ROLE OF APOBEC2 IN RHABDOMYOSARCOMA AND ITS INVOLVEMENT IN THE PANNEXIN-1 MEDIATED INHIBITION OF MALIGNANT PROPERTIES

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Abstract

Rhabdomyosarcoma (RMS), the most common soft tissue cancer in Canadian children, is an aggressive childhood malignancy with a poor prognosis. To improve the outcome of RMS patients, an enhanced understanding of the molecular mechanisms involved in RMS pathogenesis and regulating its malignant properties is imperative. RMS tumor cells are thought to arise from muscle precursors that fail to completely differentiate into skeletal muscle. Our laboratory recently identified pannexin 1 (PANX1) as a novel regulator of myogenesis. PANX1 levels are downregulated in patient-derived cell lines and primary tumour specimens as compared to differentiated skeletal muscle myoblasts and tissue, respectively. Further work demonstrated that PANX1 overexpression inhibits RMS malignant properties through a mechanism that is independent of its canonical channel function. As this was the first time that a channel-independent function had been attributed to pannexins, RNA-sequencing was performed in RMS cells expressing PANX1 compared to their control. It was found that *APOBEC2* was significantly downregulated in PANX1-expressing cells. APOBEC2 is solely found in differentiated skeletal and cardiac striated muscle and acts as a negative regulator of differentiation. We hypothesized that RMS malignant properties will decrease when APOBEC2 is downregulated and that APOBEC2 is involved in the signaling pathway by which PANX1 alleviates RMS malignancy. Here, we found that APOBEC2 is downregulated in RMS patient-derived cell lines compared to that of differentiating human skeletal muscle myoblasts. Although we found that APOBEC2 does not regulate RMS cell viability or proliferation, its overexpression promoted tumour spheroid growth. PANX1 overexpression prevents the upregulation of APOBEC2 in proliferating Rh28, Rh30, and RD but not in the other three cell lines. Our data suggests that the RMS cell lines in which PANX1 regulates APOBEC2 levels are those in which PANX1 overexpression triggers cell fusion. Notably, we found that the overexpression of APOBEC2 reversed the PANX1-mediated induction of RMS cell fusion. Together these results suggest that APOBEC2 regulates tumor growth *in vitro* in which an increase in APOBEC2 expression promotes RMS malignancy. At the same time, we identify a further role for APOBEC2 in cell fusion shedding additional light onto the mechanism by which PANX1 functions to alleviate RMS malignant properties.

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List of Abbreviations

^{10}PANX	Panx1-mimetic inhibitory peptide
2D	Two dimensional
3D	Three dimensional
5mC	5-methylcytosine
AID	Activation induce deaminase
ANOVA	Analysis of variance
ApoB	Apolipoprotein B
APOBEC	Apolipoprotein B mRNA editing enzyme catalytic polypeptide cytidine deaminase
APOBEC2	Apolipoprotein B mRNA Editing Enzyme Catalytic Subunit 2
aRMS	Alveolar rhabdomyosarcoma
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
BCA	Bicinchoninic acid assay
Bcl6	B cell lymphoma 6
bFGF	Basic fibroblast growth factor
BrdU	Bromodeoxyuridine or 5-bromo-2'deoxyuridine
BSA	Bovine serum albumin
Ca^{2+}	Calcium ion
Cdh2	Cadherin-2
CDH4	Cadherin-4 precursor
CNS	Central nervous system
Col6a1	Collagen VI
CREB	cAMP-response element binding protein
Crmp2	Collapsing response mediator protein-2
DMEM	Dulbecco's modified/eagle's medium
DNA	Deoxyribonucleic acid
dsDNA	Double stranded deoxyribonucleic acid
Eif4g2	Eukaryotic translation initiation factor 4 gamma 2

ER	Endoplasmic reticulum
eRMS	Embryonal rhabdomyosarcoma
FBS	Fetal bovine serum
FOXO1	Forkhead box protein O1
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GFP	Green fluorescent protein
HDAC	Histone deacetylase
hEGF	Human epidermal growth factor
HEK	Human embryonic kidney
HPLC	High performance liquid chromatography
HSMM	Human skeletal muscle myoblasts
IL-1β	Interleukin-1β
kDa	Kilodaltons
KO	Knockout
MEF2	Myocyte enhancer factor 2C
mg	Milligrams
MHC	Myosin heavy chain
mRNA	Messenger ribonucleic acid
MS	Mass spectrometry
MuRF1	Muscle RING-finger protein-1
Myf4	Myogenic factor 4
Myf5	Myogenic factor 5
MyoD	Myoblast determination protein 1
MyoG	Myogenin
NADPH	Nicotinamide adenine dinucleotide phosphate
NF-kB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NO	Nitric oxide
PANX/Panx	Pannexin
PANX1/Panx1	Pannexin 1
PANX2/Panx2	Pannexin 2
PANX3/Panx3	Pannexin 3

PAX3	Paired Box 3
PAX3-FKHR	Paired Box 3 – Forkhead homolog in rhabdomyosarcoma
PAX 7	Paired Box 7
PAX7-FKHR	Pared Box 7 – Forkhead homolog in rhabdomyosarcoma
PAXBP1	PAX3 and PAX7 Binding Protein 1
PBS	Phosphate-buffered saline
PBST	Phosphate-buffered saline with Tween 20
PCR	Polymerase chain reaction
PLD	Phospholipase D
Pou6f2	POU class 6 homeobox 2 protein
Pten	Phosphatase and tensin homolog
PVDF	Polyvinylidene fluoride
qPCR	Quantitative polymerase chain reaction
RMS	Rhabdomyosarcoma
RNA	Ribonucleic acid
RNA-seq	RNA sequencing
RPMI	Roswell Park Memorial Institute
RT-qPCR	Real time quantitative polymerase chain reaction
SC	Satellite cells
SDS	Sodium dodecyl sulfate
siRNA	Small interfering RNA
SIX1/4	Sine oculis homeobox transcription factors 1/4
ssDNA	Single stranded deoxyribonucleic acid
ssRNA	Single stranded ribonucleic acid
TA	Tibialis anterior
TBST	Tris-buffered saline solution
TGF-β	Transforming growth factor beta
Tgfbi	Transforming growth factor beta induced
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor alpha
Toporsa	Topoisomerase I-binding arginine/serine-rich E3 ubiquitin protein ligase

Tp53	Tumor protein 53
tRNA	Transfer ribonucleic acid
Ubc9	Ubiquitin-conjugating enzyme 9
μg	Microgram
UTP	Uridine-5'-triphosphate
WT	Wild type
Wnt	Wingless/Integrated

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1.0 Introduction

1.1 An Overview of Rhabdomyosarcoma

Rhabdomyosarcoma (RMS), the most common soft tissue sarcoma in Canadian children, is an aggressive paediatric cancer with a poor prognosis[1,2]. Worldwide approximately 5% of all childhood cancers are identified as RMS[3]. Specifically, in Canada, it is reported that 4.5 cases per million children under fourteen are diagnosed with RMS every year[3]. RMS develops more commonly in males than females with an average incidence of 1.4:4 (respectively)[1] and are typically associated with the skeletal muscle lineage, due to the thought that they originate from mesenchymal origin based on the expression of many myogenic markers including MyoD[1,4]. RMS displays 2 major histological subtypes: alveolar (aRMS) and embryonal (eRMS) rhabdomyosarcoma representing 31% and 68% of cases respectively[5]. The remaining 1% of cases are described as being classified as pleomorphic, however, this subtype is rarely identified and uniquely present in adults over the age of forty-five[1]. When examining the two major subtypes; eRMS on one hand usually affects infants or children under ten years of age and is typically associated with a loss of heterozygosity at the 11p15 locus[1,3]. Whereas aRMS mostly occurs in adolescents and young adults and is associated with a worse prognosis[3]. The majority of aRMS tumours have been characterized by chromosomal translocations occurring at t(2;13) (q35;q14) and t(1;13) (q36;q14) resulting in the fusion between the regulatory domain of the FOXO1 transcription factor gene and the DNA binding domain of either PAX3 or PAX7 (causing PAX3-FKHR or PAX7-FKHR fusions respectively)[1,6,7]. This increases PAX expression leading to an acceleration of cell proliferation and a trend towards a myogenic undifferentiated state[6,7]. In patients with this translocation, the PAX3 fusion is more common with a worse prognosis, whereas

patients without the translocation (fusion negative) tend to resemble eRMS patients presenting with a better prognosis[8].

Although these solid tumours may arise anywhere throughout the body (most often in the head and neck region, genitourinary tract, or extremities), patients are often asymptomatic unless the mass has grown to impact other nearby structures[1,5]. With this being said, the most common current RMS treatment is chemotherapy involving the use of vincristine, actinomycin-D, and/or cyclophosphamide[6]; all of which have significant side effects. Complementary treatments include radiotherapy to improve local control and surgery for primary resection of the local area[6,9]. However, both of these complementary treatments also have limitations, such as the long-term damaging effects of radiation in children[6,9]. Nevertheless, the five-year survival rate for eRMS and aRMS remains at ~65% and 50% respectively[10]. Consequentially, despite intensification of therapy, patients with recurrent or metastatic RMS experience five-year survival rates of only 30% or 17% respectively[10–12].

1.1.1 Dysregulation of Myogenic Regulatory Factors in Rhabdomyosarcoma

The largest tissue mass (approximately 35-40% of body weight) in the human body is skeletal muscle, which is composed of multinucleated contracting muscle cells (myofibers)[13–16]. Skeletal muscle plays a key role in metabolism, posture, respiration, and movement[13–16]. Muscle can be generated both embryonically and postnatally[14]. The first requirement during developmental myogenesis is for the cell to commit to a myogenic cell fate[17]. From here, the primary fibers originating from the mesoderm begin to proliferate and scaffold into secondary fibers[17]. Upon the fusion of these secondary fibers, they become multinucleated and mature, with a subset entering quiescence to remain as satellite cells (SC)[17]. SCs are myogenic stem cells (ubiquitously

expressing PAX7) which are located in myofibers between the sarcolemma membrane and the basal lamina and become activated following muscle injury[18,19]. Following their activation, SCs proliferate, exit the cell cycle, and differentiate to form new myofibers, or to repair damaged myofibers[18,19]. In this same final stage when the myofibers are maturing, the extracellular matrix is remodeled and repaired[22], and the SCs replenish their stem cell pool, through a self-renewal process to ensure that they can maintain homeostasis and are ready for the next repair event[20–22].

For myoblasts to differentiate, they must first stop proliferating and exit the myogenic cell cycle[14,23,24]. This process is tightly controlled through the binding of transcription factors to DNA[14,23,24]. The initial signaling factors required for the cell specification to muscle lineage are the sine oculis homeobox transcription factors 1/4 (SIX1/4), and the paired-homeobox transcription factors 3/7 (PAX3/7)[25]. PAX3 has a more significant role in regulating embryonic differentiation, whereas PAX7 regulates differentiation and the maintenance of satellite cells postnatally[25]. The later regulatory factor proteins that modulate myoblast differentiation include the family of basic helix-loop myogenic transcription factors consisting of MyoD, myogenic factor 5 (Myf5), myogenin (MyoG), and myogenic regulatory factory 4 (Mrf4)[26,27]. These myogenic regulatory factors initiate their functions by binding to the E-box consensus sequences on the regulatory regions of their target genes[28]. Both MyoD and Myf5 are expressed in proliferating myoblasts and target the specification of cells towards the myogenic lineage embryonically[29–31]. MyoD protein expression peaks during G1, where it prevents the cell from progressing through the cell cycle and initiates myoblast differentiation[29,32–36]. The expression of MyoD and the downregulation of Myf5 triggers the early differentiation phase to begin, upregulating myogenin, resulting in the formation of immature myofibers (which are fused myoblasts forming myotubes)[27,29,32,35–38]. Myogenin acts to commit the cell to a differentiated fate and ensures proper

muscle fiber formation during myogenesis[29,30,39,40]. The final stage of differentiation is triggered by the upregulation of Mrf4, where the myotubes fully mature into adult muscle and form multinucleated fibers[27,29,30]. At this final stage, the mature multinucleated muscles will begin to express the myosin heavy chain (MHC) protein[27,29,30]. MHC is thus used as a marker for terminally differentiated muscle, as it is a motor protein with a role in ATP hydrolysis that allows mechanical force generation in muscle[27,29,30].

When skeletal muscle growth, differentiation, and/or regeneration is disrupted, or when myogenic regulatory factors are dysregulated, diseases such as RMS may arise[6]. Indeed, RMS develops when cells are disrupted from progressing through myogenesis[6]. This can exemplify itself as the cells failing to exit the cell cycle, and are thus, found in an indefinite proliferating state where they are unable to terminally differentiate[41,42]. As a result, RMS cells typically express gene characteristics typical of early development such as MyoD, and not of mature muscle such as MHC[43]. This suggests that these dysregulated myogenic pathways may be key to understanding the molecular mechanisms by which RMS arises[43]. Additionally, it has previously been shown that increasing RMS differentiation is a method to halt tumor growth, for example by targeting the dysregulation of MyoD[44], or through the use of muscle-specific microRNAs (which promote myogenic differentiation)[45]. Therefore, gaining a further understanding of the molecular mechanisms mediating RMS malignancy may provide insight into potential novel therapeutic targets.

1.2 An Overview of the Pannexin Family

Our laboratory has identified pannexin1 as a key regulator of myogenesis[46]. Pannexins are a family (pannexin 1 (PANX1), pannexin 2 (PANX2), and pannexin 3 (PANX3)) of large single

membrane channels[47]. Pannexins were first discovered during the early 2000s due to their sequence homology to innexins, which are invertebrate gap junction proteins[48]. Unlike the vertebrate gap junction forming proteins known as connexins, in which pannexins share a similar structural topology, pannexins only form single membrane channels called pannexons[49,50]. When examining why pannexins are unable to form these same gap junctions, it has been suggested that their N-glycan chains cause steric hindrance that prevent further appositional pannexon docking[50]. The channel structure which is formed varies between the different members of the pannexin family with PANX1 forming heptamers, PANX2 forming heptamers or octamers, and PANX3 forming hexamers[47,51]. In addition to their differences in channel structure, pannexins also vary in terms of their expression profiles[47]. PANX1 (Panx1 in rodents) is ubiquitously expressed with transcripts found throughout the human body (including the sensory systems[52,53], the brain[54,55], the cardiovascular system[56–58], exocrine glands[59,60], skeletal muscle[61], prostate[62], liver[49], skin[63], and lung epithelial[64]). It has been originally suggested that PANX2 expression is limited to the central nervous system (CNS) and brain[48,65]. However, others have found that although the Panx2 transcript does appear to be limited to the CNS, upon analysis of protein levels Panx2 expression was found within all tissues (including the brain, eye, small intestine, testis, skin, heart, lung, stomach, and more) examined[66,67]. This suggested that the previously assessed transcriptional activity may not be a reliable predictor of Panx2 protein expression[66,67]. Finally, PANX3 is limited to skin[68], cartilage[69], duodenum[70], osteoblasts[69], cochlea[65], and skeletal muscle[60,61]. However, despite their various expression profiles, they have all been found to have similar channel functions[47].

To study PANX1 channel function a variety of different channel blockers and mimic peptides were identified. The most common channel inhibitor that is used is carbenoxolone[71,72].

Carbenoxolone interacts with the first extracellular loop of PANX1 to prevent its channel function, however, it also acts as a global gap-junction inhibitor at higher concentrations and therefore potentially has off-target effects[71,72]. Probenecid, which is traditionally used to treat gout, is another common PANX1 channel blocker, however, its mode of inhibition is not well characterized other than that it is selective for pannexin channels over connexin channels[73,74]. Other channel inhibitors include: ^{10}PANX, a Panx1-mimetic inhibitory peptide specific for PANX1 channels[75,76]; mefloquine, used to treat malaria, is shown to block Panx1 channels[77]; and trovafloxacin, an antibiotic, which at low doses can block PANX1 channels[78].

