical stimulation. Cells were plated onto 0.1% gelatin-coated 6-well plates. The lids of the plates were fitted with two parallel platinum wire electrodes, placed at the opposite ends of each well and extending into the media. The wires from all wells were arranged in parallel and connected to an electrical stimulator (Harvard Apparatus Canada, Saint-Laurent, Quebec, Canada). Cells were stimulated at 5 V and a frequency of 5 Hz for 4 hours/day and allowed a subsequent 20 h recovery period. Cells were collected following the recovery period throughout the 4 days of the protocol.

Soft agarose colony-formation assay. Materials: 0.7% (w/v) DNA grade Agarose, 1% (w/v) DNA grade Agar, 0.005% Crystal Violet (Sigma-Aldrich, Oakville, ON, Canada), 2X Media + 20% (v/v) FBS. After 48 h of transfection with MYOGENIN containing the S160/164A mutations or empty vector, RH30 cells were assayed for their capacity to form colonies as previously described.[45] A total of 1×10^4 cells were suspended on a layer of 0.35% agarose in DMEM (10% FBS) with or without 10 μM AR-A014418, in 6-well plates. Medium was refreshed every 3–5 days as needed and on the 22nd day, the amount of colonies were

counted using a contrast phase microscope. The relative colony sizes were calculated using ImageJ software (Scion Corporation, Frederick, MD, USA). Four independent experiments were carried out in triplicate.

Conflict of Interest

The authors declare no conflict of interest.

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Supplementary Information accompanies this paper on Cell Death and Disease website (http://www.nature.com/cddis)

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