PANX1 channels play a key role in ATP release, calcium leak channels and the movement of small molecules, and anions (up to 1 kDa)[50,79]. These various channel functions have been found to be involved in many physiologic and disease states including vasodilation[56], taste response[80], inflammatory responses[76,81], human immunodeficiency virus infection[82,83], stroke[84], diabetes[85], ischemia[86], hypertension[84], and cancer (including RMS[87], gliomas[88] and melanomas[89]). One of the main roles of PANX1 channel function is communication between cells through the release of certain molecules[47]. Therefore, it has been shown that there are a variety of different stimuli which can activate PANX1 channels including mechanical stimulation[81], calcium, ATP, and potassium concentration[90], and depolarization[91]. Upon activation, PANX1 is involved in Ca^{2+} and ATP signaling through its interaction with the purinergic receptors P2Y and P2X[92]. In the context of vasodilation, PANX1 channels have been associated with the release of ATP and further Ca^{2+} wave propagation within erythrocytes to stimulate nitric oxide (NO) in smooth muscle[56]. Alternatively, ATP release through PANX1 channels has been implicated in taste sensation through the stimulation of P2 receptors releasing serotonin[53,80]. ATP release by PANX1 channels has also been linked to inflammatory responses due to the involvement of the P_2X_7 purinergic receptors,

promoting interleukin-1 β (IL-1β) cytokine release and Toll-like receptor (TLR) -independent inflammasome activation[76,81]. Finally, in apoptosis, PANX1 has been proposed to interact with the $P2X_7$ receptors to mediate cell death signaling and becoming activated upon caspase cleavage to release ATP and UTP, which serve as a marker for phagocytosis[90,93].

PANX1 expression and function have been shown to be regulated by its post-translational modifications, including glycosylation, phosphorylation, acetylation, nitrosylation, ubiquitination, and caspase cleavage[61,94]. The main post-translational modification that pannexins undergo is N-linked glycosylation specifically by N-linked glycosidases at asparagine 254 of mouse Panx1 and 255 of human PANX1 (on the second extracellular loop)[49,95]. As a result, PANX1 is detected as multiple species by western blot, reflecting the un-glycosylated core (forming Gly0 within the endoplasmic reticulum (ER)), the high mannose species in the ER membrane (Gly1), and the complex mannose species on the plasma membrane (Gly2)[61,96]. As a result, the glycosylation status is important for PANX1 trafficking and stability within the cell[49]. The glycosylation status pannexin also plays a role in PANX1 intermixing with PANX2 or PANX3 to form different heteromeric channels[95]. Although intermixing between pannexins is possible, the channel function is compromised as this intermixing results in decreased ATP/Ca^{2+} currents and a reduction in protein stability[97,98].

Another, post-translational modification that affects PANX1's function is phosphorylation (on tyrosine, serine, or threonine sites) by Src kinases, which have been found to facilitate Panx1's channel activation and ATP release[90,99]. An example of this involves phosphorylation at Lysine-198, which has been shown to play a role in facilitating Panx1 channel release of ATP through tissue necrosis factor-alpha signaling during inflammation[100]. Panx1 can also be post-translationally modified by S-nitrosylations, which inhibits Panx1 channel function, preventing

ATP release[101]. Unlike many of the other post-translational modifications, this is a reversible modification due to the covalent bond between NO and cysteine (Cysetine-40 and Cysetine-346)[101]. Another post-translational modification that PANX1 can undergo is caspase cleavage[90]. This cleavage occurs by Caspase-3/7 at residues 164-167, and 376-379[90]. As opposed to S-nitrosylation, this cleavage results in an activation of PANX1 channels, which is involved in processes such as immune clearance due to the activated and open channels allowing for high conductance of molecules[90,102]. Finally, PANX1 can also undergo acetylation and ubiquitination, which can occur on lysine residues 381 and 409[103]. There is currently limited knowledge on the functional role of acetylation on PANX1 other than regulation of PANX1 channel expression[103]. Ubiquitination on the other hand has previously been examined by our laboratory and shown that it may regulate PANX1 trafficking by increasing cell surface expression[103] (Blinder, unpublished). Altogether, these post-translational modifications are important for PANX1 channel function in terms of gating and permeability, along with protein localization and/or degradation[103].

1.2.1. Role of PANX1 in Cancer

PANX1 has also been recently implicated in many forms of cancer (Summary found in Table 1). Interestingly, PANX1 levels are either increased or decreased depending on cancer type[89,104,105]. In the case of certain cancers including melanoma, testicular cancer, breast cancer, neuroblastoma, and hepatocellular carcinoma, the overexpression of PANX1 is associated with increased tumour aggressiveness, metastasis, and overall poor survival[89,104,105]. Although the signaling pathways involved in each cancer appear to vary, understanding these can provide additional insight into potential therapeutic targets. For example, melanoma has been shown to have a high expression of PANX1 and trigger the Wnt/β-catenin signaling pathways resulting in

increased tumour aggressiveness[89,106]. This was further supported by findings indicating that PANX1 levels were proportional to tumour aggressiveness and that knocking down PANX1 with shRNA resulted in decreased tumourigenesis (including promotion of differentiation, decreased metastasis, reduction in mobility and proliferation, and alteration of cellular morphology)[89]**.** Conversely, while the endogenous overexpression of PANX1 in testicular cancer led to increased tumourigenesis, the signaling pathway mediating this relationship varied[107,108]. Specifically, in testicular cancer, the extracellular signal-regulated kinase 1/2 signaling pathway is involved and results the protein expression of E-cadherin, vimentin, and matrix metalloproteinase-9[107,108]. Interestingly, metastatic breast cancer cells express a truncated form of PANX1 (truncated halfway through the first extracellular loop) that results in a gain-of-function channel activity mediating its tumourigenesis[110,111]. This gain-of-function channel activity resulted in increased interaction with P2Y receptors, the accumulation of ATP release, and inhibition of cell death[105]. Additionally, in breast cancer cells, PANX1 channels were found to be mechanosensitive due to an interaction between its C-terminus and proteins such as F-actin which tethered it to the cytoskeleton increasing channel activation and promoted additional tumour metastasis[109,110].

By contrast, other cancers such as glioma, RMS, and keratinocyte carcinomas show that the downregulation of PANX1 increases malignant properties[68,87,88,111]. An example of this is glioma, in which the overexpression of PANX1 reduces the malignant properties of rat C6 glioma cells[88]. PANX1 overexpression in these C6 cells acted as a tumour suppressor, resulting in increased dye uptake activity, and reduced proliferation, migration, and *in vivo* tumour formation[88]. This same expression pattern has also been observed in gallbladder adenocarcinoma where PANX1 was minimally expressed within these cell lines compared to normal human gallbladder epithelium[112]. This study additionally found a negative correlation between PANX1 expression

and tumour progression (as examined by cell proliferation)[112]. In the context of basal or squamous cell carcinomas, PANX1 is not detected within these tumours as compared to the normal skin cells (which normally express PANX1)[63]. As a result, this downregulation has been proposed to have several potential roles including protection against keratinocyte transformation, or promotion of tumorigenesis as a function of an increased rate of proliferation within these keratinocyte carcinomas[63]. Alternatively, in some cancer models, depending on the stage of the cancer, PANX1 has been shown to be differentially expressed[113]. As an example. hepatocellular carcinoma shows high expression of PANX1 in metastatic tumours, whereas non-metastatic tumours tend to show a low expression of PANX1[113]. Overall, this suggests that depending on the cancer type, the expression of PANX1 can have differing roles and effects on tumour aggressiveness and metastasis[113].

Table 1: *PANX1 in Cancers*

Cancer	***Expression of PANX1***	***Implications on Mutagenesis***
Melanoma[89,106]	High	Downregulating PANX reduced tumourigenesis (promoted differentiation, decreased migration, tumour formation, and metastatic potential)
Testicular[108]	High	Blocking channels reduced tumourigenesis (cell migration and invasion)
Breast[104,105,114]	High and truncated	High truncated expression promoted tumourigenesis (increase in ATP release, channel activity, and purinergic signaling) Blocking channels reduced tumourigenesis
Neuroblastoma[115]	High	Blocking channels reduced tumourigenesis (reduced proliferation)
Hepatocellular Carcinoma[113]	Low in non-metastatic High in metastatic	High expression promoted tumourigenesis (increased aggressiveness/ metastasis)
Glioma[88,111]	Low	Expressing PANX1 reduced tumourigenesis (suppresses tumour formation, stabilized 3D tumour structure, and upregulates actinomyosin)
Keratinocyte[63]	Low	Low expression promoted tumourigenesis (dysregulated keratinocyte differentiation)

1.2.2 Role of Pannexin1 in Myogenesis and Skeletal Muscle

Within skeletal muscle, PANX1 is expressed in satellite cells, myoblasts, myofibers, and myotubes[116]. Our laboratory has previously described a key role for PANX1 channels in myogenesis *in vitro*[46]. This previous work has shown that PANX1 levels are very low in undifferentiated primary skeletal muscle myoblasts and increase during their differentiation[46]. The increase in PANX1 expression was also observed during muscle development and regeneration *in vivo*[46]. More recently, our laboratory has identified that this increase in PANX1 expression occurs due to increased ETV4 binding specifically to the PANX1 promoter[117]. This therefore, connects historical research that has found that the overexpression of ETV4 (often by myocyte enhancer factor 2C (MEF2)) promotes differentiation, to our research on PANX1[117]. The role for PANX1

in myogenesis was further corroborated by blocking PANX1 channel activity which was found to inhibit myoblast differentiation and fusion, while overexpressing PANX1 promoted this process[87]. Further, a link between PANX1 with skeletal muscle regeneration and myoblast fusion has been recently identified with the findings that Panx1$^{-/-}$ mice display evidence of deficits in these processes[118]. This study identified the PANX1s interaction with P_2X_7 receptors to be responsible for release of ATP which in turn induced both membrane blebbing and migration, and identified the involvement of lipid intermediates including Phospholipase D (PLD) and A_2 in these signaling pathways[118].

Based on the known role for PANX1 channels and its localization within the sarcolemma and in the t-tubules of muscles near P2 receptors, PANX1 has been identified to be involved in skeletal muscle properties including contraction and plasticity[116,119]. In the context of contraction, it has been shown that PANX1 is involved in the regulation of membrane depolarization and potentiation, however, this effect varied between slow and fast-twitch fibers[46,63]. In slow-twitch muscles, PANX1 activates $P2X_4$ receptors increasing calcium ion concentration intracellularly[120]. Whereas in fast-twitch muscles, PANX1 activated P2 receptors increasing extracellular ATP concentrations[116,121]. To verify this, Panx1$^{-/-}$ mice have been used to identify that muscles from these mice had reduced contraction following electrical stimulation that was associated with a reduction in ATP release[116]. Alternatively, in skeletal muscle plasticity (the ability for skeletal muscles to adapt and remodel based on their environmental and functional demands), PANX1 has been linked to the capability of skeletal muscle to switch from fast to slow-fiber type[122]. Here, PANX1 releases ATP which further increases calcium ion concentration and initiates transcriptional changes required for this fiber-type determination[123]. As a result of the key role that PANX1 plays in skeletal muscle, alteration in expression can result in negative physiological

outcomes. For example, our laboratory has shown that Panx1 levels are downregulated in a severe mouse model of Duchenne muscular dystrophy[124]. More recently, it has also found by others that PANX1 is involved in skeletal muscle inflammation in the setting of obesity[125]. In this report, mice fed with a high-fat diet had higher levels of ATP concentration extracellularly associated with increased PANX1 levels as compared to mice fed a normal diet[125]. Thus, they proposed that the overexpression of PANX1 resulted in increased ATP release which as a result promoted not only an inflammatory muscle state but also insulin resistance within the obese mice[125].

1.2.3 Pannexin1 in RMS

Based on the regulation of PANX1 levels during myogenesis, our laboratory explored whether PANX1 expression is altered in RMS and if so, whether restoration of its expression could reduce RMS malignant properties. We found that like undifferentiated myoblasts, PANX1 transcript and protein levels were downregulated in our panel of six patient-derived RMS cell lines (3 eRMS and 3 aRMS) as compared to normal differentiated skeletal muscle cells[87]. PANX1 levels were also examined in thirteen human primary RMS and seven normal skeletal muscle tissues from patients at our institution and found that all tumour specimens tested showed a drastic downregulation of PANX1 levels[87]. More recently our laboratory found that through RNA-sequencing and protein expression that the PANX1 transcripts expressed within the Rh18 and Rh30 cell line lacked the 5' untranslated region[117]. Upon further investigation, our laboratory was able to identify specific consensus sequences on the *PANX1* promoter that correspond to the transcription factors CREB and ETV4[117]. Through determining the effects of deletions within these sites, it was suggested that the transcription factors ETV4 plays a key role in regulating the expression of the 5' untranslated region[117].

PANX1 was overexpressed in RMS cells to determine whether it could reduce their malignant properties[87]. PANX1 overexpression resulted in an induction of partial differentiation of Rh30 (aRMS) cells and abrogated the proliferative and migratory potential of Rh18 (eRMS) and Rh30 (aRMS) cell lines in 2D culture[87]. Additionally, using Rh18 and Rh30 cell lines, we showed that PANX1 overexpression inhibited tumour spheroid growth and induced regression of established spheroids through the induction of apoptosis[87]. Remarkably, while control tumours grew rapidly in mice, PANX1 overexpression significantly reduced RMS (Rh18 and Rh30) growth[87]. As increasing PANX1 levels would thus be of therapeutic benefit, our laboratory has attempted to identify a drug that could increase the endogenous levels of PANX1 in RMS[117]. As a result, treatment with quercetin (a natural flavonoid) had not only a positively correlated dose-dependent increase in PANX1 expression, but also was able to re-express the 5' untranslated region[117]. When examining the effect of quercetin in both Rh18 (eRMS) and Rh30 (aRMS), the cells appeared elongated (a marker of myogenic differentiation), showed increased expression of MyoD and MyoG, prevented the formation of spheroids in culture, and induced the regression of established spheroids[117]. Through the use of PANX1 siRNA, these properties were determined to be specifically PANX1-dependent[117]. Upon further investigation of this process, it was found that when examining ETV4 binding to the promoter of PANX1, treatment with quercetin further promotes this binding allowing for translation of the downstream sequence and thus an mRNA transcript of PANX1 that contains the proper 5' untranslated region[117]. Therefore, through quercetin treatment, we were able to again mitigate the malignant properties of RMS including preventing tumour formation and inducing the regression of spheroids[117].

We investigated the mechanism by which ectopic PANX1 reduces RMS malignant properties and found that it was independent of its canonical channel function[87]. This was

established using PANX1 channel blockers and using PANX1 mutants that are devoid of its channel activity[87]. Moreover, ectopic PANX1 was unable to form functional channels based on our dye uptake assays[87,126,127]. These studies provided the first evidence of the existence of PANX1-mediated functions independent of its canonical channel activity[87]. Recently, mechanisms external to ATP release and PANX1's canonical channel function have been of increasing interest[115,128]. Some alternative mechanisms (to PANX1s channel function) include PANX1 interactions with actin-related proteins 2/3 for the generation of force, or interactions with collapsing response mediator protein-2 (Crmp2) for the remodeling of microtubules[115,128]. Deciphering the molecular pathways by which PANX1 expression alleviates RMS malignant properties may therefore reveal new potential therapeutic targets for RMS, in addition to shedding light on the mechanism by which PANX1 functions.

1.2.4 Identification of the PANX1 Interactome and Transcriptome

As there has been limited research into PANX1's function aside from its channel function, our laboratory wanted to take an unbiased approach to identify the potential mechanism behind the PANX1-mediated inhibition of RMS[129]. This was specifically done with an RMS patient-derived cell line (Rh18 and Rh30) by determining the PANX1 interactome using BioID[129]. After capturing the biotinylated proteins and submitting them for HPLC-ESI-MS/MS analysis, 240 from the Rh18 cell line and 238 proteins from the Rh30 cell line were labeled as interactors[129]. When further classifying these interactors it was found that the majority were localized at or near the plasma membrane, including in the cytosol and cytoskeleton[129]. To further verify this interactome, co-immunoprecipitations were performed using PANX1-enriched membrane fractions, which identified 146 interactors within the Rh18 cell line and 202 interactors within the Rh30 cell line[129].

When comparing the top 50 interactors, 27 and 26 were overlapping within the Rh18 and Rh30 cell lines, respectively[129]. When examining these overlapping interactors, the three main pathway clusters identified included proteins associated with the plasma membrane, the actin filament, and the microtubules[129]. Interestingly, 43 of the top 50 hits from the BioID between Rh18 and Rh30 cell lines were identical, suggesting similar mechanisms of PANX1-mediated inhibition of malignant properties between the two RMS subtypes[129].

The top hit identified by both BioID and co-IP using PANX1 enriched membrane fractions, was AHNAK[129]. AHNAK, also known as desmoyokin, is a large scaffolding protein[130,131]. AHNAK is typically localized to the plasma membrane and plays a major role in muscle and epithelial cells in terms of organization, as well as structural support[130,132]. Additionally, the overexpression of AHNAK has been found to promote cell migration and invasion when examining several cancer models including mesothelioma[132,133]. The reverse has also been found to be true in both mammary carcinoma cell lines, in which the downregulation of AHNAK led to a reduction in tumour proliferation and invasion[133]. Our laboratory found that the knockdown of AHNAK expression in Rh18 and Rh30 cell lines reversed the PANX1-mediated reduction in cell viability and migration, and induction of anoikis, indicating that PANX1 inhibits RMS malignancy through a mechanism that involves its interaction with AHNAK[129].

To gain additional insight into the PANX1-mediated reduction of RMS malignant properties mechanism, our laboratory also identified the genes that are regulated by ectopic PANX1 in Rh30 cell lines[129]. Thus, RNA-sequencing analysis was conducted on the Rh30 cell line overexpressing PANX1 or the empty vector[129]. This analysis discovered 1273 genes (5.2%) that were significantly altered (898 upregulated and 39 downregulated) upon the overexpression of PANX1[129]. These genes were classified by gene ontology, which determined several common

pathways including the regulation of apoptosis, and migration[129]. Amongst these genes, *APOBEC2* was found to be downregulated, which was validated by RT-qPCR, in PANX1-expressing cells[129].

1.3 An Overview of the AID/APOBEC Family

APOBEC2 is the oldest member of the activation-induced deaminase/apolipoprotein B mRNA editing enzyme catalytic polypeptide cytidine deaminase family, which are all closely related to one another based on sequence homology and conservation of structure[134–137]. This family of enzymes was initially discovered in 1987 through an examination of the apolipoprotein B (ApoB) mRNA transcript[138,139]. This analysis found a specific cysteine to uracil post-translational modification that altered the ApoB mRNA transcript forming a premature stop codon[138,139]. However, it was not until 1995, that the first family member identified, APOBEC1 was named for this function in editing ApoB, which is a lipoprotein structural protein[140,141]. ApoB has historically been known for its role in the transport of both lipids and cholesterol to the body from the digestive system, as well as the liver[142]. Eventually, the rest of the family members were discovered based on their similar topology, sharing at least one secondary protein structure[143]. This structure is additionally shared through the formation of at least one cytidine deaminase CDA domain[143]. This family of eleven proteins although expressed in different tissues with different substrates and functions have all been known to edit DNA and/or RNA as they are zinc-dependent deaminases[26,134,135]. This allows these proteins to remove the amino group converting a cytidine (on either cytosine, free cytidine, or deoxycytidine triphosphates) to a uridine base, resulting in a change in the expression of the gene products[137].

The members of the AID/APOBEC family include Activation-induced deaminase (AID), APOBEC1, APOBEC2, APOBEC3 (A-D and F-H), and APOBEC4. The different members of the

family are all related to each other and are believed to have initially originated from the tRNA adenosine deaminase/adenosine deaminase tRNA Specific 2 family which function to convert adenosine to inosine[137]. From here they split into two branches; the first being APOBEC4-like (including APOBEC1, 3, and 4) and the second being AID-like (including AID and APOBEC2)[137]. Each of the members of the APOBEC/AID family has a unique function within the body[137]. APOBEC1 has been linked to RNA editing, specifically within translation and the editing of messenger RNA, often within the 3' untranslated regions of genes in the small intestine[144,145], liver[144,145], macrophages within the immune system[146–148], and microglia within the central nervous system[149]. APOBEC1's function originated within the small intestine, as previously discussed, where it was found that APOBEC1 had the ability to convert a cysteine to an uracil in the ApoB mRNA. This resulted in the generation of a premature stop codon within the ApoB mRNA preventing its function in lipid metabolism[140,147,149]. APOBEC3 on the other hand can mutate DNA, forming novel gene variants specifically within the innate immune system, playing a key role in the editing of retroelements and viruses such as human immunodeficiency virus[150,151]. One of the most well-researched APOBEC3s is APOBEC3G which hypermutates viral DNA and therefore is being used as a potential therapeutic target within human immunodeficiency virus as APOBEC3G has been found to mutate viral DNA during reverse transcription events[152–154]. Additionally, AID has been linked to DNA editing, where it has been found to play a role in DNA demethylation and the deamination of deoxycytidine to deoxyuridine[155,156]. As a result, AID's expression profile has been linked to adaptive immunity and activated B cells, with additional findings linking a potential role in the maturation of genomic immunoglobulins[157–159]. Finally, APOBEC4's function is largely unknown due to minimal conducted research as unlike the other members of its family it lacks the ability to deaminate DNA[160–162]. However, it is known that APOBEC4 is localized solely within

the testes[160–162]. Overall, this family of enzymes is generating increasing interest as more is discovered and their role and involvement in many biological processes becomes further understood.

1.3.1 APOBEC2's Proposed Mechanisms of Action

APOBEC2 is the most evolutionarily conserved member of its family, however due to a retro-transpositional event, has an altered N-terminal exon and deaminase region introns[26,134,135,137,163]. As a result, it is unique from its other family members as it does not contain the same DNA/RNA binding motif[26,134,135,137,163]. Hence, as it does not have the cysteine to uracil catalytic activity (as tested through mutation assays[161,164] and biochemical DNA deamination assays[165,166]) nor the ability to bind to ssDNA or ssRNA, it is thus unable to act like the other members of its family[26,134,135,137,163]. Instead, it may be involved in DNA demethylation specifically through an alternative mechanism involving its zinc-dependent cytidine deaminase activity[26,134,135,163]. Thus, APOBEC2 may be linked to cell differentiation as DNA methylation (5mC removal) is a form of epigenetic modification that can be used to suppress gene transcription, with demethylation allowing for gene expression[167,168]. In examining retina regeneration in zebrafish, it was found that APOBEC2 closely interacts with the chaperone protein ubiquitin-conjugating enzyme 9 (Ubc9), the POU class 6 homeobox 2 protein (Pou6f2), and the topoisomerase I-binding, arginine/serine-rich, E3 ubiquitin-protein ligase (Toporsa)[165,169–172]. In the zebrafish, expression of APOBEC2 has been shown to increase DNA demethylation of the genome which promoted the conversion of both neurons and glia to a stem cell fate allowing for optic nerve regeneration[155,172]. This demethylation is needed for the induction of cell differentiation and regeneration, which is done through revealing the DNA promoter regions that were suppressing

transcription to allow for the promotion of gene expression[167,173,174]. Furthermore, the methylation/demethylation status can be used to regulate muscle development, mature muscle status, and the respective changes needed in myogenesis[170]. This is often observed during myogenesis as there is a significant reduction in methylation status upon the induction of differentiation, as seen during both in early developmental differentiation and muscle regeneration[173–175]. Indeed, within the zebrafish model, the knockdown of APOBEC2 resulted in a dystrophic phenotype in skeletal muscle, adding to the key role of APOBEC2 within development and myogenesis[176]. Finally, APOBEC2 is preferentially expressed in slow-type muscles over fast-type muscles demonstrating a potential reliance on the oxidative metabolic pathway which is controlled by mitochondria[170]. One study that specifically looked at the impact on the mitochondria as a result of a knockout of APOBEC2, found that this knockout resulted in enlarged mitochondria, activated autophagic vacuoles causing mitophagy (mitochondrial autophagy), and generation of reactive oxygen species[135]. These mitochondrial defects resulted in muscle dysfunction and accumulation of nuclear abnormalities[135].

APOBEC2 has been observed to play a role in many additional processes such as left-right axis specification in *Xenopus* [171,176,177]. This study in *Xenopus* found that when examining left-right axis specification in early embryogenesis APOBEC2 expression is regulated by transforming growth factor β (TGF-β), with the downregulation of APOBEC2 being able to inhibit TGF-β through a feedback mechanism[177]. Additionally, it was found that this regulation occurred through an interaction with APOBEC2's cytidine deaminase enzymatic domain, suggesting for the first time, that in some species APOBEC2 does have cytidine deaminase activity[177]. The other potential pathway that APOBEC2 has been found to play be involved in is that of tumour necrosis factor-alpha (TNF-α) and interleukin-1β[178]. APOBEC2 expression increases upon stimulation from both

of these factors[178]. This was further verified through blocking NF-kappaB expression upstream of this pathway, which proved to prevent any further upregulation of APOBEC2 from TNF-α[178]. When examining APOBEC2's in cancer, overexpression of APOBEC2 in hepatocytes resulted in the development of tumours within both the liver and lungs before the age of two within transgenic mice[179]. Upon further examination, there was an accumulation of nucleotide alterations, specifically at the mRNA level, in tumour suppressing genes Pten and Tp65, which may have resulted in the increased rate of tumourigenesis[179]. Despite the above research, there is still no known consensus as to the mechanism by which APOBEC2 achieves its function; with theories including through RNA editing[134,179], DNA demethylation[155,156,171], binding to chromatin[180] or none of the above[170,176,177]

1.3.2 APOBEC2 as a Negative Regulator of Myoblast Differentiation

APOBEC2 is localized next to the sarcomere Z lines specifically in differentiated skeletal and cardiac striated muscle of mammals and chicken[169]. During muscle development and regeneration, APOBEC2 levels increase during early differentiation while it decreases as differentiation occurs (at times translocating in myoblasts from the nucleus to the cytoplasm)[26]. To support this, myoblasts isolated from APOBEC2 knockout mice have been used to determine that APOBEC2 does not function within the proliferative phase of myogenesis nor during terminal differentiation, rather solely playing a role early during differentiation[26]. Therefore, it is seen as a negative regulator of differentiation, both during development and regeneration after injury[26,177]. To aid this theory, it has been found that a lack of APOBEC2 promotes myoblast differentiation[26,178]. This can shorten the inflammation phase of muscle regeneration since APOBEC2 expression is induced by tumour necrotic factor-α which is triggered during the

inflammation phase by activated macrophages[26,178]. The effect of this, however, has been shown to accelerate the repair phase of muscle regeneration and thereby also shortening the destruction/inflammation phase[26,178]. The repercussions of such can result in an impairment of functional muscle recovery or a reduction in the diameter of myofibers[26]. Previous studies have found that deficiency of APOBEC2 results in a shift from fast to slow type muscle fibers, myopathy, dystrophic phenotypes, decreased muscle mass, and the promotion of muscle regeneration[26,135,170,176].

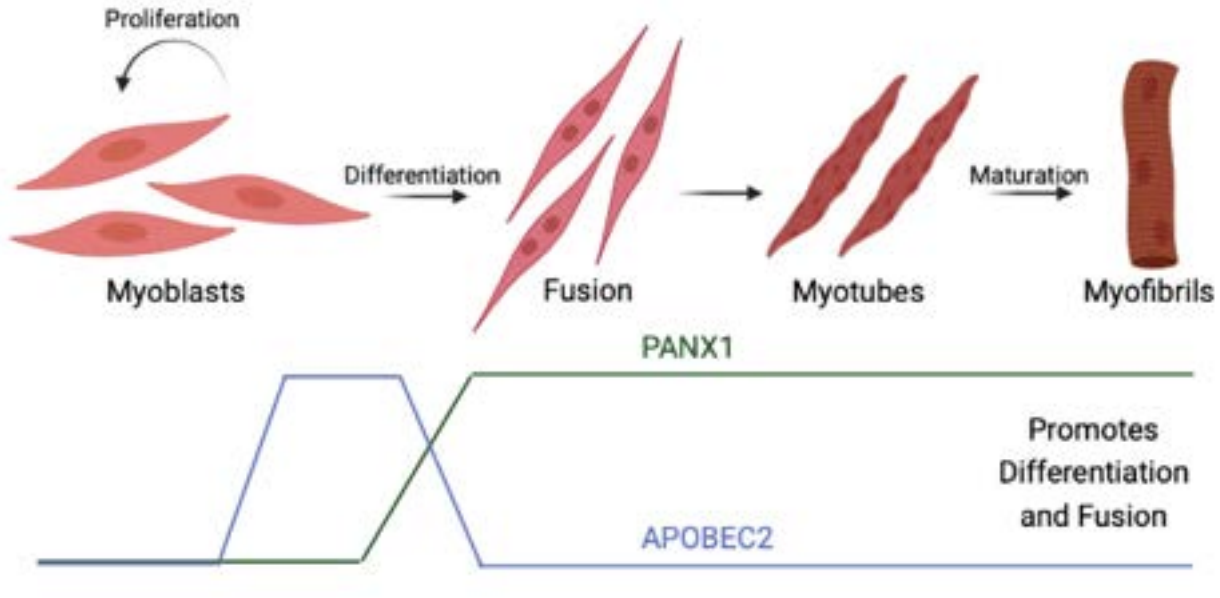

Figure 1: *Regulation of APOBEC2 and PANX1 Levels Throughout Myogenesis*
APOBEC2 levels have previously been reported to rise during proliferation of myoblasts, with expression peaking during early differentiation[26]. However, as differentiation progresses APOBEC2 levels begin to decrease to the point in which APOBEC2 is not expressed in the terminal stages of differentiation[26]. It is this low expression of APOBEC2 however that has been determined to promote both differentiation and fusion, thus determining APOBEC2 as a negative regulator of differentiation[26,177]. By contrast, PANX1 expression is low during the proliferative and undifferentiated phases of myogenesis and increases during differentiation[46]. In the case of PANX1, its high expression has been found to promote both myoblast differentiation and fusion[46]. Created with BioRender.

Although muscles from APOBEC2 knockout mice have normal morphology, they display an earlier progression of differentiation and fusion[26]. This was associated with an increase in the expression of myosin heavy chain, cooperating factor MEF2C, and myogenin[26]. Additional effects of the upregulation of myogenin are the upregulation of E3 ubiquitin ligases atrogen-1 and MuRF-1 both of which promote ubiquitin proteolysis[26,181]. This ubiquitin proteolysis could result in muscle atrophy through myogenin-atrogin-1-MuRF-1 signaling, however, it has been also associated with the promotion of myoblast differentiation through the degradation of differentiation inhibitory factors[26,181]. Thus, reduction or lack of APOBEC2 enhances the fusion, differentiation, and maturation of myoblasts[26]. Upregulation was also observed for MyoD and myf5 in APOBEC2 deficient myoblasts during myoblast proliferation, suggesting that the APOBEC2 deficiency aids in the determination of myogenic fate through enhancing the formation of myotubes and suppressing further growth[26]. One potential mechanism as to how APOBEC2 regulates myoblast differentiation may be through acting as a transcriptional repressor[182]. An analysis of genes that are repressed during differentiation found that APOBEC2 occupies their promoter regions by binding to chromatin, while at the same time interacting with HDAC transcriptional corepressor complexes (HDAC and CHD4)[182]. Hence, APOBEC2's role in the transcriptional control of myogenesis makes it a unique member of its family through its ability to modify cellular differentiation and regeneration[182].

1.3.3 Role of PAX7 in Myogenesis Through Regulating APOBEC2 Levels

While the mechanism regulating APOBEC2 expression remains largely unknown, some research has come to light recently suggesting the involvement for PAX7[183]. PAX7 is a transcriptional factor involved in embryonic myogenesis, the regulation of satellite cells, and the

maintenance of adult muscle regeneration[184,185]. For myogenic differentiation to occur, PAX7 must be downregulated to allow the necessary myogenic regulatory factors to be synthesized, which results in the myogenic precursor cells or satellite cells to enter differentiation[186–188]. PAX7 can be expressed in both quiescent satellite cells and early differentiating cells, regulating the balance between self-renewal and differentiation[189,190]. Specifically, it has been found that one event required for commitment to muscle cell identity and the activation of satellite cells is that of PAX7 mediated DNA demethylation[191]. Upon further examination, it was found that APOBEC2 played a key role in this process, given that when APOBEC2 is downregulated within myogenic precursors there was a reduction in the expression of myogenic regulatory factors including myogenin[191]. PAX7 may also regulate cell proliferation, where depending on the model system, the overexpression of PAX7 results in an increase[192–194] or a decrease in proliferation[195–197]. Notably, within PAX7$^{-/-}$ teratomas, there was a resultant downregulation of APOBEC2 expression[183]. Based on what is observed upon the upregulation of PANX1, this subsequent downregulation of APOBEC2 and inhibition of cell proliferation therefore appears to be potentially linked to the regulation of PAX7[129,183].

1.4 Project Rationale

As stated earlier, our laboratory has previously demonstrated that overexpression of PANX1 inhibits eRMS and aRMS malignant properties *in vitro* and *in vivo*[87]. Overexpression of PANX1 in aRMS Rh30 cells leads to a decrease in *APOBEC2* transcription levels[129]. APOBEC2 has been shown to act as a negative regulator of myogenesis, suggesting that the reduction of APOBEC2 expression may increase the differentiation, fusion, and maturation of myoblasts[26,170]. While this downregulation of APOBEC2 in RMS may be beneficial by promoting processes such

as cell differentiation, the function of APOBEC2 in RMS and its potential function in the PANX1-mediated inhibition of RMS malignant properties has yet to be investigated[87].

1.4.1 Hypothesis

Based on the role of APOBEC2 in myogenesis, we hypothesize that the downregulation of APOBEC2 will decrease RMS malignant properties and that APOBEC2 is involved in the signaling pathways by which PANX1 alleviates RMS malignancy.

1.4.2 Research Aims

1) Determine the endogenous expression of APOBEC2 in aRMS and eRMS patient-derived cell lines.
2) Examine the functional relationship between APOBEC2 and PANX1.
3) Determine the role of APOBEC2 in the PANX1-mediated inhibition of RMS progression.

2.0 Materials and Methods

2.1 Cell Lines and Cell Culture

Patient-derived RMS cell lines for representative eRMS (Rh18, Rh28, Rh41) and aRMS (Rh30, and Rh36) were obtained from Dr. P. Houghton (St. Jude Children's Hospital, Memphis, TN, USA). The last aRMS representative cell line RD was from the American Type Culture Collection (ATCC). These cells were cultured in RPMI-1640 media (Gibco, Massachusetts, USA, #11875093) supplemented with 10% fetal bovine serum (FBS) (Sigma-Aldrich, Massachusetts, USA, #F1051-500), and 1% penicillin/streptavidin (Thermo Fisher, Massachusetts, USA, #SV30010). HEK293T cell lines were from ATCC and cultured in complete DMEM high glucose media (HyClone, Utah, USA, #SH30022.01) supplemented with 10% FBS, and 1% penicillin/streptavidin. HSMM cell lines were from Dr. B. Chazaud (Institut NeuroMyoGene, Universite Claude Bernard Lyon, Lyon, France). These HSMM cell lines were grown in HAMs F14 Media (MultiCell, Rhode Island, USA, #318014-Cl) supplemented with 30% FBS, 10 μg/mL Insulin (Sigma-Aldrich, #16634), 25 ηg/mL basic fibroblast growth factor (bFGF) (Sigma-Aldrich, #SRP4037), 10 ηg/mL human epidermal growth factor (hEGF) (Sigma-Aldrich, #E9644), 2 μg/mL amphotericin (Sigma-Aldrich, #A2942), 1% penicillin/streptavidin, at a pH of 7.4[198]. The HSMM cell lines were differentiated for up to ten days in DMEM high glucose media supplemented with 5% horse serum (Gibco, #16050111).

The cumate-inducible RMS stable cell lines for the expression of PANX1 using the SparQ Cumate Switch Inducible System (System Bioscience, #QM600A-1) were made by Dr. Xiao Xiang from our laboratory and have been previously described (Children's Hospital of Eastern Ontario, Ottawa, ON, Canada)[87]. These stable cells were cultured in RPMI-1640 media supplemented with 10% FBS, 1% penicillin/streptavidin, 100 μg/mL geneticin (Gibco, #10131-

035), and 2 μg/mL puromycin (Sigma-Aldrich, #P8833). PANX1 expression was inducible upon 30 μg/mL of cumate (SBI, Califonia, USA, #QM150A-1). If cultured for longer than 24 hours, cumate was refreshed every other day. Rh18 and Rh30 stable cell lines for the AHNAK or NTC shRNA expression were made by Dr. Xiao Xiang from our laboratory and were cultured with the RMS stable cell lines media with the further addition of 50 μg/mL hygromycin (Sigma-Aldrich, #400053), as we previously described[129]. To induce the shRNA-based knockdown, these stable cells would have been incubated for 72 hours in 50 ηg/mL doxycycline (Sigma-Aldrich, #D3447). As these cells were made in the same backbone as the previously described stable PANX1 expression RMS cells, upon the addition of 30 μg/mL of cumate for at least 24 hours PANX1 expression was also inducible. If cultured for longer than 24 hours, cumate and doxycycline were refreshed every other day.

To generate the APOBEC2 overexpression stable cell lines, lentiviral vectors were obtained from Vector Builder for both the overexpression of APOBEC2, pLV[Exp]-Bsd-TRE>hAPOBEC2[NM_006789.4] (Vector ID: VB210721-1157dvf), and the empty vector pLV[Exp]-Bsd-TRE>ORF_Stuffer (Vector ID: VB210809-1167qsh). These vectors were packaged into lentiviruses following supplier protocols (SBI, #LV100A-1). Once the lentiviruses were generated, they were then tittered using the Global UltraRapid Lentiviral Titer Kit (SBI, #LV961A-1) following the manufacturer's protocols. Based on the generated MOI values, the lentiviruses were transduced into Rh30 cells using a range of MOIs from 0.05 to 250, to determine an MOI value that was physiologically relevant based on the endogenous APOBEC2 expression in differentiating HSMM cells. These viruses were then transduced at an MOI of 100 into Rh30 cells stably inducible PANX1 or the empty vector (described above). The cells were then cultured with the RMS PANX1 stable cell line media with the further addition of 3μg/mL blasticidin for

selection (Milipore, Massachusetts, USA, #203350). If cultured for longer than 24 hours, cumate was refreshed every other day.

All cell cultures were maintained at 37°C and 5% CO_2.

2.2 RNA Extraction, Reverse Transcription, and Quantitative PCR

When examining APOBEC2 transcript levels endogenously, $8.0x10^5$ cells were plated into a 6-well plate to be cultured in their respective media, and then collected after 24 hours. When examining APOBEC2 transcript levels upon the overexpression of PANX1, the same number of cells would be plated into a 6-well plate, and then the cells were collected after 8 days after cumate induction (chosen as a respective time point that showed consistent regulation of APOBEC2 by PANX1). Using an RNeasy Mini Kit (Qiagen, Maryland, USA, #74104) total RNA was extracted and purified using the TURBO DNA-free kit (Invitrogen, Massachusetts, USA, #AM1907). A High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher, 4368814) was then used to perform reverse transcription. Finally, the synthesized cDNA was used as a template with the SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad, California, USA, #172-5271) for quantitative PCR on Mastercycler ep-*realplex* (Eppendorf, Hamburg, Germany) real-time PCR system using 50ηg of cDNA. The primers used for qPCR included gene-specific validated primers for APOBEC2 and GAPDH (Origene, Sequences can be found in Table 2). Relative expression and fold change were then determined using a comparative Ct method.

Table 2: *Sequences of the primers used for qPCR analysis*

Construct	***Direction of Primer***	***Primer Sequence (5' to 3')***
APOBEC2	Forward	AGAACCTGGACGACCCTGAGAA
	Reverse	CAACCACATAGCAGAGGAAGGTC
GAPDH	Forward	CAAGACCTTGGGCTGGGAC
	Reverse	AGGCTGCGGGCTCAATTTAT

2.3 Antibodies

Primary antibodies: anti-APOBEC2 (Sigma-Aldrich, #HPA017957), anti-PANX1 (Sigma-Aldrich, #HPA016930), anti-MHC (R&D Systems, Minnesota, USA, #MAB4470), anti-MyoD (Novus, Missouri, USA, #NBP1-54153SS), anti-GAPDH (Advanced ImmunoChemistry, California, USA, Mab 6C5), anti-GAPDH (Cell Signaling, Massachusetts, USA, #14C10), anti-PAX7 (DSHB, Iowa, USA, AB_528428) and anti-myogenin (Santa Cruz Biotechnology, Texas, USA, #sc-12732). Secondary antibodies used for immunofluorescence included: anti-rabbit Alexa Fluor 488 (Invitrogen, #A11008 or #A11017), anti-mouse Alexa Fluor 546 (Invitrogen, #A11003) and anti-mouse Alexa Fluor 594 (Invitrogen, #A11012 or #A11005). Secondary antibodies used for western blotting included: anti-rabbit Alexa Fluor 680 (Invitrogen, #A21109) and anti-mouse Alexa Fluor 800 (LI-COR, Nebraska, USA, #925-32210). A summary table with respective dilutions for both western blot analysis and immunofluorescence can be found in Table 1.

Table 3: *List of Primary and Secondary Antibodies Utilized for Western Blot Analysis and Immunostaining*

Antigen	***Supplier***	***Ig Type***	***Dilution Buffer***	***Dilution for Western Blot***	***Dilution for Immunofluorescence***
APOBEC2	Sigma-Aldrich	Rabbit	10% Skim Milk	1:1000	1:200
GAPDH	Cell Signaling	Rabbit	5% BSA	1:5000 or 1:10000	N/A
GAPDH	Advanced ImmunoChemistry	Mouse	5% BSA	1:5000 or 1:10000	N/A
MHC	R&D Systems	Mouse	5% BSA	1:5000	1:100
Mouse 546	Invitrogen	Goat	3% BSA	N/A	1:500
Mouse 594	Invitrogen	Goat	3% BSA	N/A	1:500
Mouse 800	LI-COR	Goat	5% BSA	1:5000 or 1:10000	N/A
MyoD	Novus	Rabbit	5% BSA	1:1000	1:100
PANX1	Sigma-Aldrich	Rabbit	5% BSA	1:2000	1:200
PAX7	DHSB	Mouse	5% BSA	1μg/mL	0.3μg/mL
Rabbit 488	Invitrogen	Goat	3% BSA	N/A	1:500
Rabbit 680	Invitrogen	Goat	5% BSA	1:5000 or 1:10000	N/A

2.4 Western Blotting

When examining protein expression endogenously (APOBEC2, myogenic regulatory factors, or PAX7), $8.0x10^5$ cells would be plated into a 6-well plate to be cultured in their respective media, and then collected after 24 hours. When examining protein expression (APOBEC2, myogenic regulatory factors, or PAX7) over time, the same number of cells would be plated into

a 6-well plate, and the cells would be collected at regular time intervals after cumate induction (0hr (cells collected just prior to cumate induction), 2 days, 4 days, 8 days, and 10 days). When examining AHNAK protein expression, cells (WT Rh30, Rh30 cells cumate inducible to over-express PANX1 with doxycycline-inducible expression of NTC shRNA, and Rh30 cells cumate inducible to over-express PANX1 with doxycycline-inducible expression of AHNAK shRNA) were plated in a 6-well plate with $8.0x10^5$ cells per well. Twenty-four hours after initial seeding, the cells were induced with doxycycline to promote the knockdown of AHNAK and left in culture for seventy-two hours. The cells were then treated with cumate (to induce the overexpression of PANX1) for 24 hours and then collected.

Cells were washed in cold phosphate-buffered saline (PBS) and solubilized in lysis buffer (150mM NaCl (Fisher Scientific, #BP358), 10mM Tris-HCl (pH 7.4), 1mM EDTA (Sigma-Aldrich, #E5134), 1mM EGTA, (Sigma-Aldrich, #E3889) 0.5% Nonidet P-40 (Fluka, North Carolina, USA, #74385), and 1% Triton X-100 (Fisher Scientific, #AC327271000)) which was supplemented with protease/phosphatase inhibitor (Cell Signaling, #5872S).

The protein concentrations were determined through a microBCA (Thermo Scientific, #23225). Proteins were denatured by boiling in Laemmli buffer (β-mercaptoethanol 1.25M Tris-HCl pH 6.8, Glycerol, SDS, and Bromophenol blue) for 5 minutes. Proteins were then separated by SDS-PAGE on a 12% gel (APOBEC2) or a 10% gel (Myogenic regulatory factors and PAX7) or a 6% gel (AHNAK) and then transferred onto membranes (nitrocellulose for APOBEC2 and myogenic regulatory factors, and PVDF for AHNAK and PAX7). The nitrocellulose membranes were then blocked with 10% skim milk in tris-buffered saline (TBST) for two hours, whereas the PVDF membranes were blocked with 5% bovine serum albumin (BSA) in phosphate-buffered saline solution (PBST) for one hour. The membranes were then immunoblotted with anti-

APOBEC2 (1:1000), anti-PANX1 (1:2000), anti-MyoD (1:1000), anti-Myogenin (1:1000), anti-AHNAK (1:1000), anti-MHC (1:5000) or anti-PAX7 (1μg/mL) overnight at 4°C. Membranes were washed with PBST and then immunolabeled at room temperature for one hour with secondary antibodies (1:5000). Membranes were then re-probed using anti-GAPDH (1:5000 or 1:10000) for one hour at room temperature for normalizing protein loading. Western blots were then imaged and quantified using the Odyssey CLx Imaging System (Li-Cor). When quantifying the blots, the relative intensities were determined based on the average of western blots from three independent experiments.

2.5 Immunofluorescence Microscopy

When examining protein expression endogenously, $8.0x10^5$ cells would be plated into a 6-well plate on coverslips to be cultured in their respective media, and then fixed after 24 hours. When examining protein expression over time, the same number of cells would be plated into a 6-well plate onto coverslips, and the cells would be fixed at regular time intervals after cumate induction (0hr (cells collected just prior to cumate induction), 2 days, 4 days, 8 days, and 10 days). Cells were fixed onto coverslips (Fisher Scientific, #12-545-80) using a solution of 80% methanol and 20% acetone for 30 minutes at 4°C (for APOBEC2) or using a solution of 3.7% formaldehyde for 30 minutes at room temperature (for myogenic regulatory factors and PAX7). Permeabilization was done with 0.1% Triton X-100 (Sigma-Aldrich, #9036-19-5) and 0.1M glycine (Sigma-Aldrich, G8898) in PBS for up to 30 minutes. Blocking was then done for one hour in a solution of 3% BSA (Sigma-Aldrich, #A7906) in PBS. Cells were then incubated with primary antibodies for anti-APOBEC2 (1:200), anti-myogenin (1:100), anti-myoD (1:100), anti-MHC (1:100), and anti-PANX1 (1:200) for one hour at room temperature. However, anti-PAX7 (0.3μg/mL) was incubated at 37°C for one hour. Cells were then washed with PBS and then treated with secondary

antibodies for another hour at room temperature. Cells were washed again with PBS, then distilled water, and mounted with DAPI Fluoromount G (SouthernBiotech, Alabama, USA, #0100-20). Representative images were collected sequentially with the Olympus Fluoview FV-1000 Laser Confocal Microscope or the EVOS Cell Imaging System microscope maintaining microscope settings. When comparing myogenic regulator factor expression, images of fifteen random fields were collected at x40 objective and were sequentially counted. When evaluating PAX7 expression, images of ten random fields were collected at x60 objective and were sequentially counted. Quantifications from at least three independent experiments were made using ImageJ software.

2.6 Evaluation of Multinucleation Status

To assess the multinucleation status of Rh28, Rh36, and Rh41 upon overexpression of PANX1, endogenous cells were seeded in 6-well plates at $8.0x10^5$ cells per well onto coverslips. Twenty-four hours post-seeding, these cells were transiently transfected with PANX1 cDNA using Lipofectamine 2000 Reagent (Invitrogen, #52887) given the manufacturer's protocol. Control cells were transfected with the empty vector. Twenty-four hours post-transfection the cells were fixed using 3.7% formaldehyde and stained for PANX1 protein expression using the methods that are described above. The coverslips were then analyzed using the Olympus Fluoview FV-1000 Laser Confocal Microscope at x60 objective, taking images of fifteen random fields. The multinucleation status was determined by sequentially counting the number of nuclei within a transfected cell.

This experiment was later repeated using the APOBEC2 overexpression stable cells. To assess the multinucleation status, either the stable cells constitutively overexpressing APOBEC2 or the empty vector were seeded in a 6-well plate at $8x10^5$ cells per well onto coverslips. Twenty-

four hours post-seeding cells were treated with or without the presence of 30 μg/mL cumate (to induce the overexpression of PANX1) and then cultured for an additional forty-eight hours. At this point, the cells were fixed with a solution of 80% methanol and 20% acetone and stained for APOBEC2 protein expression using the same methods as described above. The coverslips were then analyzed using the Olypmus Fluoview FV-1000 Laser Confocal Microscope at x60 objective, taking images of fifteen random fields. Multinucleation status was again classified by sequentially counting the number of nuclei within a transfected cell.

2.8 BrdU Proliferation Assay

To assess cell proliferation, the stable APOBEC2 overexpression or empty vector cells were seeded into a 96-well plate at 5,000 cells per cell. Twenty-four hours later, cells were treated with either 0.06 μg/mL or 30 μg/mL cumate to induce overexpression of PANX1. These plates were then incubated for an additional two or four days. At the end of this period, the wells were then treated with 10μM BrdU incorporating reagent for two hours (this time was determined based on the doubling time of parental Rh30 being 38.8 ± 5.6 hours) and then analyzed for proliferation following the protocol defined in the BrdU Cell Proliferation ELISA Kit (abcam, Cambridge, UK, #ab126556). Results were analyzed using a single read at 450 ηm on a Synergy HTX plate reader (BioTek, Vermont, USA).

2.9 Trypan Blue and Alamar Blue Viability Assays

Constitutively overexpressing APOBEC2 and empty vector stable cells were seeded in 6-well plates at $8x10^5$ cells per well. 24 hours after seeding the cells were treated with or without the presence of 30 μg/mL cumate for two, four, or eight days. At this point, the cells and supernatant

were then combined with equal parts of 0.4 % Trypan Blue stain (NanoEntek, Massachusetts, USA, #EBT-001). The live cells were then counted on an Invitrogen Countess.

For the Alamar Blue viability assay, constitutively overexpressing APOBEC2 and empty vector stable cells were seeded in 96-well plates at $5x10^4$ cells per well. 24 hours after seeding the cells were treated with or without the presence of 30 μg/mL cumate for two, four, or eight days. At the end of this incubation period, the cells were treated with 0.15 mg/mL Resazurin sodium salt (Sigma-Aldrich, #R7017-5G) for an additional twelve hours and then read on a Synergy HTX plate reader (BioTek) with an excitation: emission filter (530/25; 590/25 ηm).

2.10 Spheroid Formation and Regression Assays

3D spheroid assays were all performed and quantified using the IncuCtye Zoom Live Cell Imaging System (Essen Bioscience, Michigan, USA). For spheroid formation assays, constitutively overexpressing APOBEC2 and empty vector stable cells were pretreated with or without 0.06 μg/mL or 30 μg/mL cumate for twenty-four hours and then seeded into ultra-low adhesion plates at 5,000 cells per well (Costar, Washington, D.C., USA, #7007). For spheroid regression assays, constitutively overexpressing APOBEC2 and empty vector stable cells were seeded into the same ultra-low adhesion plates, and then select cells were treated with 0.06 μg/mL or 30 μg/mL cumate after 48 hours when spheroids had formed. For both assays, spheroids were analyzed by mean integrated intensity using the same cell imaging system every two hours for two hundred and forty hours.

2.11 Statistics

Each experiment was performed three times (n=3) and for each assay, the cells were plated in triplicates for the validity of the results. Statistical significance was later determined through the use of either an Analysis of Variance (ANOVA) or unpaired two-tailed Student's t-test using a Tukey's or Bonferroni post hoc test. Results are all described and presented as mean ± sd, with results-producing a $P<0.05$ deeming statistically significant.

3.0 Results

3.1 Endogenous Expression of APOBEC2 in aRMS and eRMS Patient-Derived Cell Lines

As APOBEC2 expression has never been examined within RMS, we first determined its endogenous levels in a panel of patient-derived aRMS (Rh28, Rh30, and Rh41) and eRMS (Rh18, Rh36, and RD) cell lines. *APOBEC2* expression was first examined at the RNA level through RT-qPCR analysis (Figure 2A) and then through Western Blotting (APOBEC2 was detected as one band ~25kDa, which is its expected molecular weight) examining protein levels (Figure 2B and 2C). APOBEC2 expression was compared to that of undifferentiated and differentiating human skeletal muscle myoblasts (HSMM).

These results showed several key findings about APOBEC2 expression. The first of which was that APOBEC2 expression levels were significantly increased in differentiating (6 days) HSMM compared to undifferentiated HSMM (Figure 2A and 2B). Secondly, that APOBEC2 levels are low in all the RMS cell lines and comparable to that of undifferentiated HSMM (Figure 2A and 2B). Finally, it was found that there was no significant difference between APOBEC2 levels in aRMS versus eRMS (Figure 2D). Overall, these results show that the transcript and protein of APOBEC2 are low in all the RMS patient-derived cell lines assessed and more comparable to that of undifferentiated HSMM than differentiating HSMM.

Confocal immunofluorescence microscopy was next performed for APOBEC2 and detected it as a punctate labeling with intracellular localization, the majority of which was within the cytosol, however with some staining within the nucleus (Figure 2E). The localization that was observed was expected, as it has been predicted and observed that APOBEC2 can translocate between the nucleus and the cytoplasm[26]. When comparing undifferentiated HSMM, differentiated HSMM, and the six RMS cell lines, this localization pattern appears to remain the same. The only

common factor is that when the expression of APOBEC2 increases so does staining observed within the nucleus. This immunolabeling analysis revealed that APOBEC2 is expressed at low levels in the RMS cell lines and undifferentiated HSMM but is highly expressed in differentiating HSMM (Figure 2E).

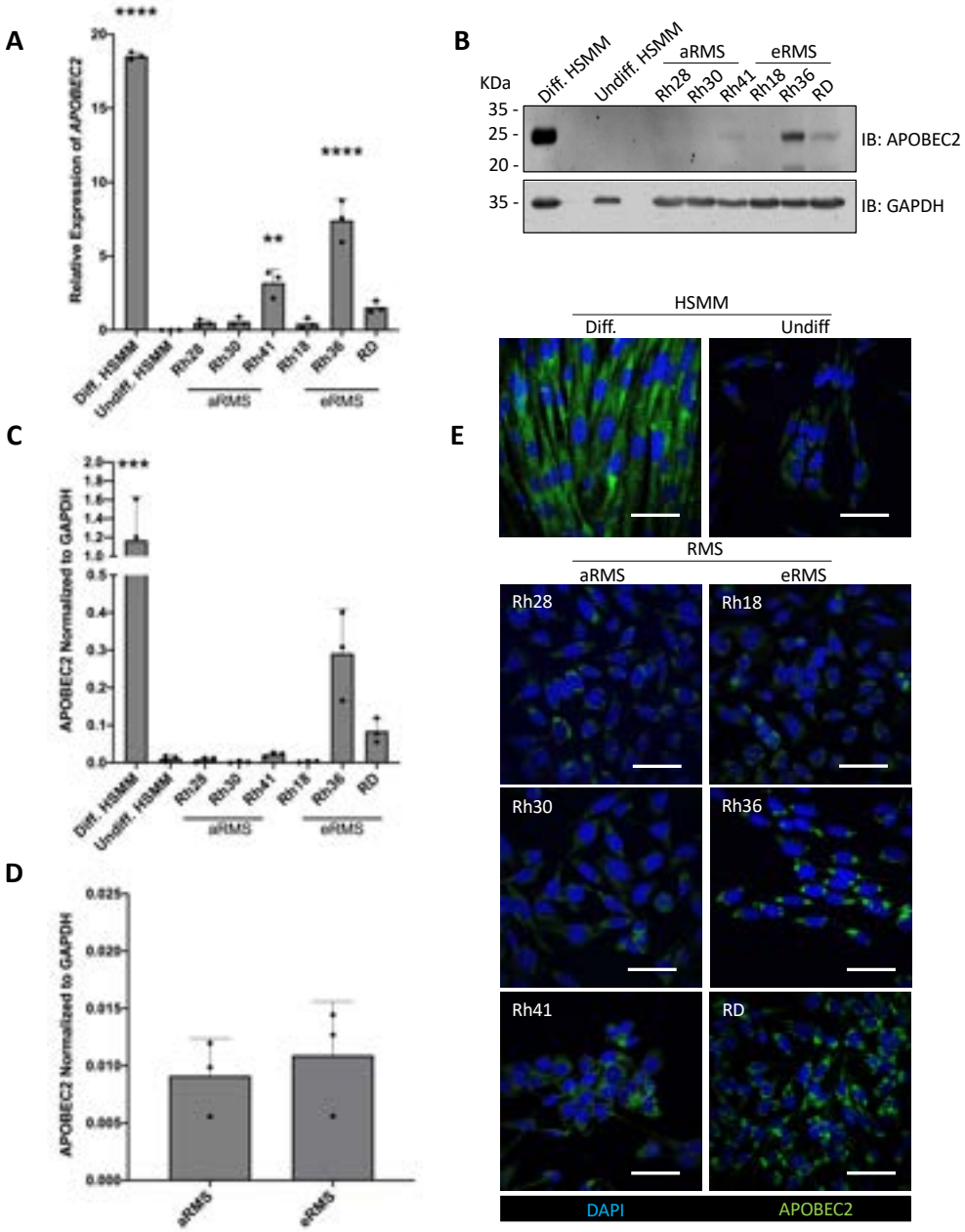

Figure 2: *Expression of APOBEC2 in patient-derived aRMS and eRMS cell lines.*
(A) RT-qPCR relative expression analysis of *APOBEC2* transcript levels. A one-way ANOVA test (Tukey post-hoc test) was used to compare the 6 RMS cell lines, with a separate test comparing Diff. HSMM to the 6 RMS cell lines. (B) Representative Western Blot of APOBEC2 protein in the various RMS cell lines, as well as undifferentiated and differentiated HSMM. GAPDH was used as a loading control. (C) Quantification of APOBEC2 protein levels normalized to GAPDH for each cell line (n=3). A one-way ANOVA test (Tukey post-hoc test) was used to compare the 6 RMS cell lines individually to differentiating and undifferentiated HSMM. (D) Quantification of APOBEC2 in the aRMS (Rh28, Rh30 and Rh41) cell lines compared to that of the eRMS (Rh18, Rh36 and RD) cell lines (n=3). (E) Immunofluorescent labelling of APOBEC2 (green) in the RMS cell lines, undifferentiated (undiff.) and differentiating (Diff.) human skeletal muscle myoblasts (HSMM). (Blue = nuclei, bars = 30µm.) HSMM differentiating for six days were used as a positive control. ** $P < 0.01$, *** $P<0.001$, **** $P<0.0001$ as determined by one-way ANOVA (Tukey post-hoc test). Results are expressed as mean ± sd (n=3)

3.2 Downregulation of APOBEC2 upon the Upregulation of PANX1 in a Panel of Cumate-inducible RMS Cells.

It has previously been found by an RNA-sequencing analysis (and validated through RT-qPCR) of Rh30 cells either expressing ectopic PANX1 or GFP that *APOBEC2* expression was significantly downregulated upon the upregulation of *PANX1*[129]. To determine whether this results in downregulation at the protein levels and to expand on these results, APOBEC2 was analyzed by Western Blotting in a panel of stable aRMS (Rh28, Rh30, and Rh41) and eRMS (Rh18, Rh36, and RD) cell lines in which PANX1 is overexpressed under the control of the cumate switch system[87]. When initially examining just the GFP control cells in all six RMS cell lines (without the overexpression of PANX1) it is observed that APOBEC2 expression increases in the control cells 10 days in culture (Figure 3 A-F). Three of the six cell lines (Figure 3A. Rh28, B. Rh30, and F. RD) showed a significant downregulation of APOBEC2 upon PANX1 upregulation, compared to their empty vector control cells after a 10 day induction period.

Using RT- qPCR analysis, we next wanted to verify our RNA-sequencing results and to determine if the regulation of APOBEC2 by PANX1 is the result of protein degradation or errors in translation[129]. As a result, APOBEC2 mRNA transcript levels (specifically after eight days in culture as this was the time point of statistically significant APOBEC2 downregulation by PANX1) were measured in our stable RMS cell lines overexpressing PANX1. As shown in Figure 4, per the Western Blotting analysis, amongst the six cell lines only Rh28, Rh30, and RD cell lines showed significant downregulation of *APOBEC2* transcript upon PANX1 upregulation. These results suggest that the regulation of APOBEC2 by PANX1 is at the transcriptional level and not the result of primarily protein degradation or errors in translation.

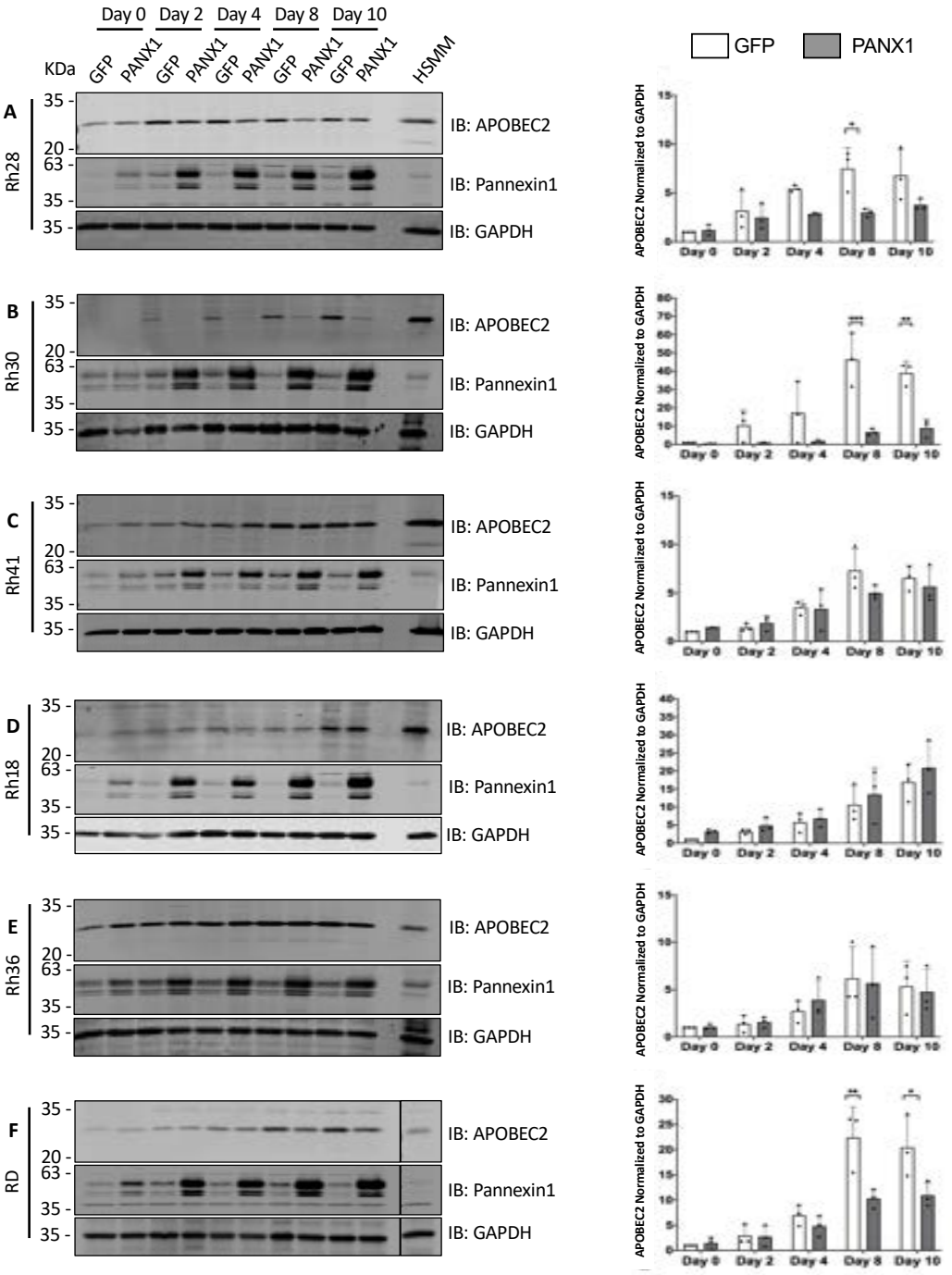

Figure 3*. Western Blot analysis of APOBEC2 levels upon induction of PANX1 in our six RMS cell lines.*

Protein was extracted from three aRMS (A. Rh28, B. Rh30 and C. Rh41) and three eRMS (D. Rh18, E. Rh36 and F. RD) cell lines over-expressing PANX1 or the GFP control after induction with cumate over a time course of eight days. GAPDH was used as a loading control. HSMM differentiating for six days were used as a positive control. * $P < 0.05$, ** $P<0.01$, *** $P<0.001$ compared to GFP as determined by two-way ANOVA and unpaired students T-tests. Results are expressed as mean ± sd (n=3).

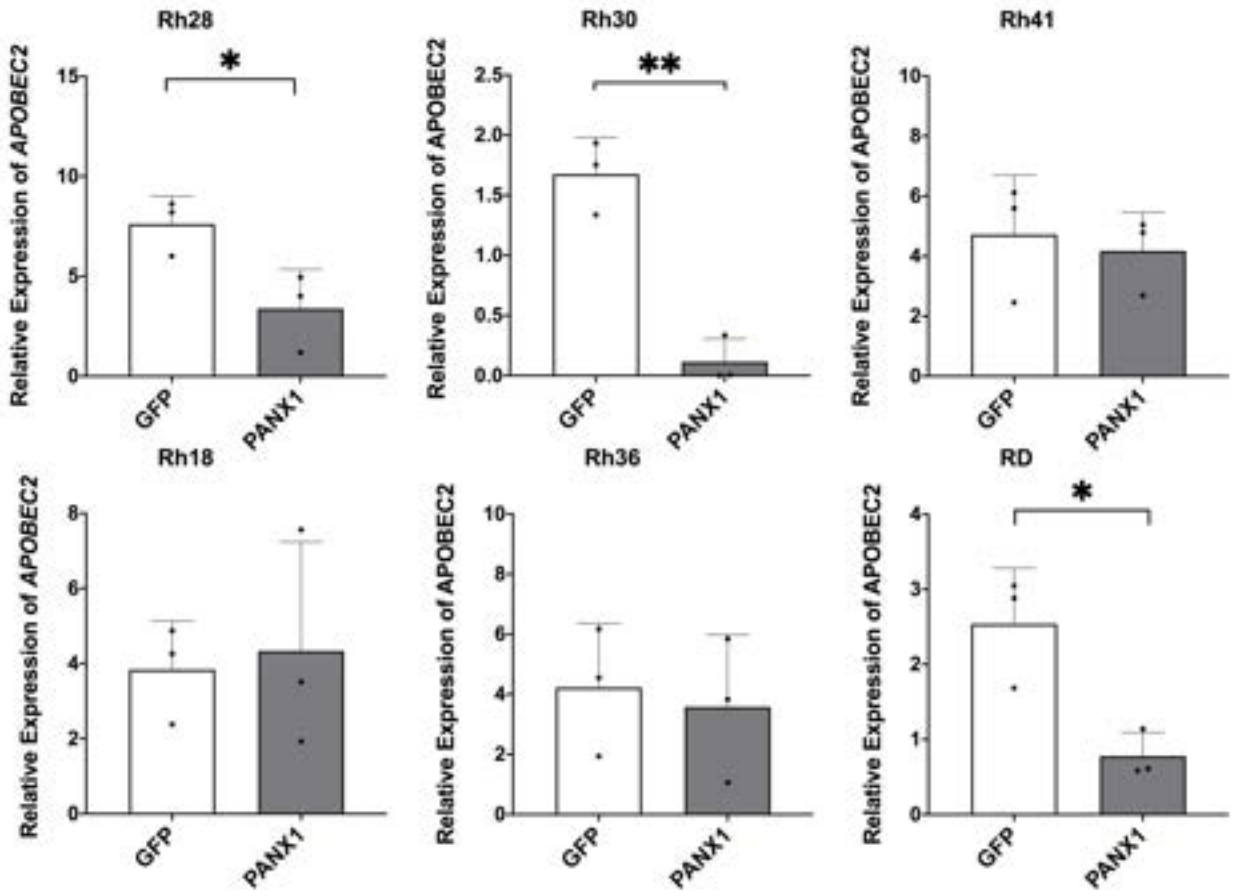

Figure 4*: RT-qPCR analysis of APOBEC2 transcript levels upon induction of PANX1 in our six RMS cell lines.*

RNA was extracted from three aRMS (Rh28, Rh30 and Rh41) and three eRMS (Rh18, Rh36 and RD) over-expressing PANX1 or the GFP control after induction with cumate for eight days. * $P < 0.05$, ** $P<0.01$ compared to GFP with cumate as determined by unpaired students T-tests. Results are expressed as mean ± sd (n=3).

3.3 Endogenous Expression of Myogenic Markers in eRMS and aRMS Cell Lines.

When examining the initial patient and tumour characteristics, we were unable to find any noticeable link between the three patient-derived cell lines (Rh28, Rh30, and RD) that display APOBEC2 downregulation upon PANX1 overexpression that could potentially explain why this was not observed in the other three cell lines. Due to the potential role of APOBEC2 in muscle differentiation and the known disruption of myogenic regulatory factors seen in RMS, we next wanted to look at the endogenous expression of myogenic markers in the six RMS cell lines (Figure 5, MyoD and myogenin shown, with MHC previously completed by our laboratory but unpublished). All six cell lines show similar levels of MyoD and myogenin. Previous work from our laboratory has also illustrated that MHC expression is very low or not detectable in all six RMS cell lines [124]. Altogether this data indicated a similar baseline in terms of myogenic markers, thus suggesting that the effect seen in Rh28, Rh30, and RD cell lines is not due to their differentiation status.

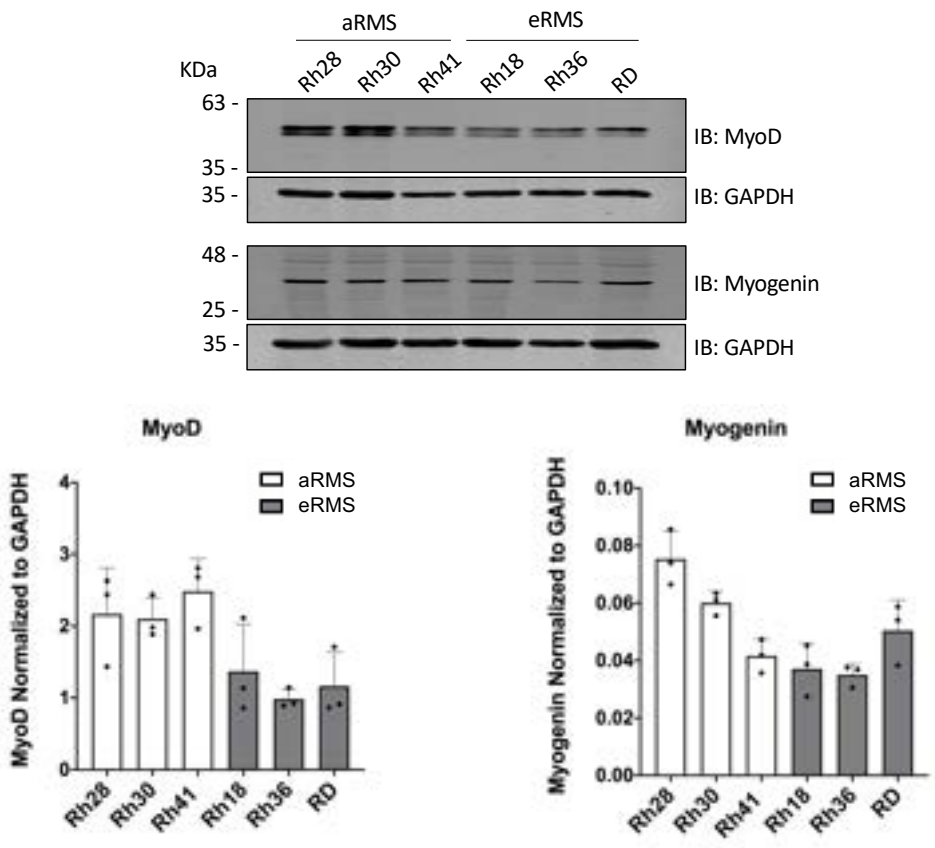

Figure 5*. Western blot analysis of endogenous MyoD and Myogenin levels in RMS cell lines.* MyoD and myogenin levels were examined in our panel of six RMS cell lines (three aRMS (Rh28, Rh30 and Rh41) and three eRMS (Rh18, Rh36 and RD)), with GAPDH used as a loading control. Results are expressed as mean ± sd (n=3).

3.4 Regulation of Myogenic Markers Upon the Upregulation of PANX1 in a Panel of Cumate-inducible RMS Cells.

Our laboratory has previously shown that the percentage of myogenin-positive cells and multinucleation status is increased in Rh30 cells overexpressing PANX1 over ten days[87]. However, these effects were not observed in Rh18 cells[87]. As this appeared to correlate preliminarily with the cell lines in which APOBEC2 levels are regulated by PANX1, we wanted to observe if the regulation of APOBEC2 by PANX1 is seen only in cells in which PANX1 induces partial differentiation or fusion. Thus, we looked at the expression of these myogenic markers (MyoD (Figures 6 and 7), myogenin (Figures 8 and 9), and MHC (Figures 10 and 11)) over the same ten-day cumate induction of PANX1 in our four other RMS stable cell lines (Rh28, Rh26, Rh41, and RD) through both immunofluorescence microscopy and Western blot analysis. When examining MyoD expression, there were no morphological changes observed and no significant differences in the percent of cells staining positive for MyoD as a result of the overexpression of PANX1 (as compared to the GFP control) as seen by immunofluorescence microscopy on any of the time points for any of the RMS cell lines observed (Figure 6). When analyzing MyoD protein expression by Western Blot analysis, these previously stated results were confirmed, showing no significant differences within ten days of cumate treatment between the control GFP vector and the cumate induced PANX1 overexpressing vector for any of the cell lines (Figure 7). When subsequently examining myogenin expression by immunofluorescence (Figure 8) and protein expression by Western Blot analysis (Figure 9), the same results as observed for MyoD were seen (showing no significant difference in myogenin expression between cells overexpressing PANX1 compared to the control in any of the RMS cell lines observed, at any time point). This same observation was additionally later observed for MHC expression by immunofluorescence (Figure

10) and protein expression by Western Blot analysis (Figure 11) showing no significant difference in MHC expression between cells overexpressing PANX1 and the control at any of the time points, in any of the RMS cell lines. Thus, the levels of the myogenic regulatory factors remained relatively unchanged when comparing the PANX1 over-expressing cells and the GFP controls over the ten-day cumate induction period, concluding that the regulation of APOBEC2 by PANX1 does not appear to be only observed in cells in which PANX1 induced partial differentiation.

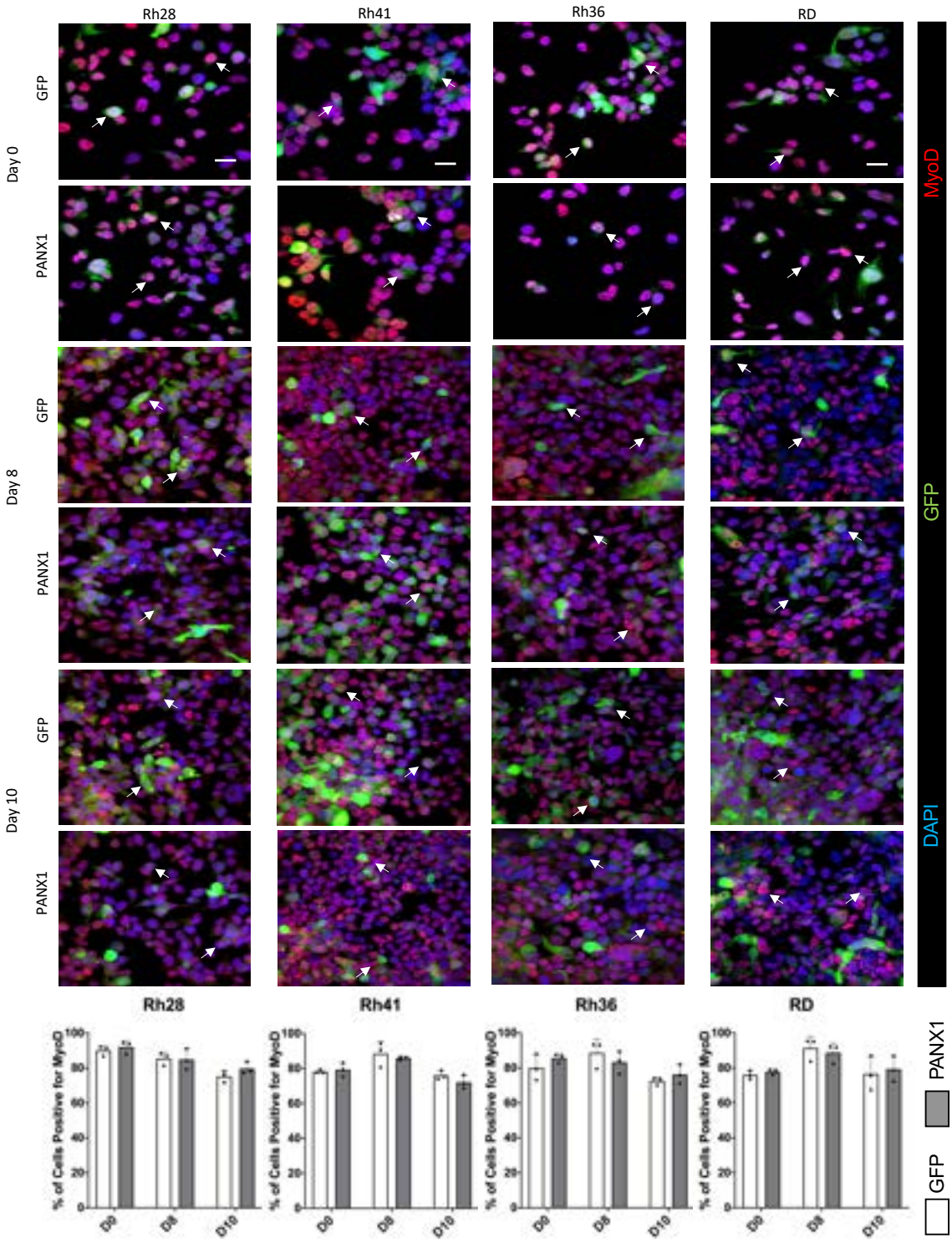

Figure 6: *MyoD levels are not Regulated by PANX1 in Rh28, Rh41, Rh36 and RD RMS cell lines upon induction of PANX1 based on Immunofluorescence Analysis.*
Inducible stable cell lines over-expressing PANX1 or the control vector GFP after treatment with cumate over ten days in culture have been labeled for MyoD (red). Representative cells expressing MyoD have been identified with an arrow. Blue = nuclei, bars 30μm. Results are expressed as mean ± sd.

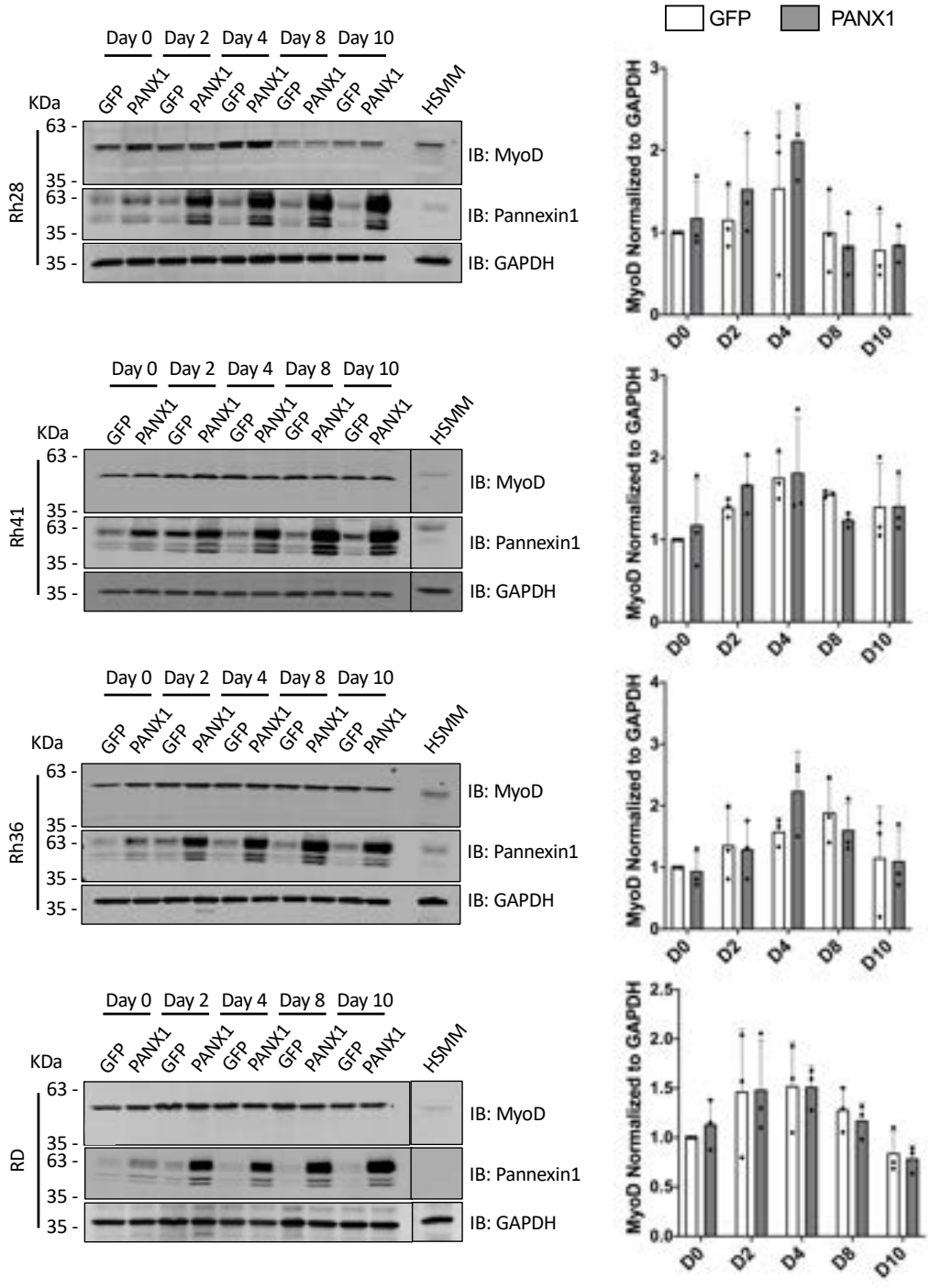

Figure 7: *MyoD levels are not Regulated by PANX1 in Rh28, Rh41, Rh36 and RD RMS cell lines upon induction of PANX1 based on Western Blot Analysis.*

Protein lysates were collected from the inducible stable cell lines over-expressing PANX1 or the control vector GFP after treatment with cumate over ten days in culture. Results are expressed as mean ± sd. GAPDH was used as a loading control. Differentiating HSMM for six days was used as a positive control.

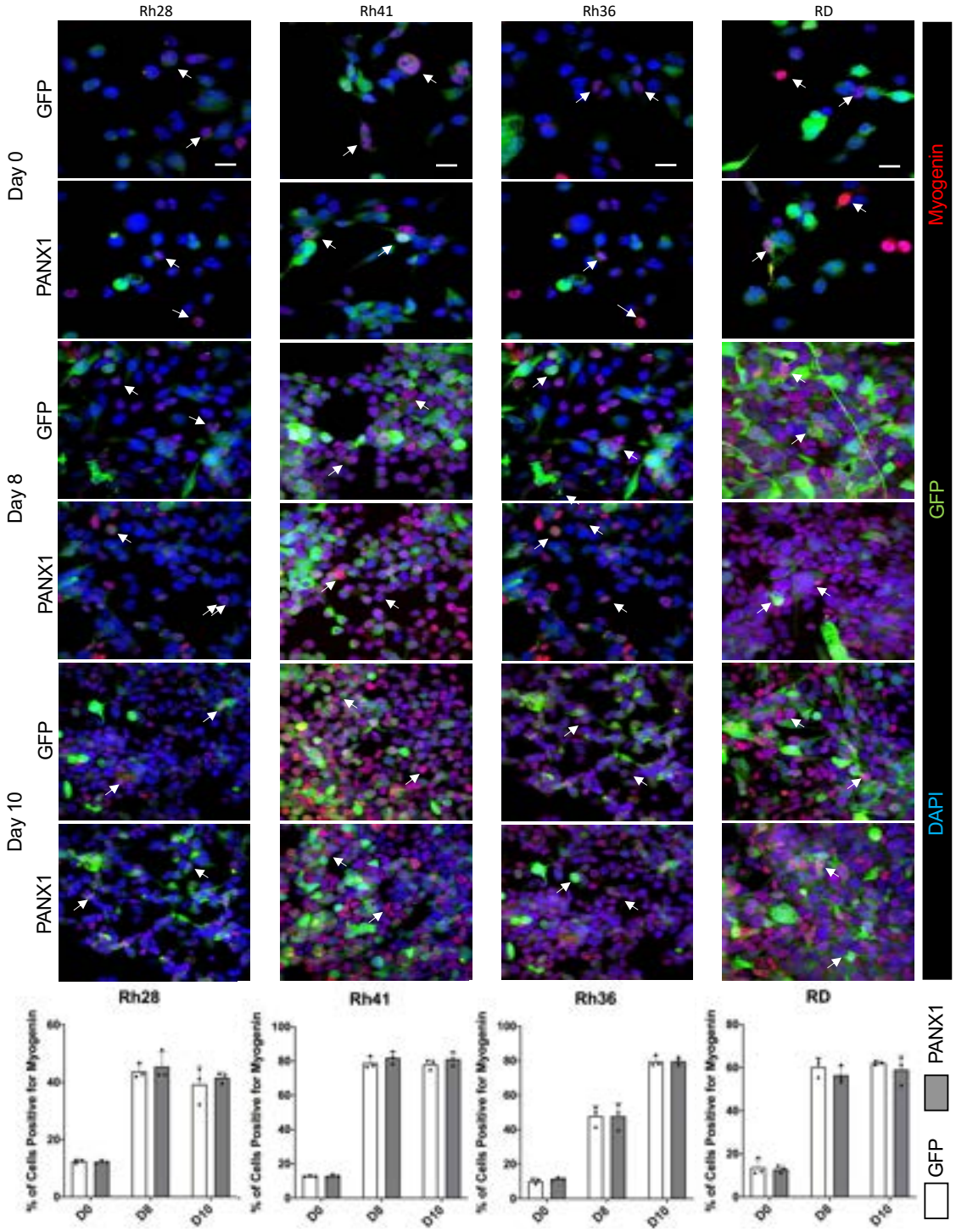

Figure 8*: Myogenin levels are not Regulated by PANX1 in Rh28, Rh41, Rh36 and RD RMS cell lines upon induction of PANX1 based on Immunofluorescence Analysis.*
Inducible stable cell lines over-expressing PANX1 or the control vector GFP after treatment with cumate over ten days in culture have been labeled for myogenin (red). Representative cells expressing myogenin have been identified with an arrow. Blue = nuclei, bars 30μm. Results are expressed as mean ± sd.

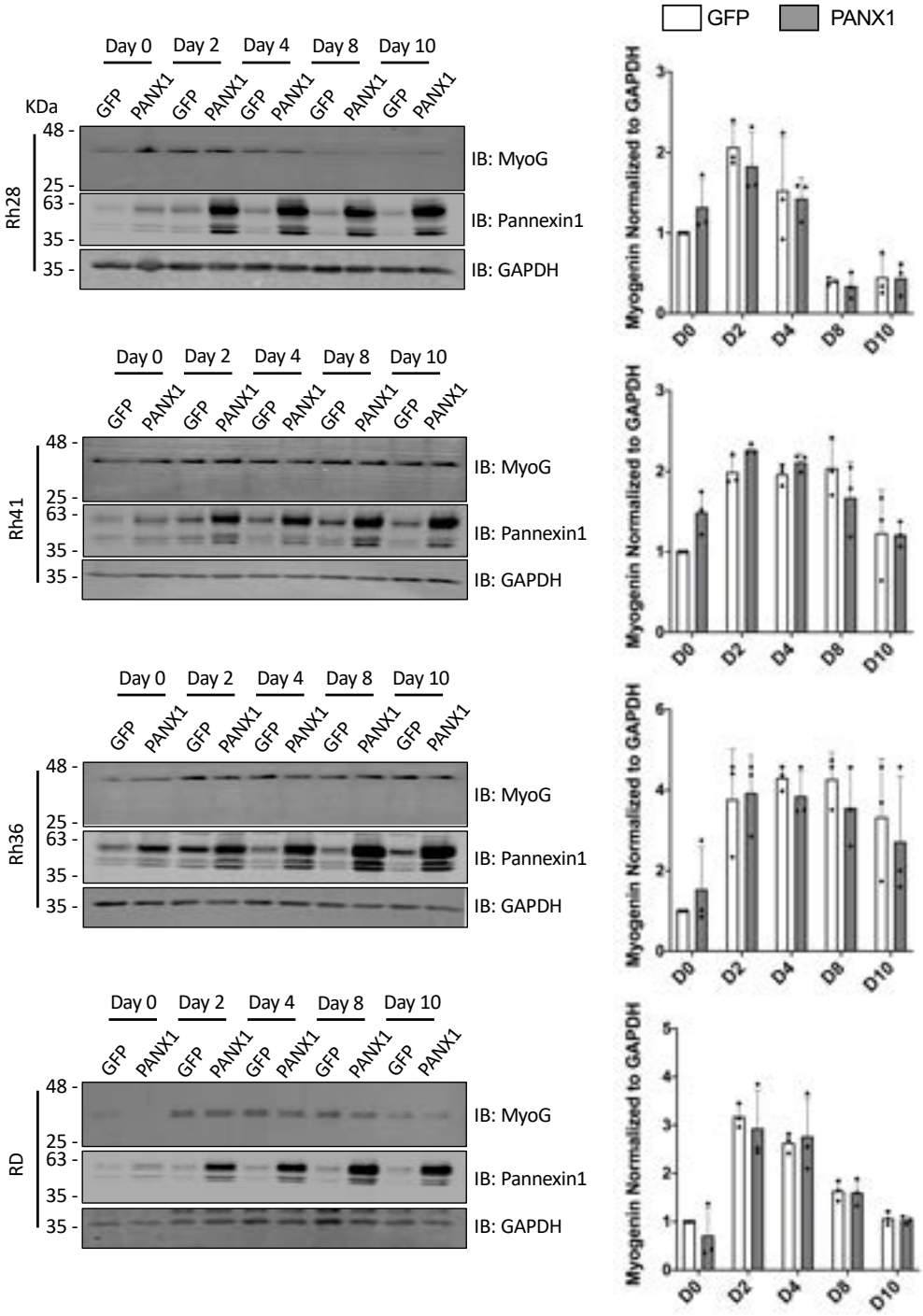

Figure 9: *Myogenin levels are not Regulated by PANX1 in Rh28, Rh41, Rh36 and RD RMS cell lines upon induction of PANX1 based on Western Blot Analysis.*
Protein lysates were collected from the inducible stable cell lines over-expressing PANX1 or the control vector GFP after treatment with cumate over ten days in culture. Results are expressed as mean ± sd. GAPDH was used as a loading control. Differentiating HSMM for six days was used as a positive control.

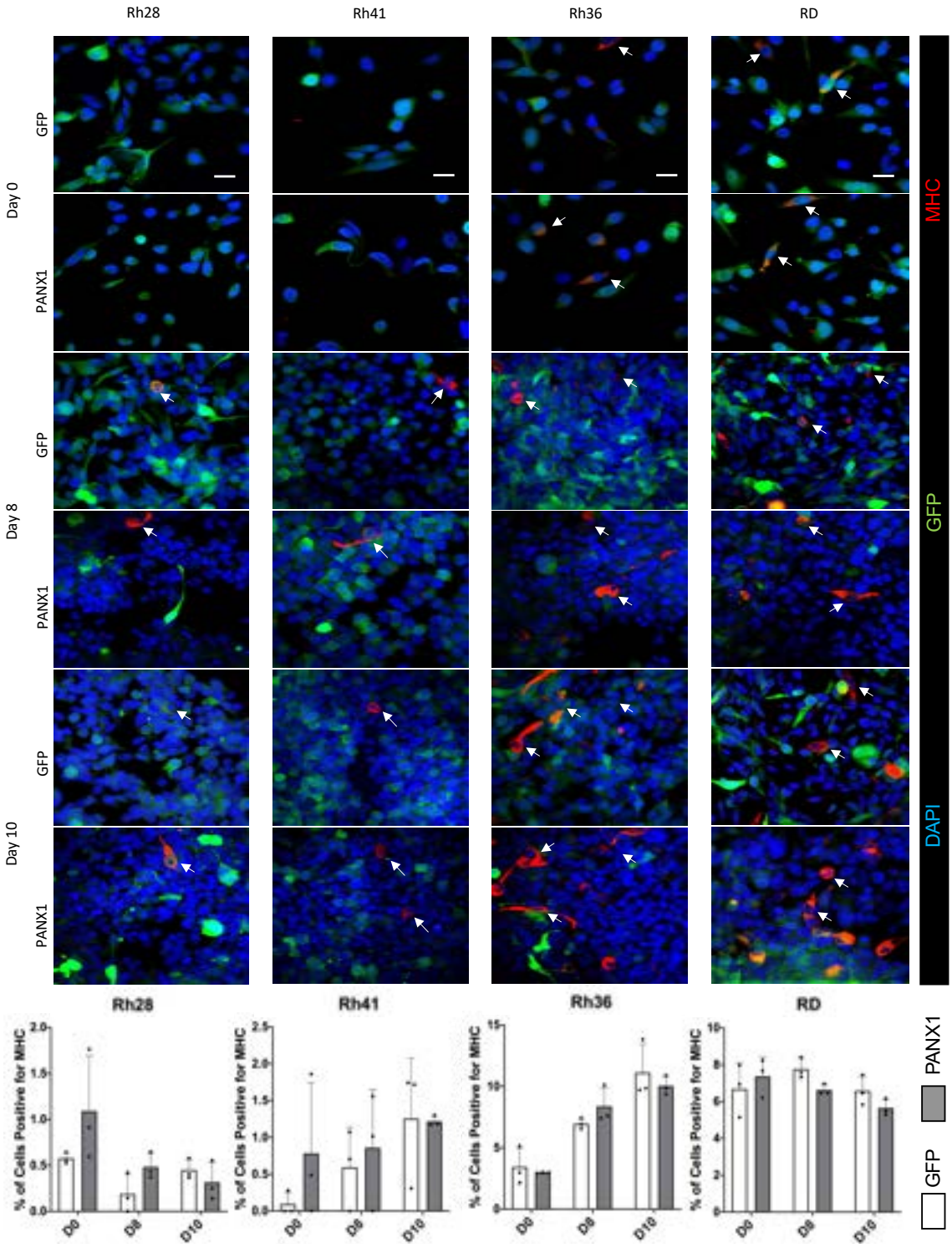

Figure 10: *MHC levels are not Regulated by PANX1 in Rh28, Rh41, Rh36 and RD RMS cell lines upon induction of PANX1 based on Immunofluorescence Analysis.*
Inducible stable cell lines over-expressing PANX1 or the control vector GFP after treatment with cumate over ten days in culture have been labeled for MHC (red). Representative cells expressing MHC have been identified with an arrow. Blue = nuclei, bars 30μm. Results are expressed as mean ± sd.

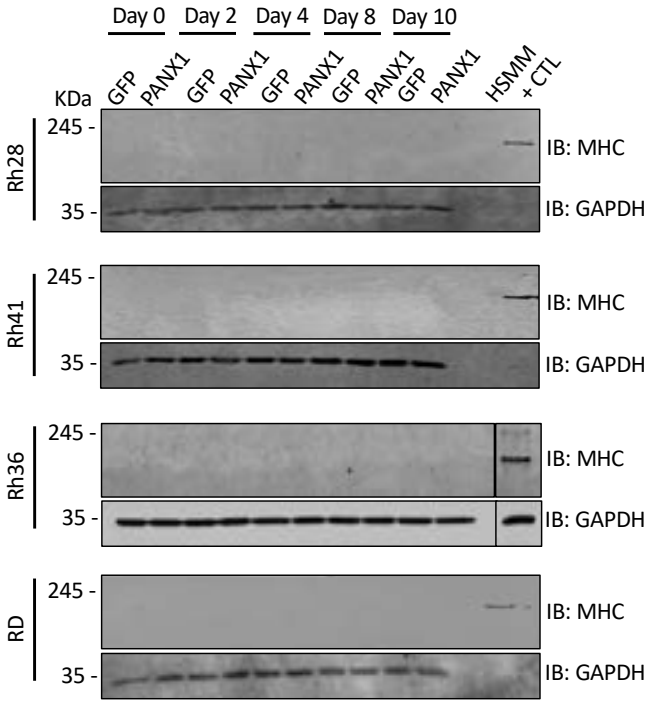

Figure 11: *MHC levels are not Regulated by PANX1 in Rh28, Rh41, Rh36 and RD RMS cell lines upon induction of PANX1 based on Western Blot Analysis.*

Protein lysates were collected from the inducible stable cell lines over-expressing PANX1 or the control vector GFP after treatment with cumate over ten days in culture. Results are expressed as mean ± sd. GAPDH was used as a loading control. Protein lysates from a wild-type male mouse *tibialis anterior* (TA) muscle were used as a positive control.

3.5 PANX1 Overexpression Increases Multinucleation in the Same Cell Lines in Which it Downregulates APOBEC2

To gain more insight into the relationship between APOBEC2 and PANX1 we wanted to further investigate a potential link with cell fusion and multinucleation status. We have previously shown that PANX1 overexpression induces multinucleation in Rh30 cells and that PANX1 expression promotes both myoblast differentiation and fusion[46,87]. Thus, we wanted to determine if there is a difference in multinucleation status between the six different patient-derived cell lines upon the overexpression of PANX1 (Figure 12). Previously our laboratory has transfected Rh18 and Rh30 RMS cell lines with GFP or PANX1 (and the immunolabeled for PANX1 and DAPI) intending to count the number of nuclei within the transfected cells[87]. This study found that in Rh30 cells, but not Rh18 cells, there were significantly more PANX1-expressing cells that were multinucleated[87]. This was then repeated previously in our laboratory (but unpublished) with RD cells, which showed the same results which were observed in Rh30 cells with more PANX1-expressing multinucleated cells. Based on these results, we then wanted to assess the remaining three RMS cell lines, Rh28, Rh41, and Rh36. When examining Rh28, similar results to that of Rh30 and RD cell lines were observed, in which significantly more PANX1-expressing cells were multinucleated compared to the control (Figure 12). In contrast, when examining Rh36 and Rh41, there was no significant difference in multinucleation status between cells expressing PANX1 and the control cells. Based on previous results and my current data, the cell lines in which the overexpression of PANX1 downregulates APOBEC2 (Rh28, Rh30, and RD) have significantly more multinucleated PANX1-expressing cells than the control[87]. This can be compared to the remaining three RMS cell lines (Rh18, Rh36, and Rh41) in which there was no significant difference in multinucleation status between the control and PANX1-expressing RMS cells[87].

Taken together, these results suggest that the regulation of APOBEC2 by PANX1 maybe only in cells in which PANX1 is capable of inducing fusion.

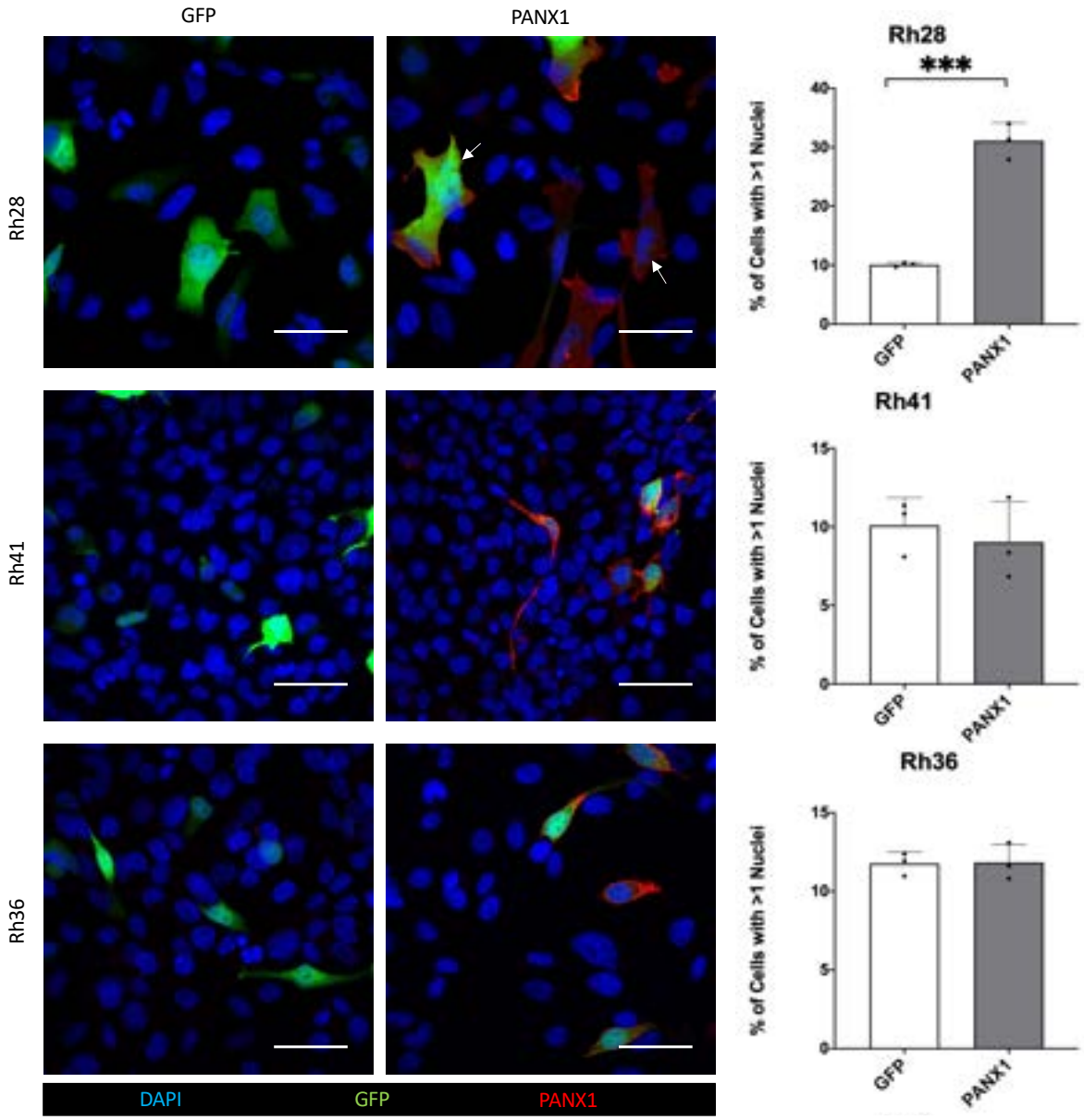

Figure 12: *PANX1 Expression Induces Multinucleation in some RMS Cell Lines based on Immunofluorescence Analysis.*
PANX1 (red) immunofluorescence labeling in Rh28, Rh36 and Rh41 cell lines transfected with PANX1 or the control vector of GFP. Nuclei (blue) have been labeled with DAPI. Representative multinucleated cells are indicated with an arrow. Bars = 30μm. ***P< 0.001 compared to GFP (n=3).

3.6 Investigation into the Involvement of AHNAK in the Mechanism Mediating the Regulation of APOBEC2 by PANX1

We then wanted to evaluate the potential mechanism mediating the regulation of APOBEC2 levels by PANX1. Previously, our lab has identified, using BioID together with co-immunoprecipitation of PANX1-enriched fractions, the PANX1 interactome in Rh18 and Rh30 cell lines[129]. The top PANX1 interactor in Rh30 and Rh18 cells was found to be AHNAK, a large scaffolding protein that plays a known role in cancer migration and invasion[130]. Additionally, through regulating the levels of AHNAK expression in Rh30 and Rh18 cells, our lab has been able to identify a role for AHNAK in the PANX1-medicated inhibition of RMS malignant properties[129]. Thus, we wanted to evaluate whether AHNAK plays a role in the PANX1-mediated downregulation of APOBEC2. To this end, AHNAK was targeted using shRNAs in our Rh30 cell lines in which PANX1 overexpression was induced by cumate for forty-eight hours and then examined for APOBEC2 levels. We found that the downregulation of AHNAK increased APOBEC2 levels (Figure 13). However, since this effect was observed both in the absence and presence of cumate, it may be independent of PANX1 overexpression. Despite this, the cells were only cultured in the presence of cumate for twenty-four hours, and as we are aware that the regulation of APOBEC2 by PANX1 is time-course dependent, this experiment should be repeated in the future keeping the cells in the presence of cumate for between four to eight days. This would be done to ensure that there was enough time to observe the regulation of APOBEC2 by PANX1 prior to a definitive conclusion being made.

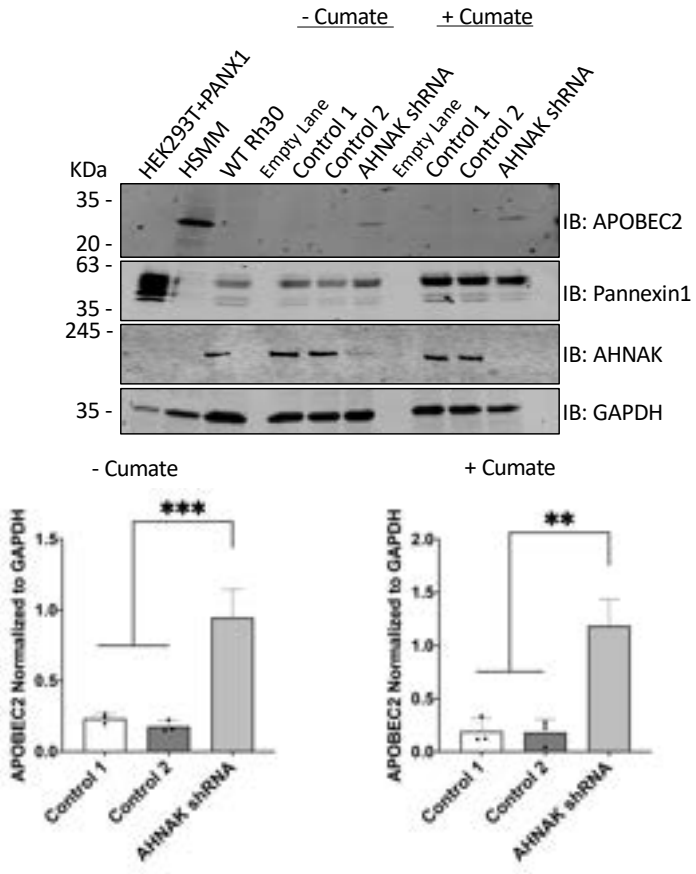

***Figure 13**: Regulation of APOBEC2 in Rh30 stable cells downregulating AHNAK May Be Independent of PANX1 Overexpression.*

Cell lysates were collected after induction of AHNAK shRNA with doxycycline for 72 hours and then cumate for 48 hours to overexpress PANX1. GAPDH was used as a loading control, HEK293T overexpressing PANX1 were used as a PANX1 positive control. HSMM differentiated for six days was used as an APOBEC2 positive control. Rh30 cells were used as an AHNAK positive control. ** $P < 0.01$, *** $P < 0.001$ compared to GFP with cumate as determined by two-way ANOVA. Results are expressed as mean ± sd (n=3).

3.7 Investigation into the Involvement of PAX7 in the Mechanism Mediating the Regulation of APOBEC2 by PANX1

Previous studies have found that the downregulation of PAX7 results in a subsequent downregulation of APOBEC2 levels leading to an increase in myoblast differentiation[183]. Thus, I wanted to evaluate the potential role of PAX7 in the PANX1-mediated downregulation of APOBEC2. To address this, we first measured the endogenous expression of PAX7 in our six RMS cell lines to determine if there are any correlations between the three cell lines in which APOBEC2 levels are regulated by PANX1 (Rh28, Rh30, and RD) as compared to the rest of the RMS cell lines. This examination found that PAX7 was most expressed in the Rh36 and RD cell lines, with some expression in the Rh41 cell line (Figure 14). However, there was minimal to no expression of PAX7 within the remaining three RMS cell lines, that being Rh28, Rh30, and Rh18 (Figure 14). Thus, the overall levels of endogenous PAX7 show the same relative distribution of endogenous APOBEC2 expression. Indeed, both APOBEC2 and PAX7 are expressed more in Rh41, Rh36, and RD cell lines, than in Rh28, Rh30, and Rh18 cell lines.

These results appeared promising for a potential mechanistic link between PAX7 and APOBEC2 and so we proceeded to evaluate the expression of PAX7 upon PANX1 overexpression by immunofluorescence (Figure 15) and Western Blot (Figure 16) analysis. When initially examining immunofluorescence microscopy (Figure 15), there were no significant differences in the percent of cells staining positive for PAX7 upon the induction of PANX1 compared to the GFP control. This was observed throughout all time points and cell lines examined. When further analyzing PAX7 protein expression by Western Blot analysis, these previously observed results were corroborated, showing no significant differences between the cells over-expressing PANX1 compared to the controls at any timepoint of the PANX1 induction nor within any of the RMS cell

lines (Figure 16). Taken together, there was no significant regulation of PAX7 levels by PANX1 overexpression in any of the six RMS cell lines examined. Therefore, this suggests that the PANX1-mediated regulation of APOBEC2 may not be a result of direct regulation from PAX7 expression.

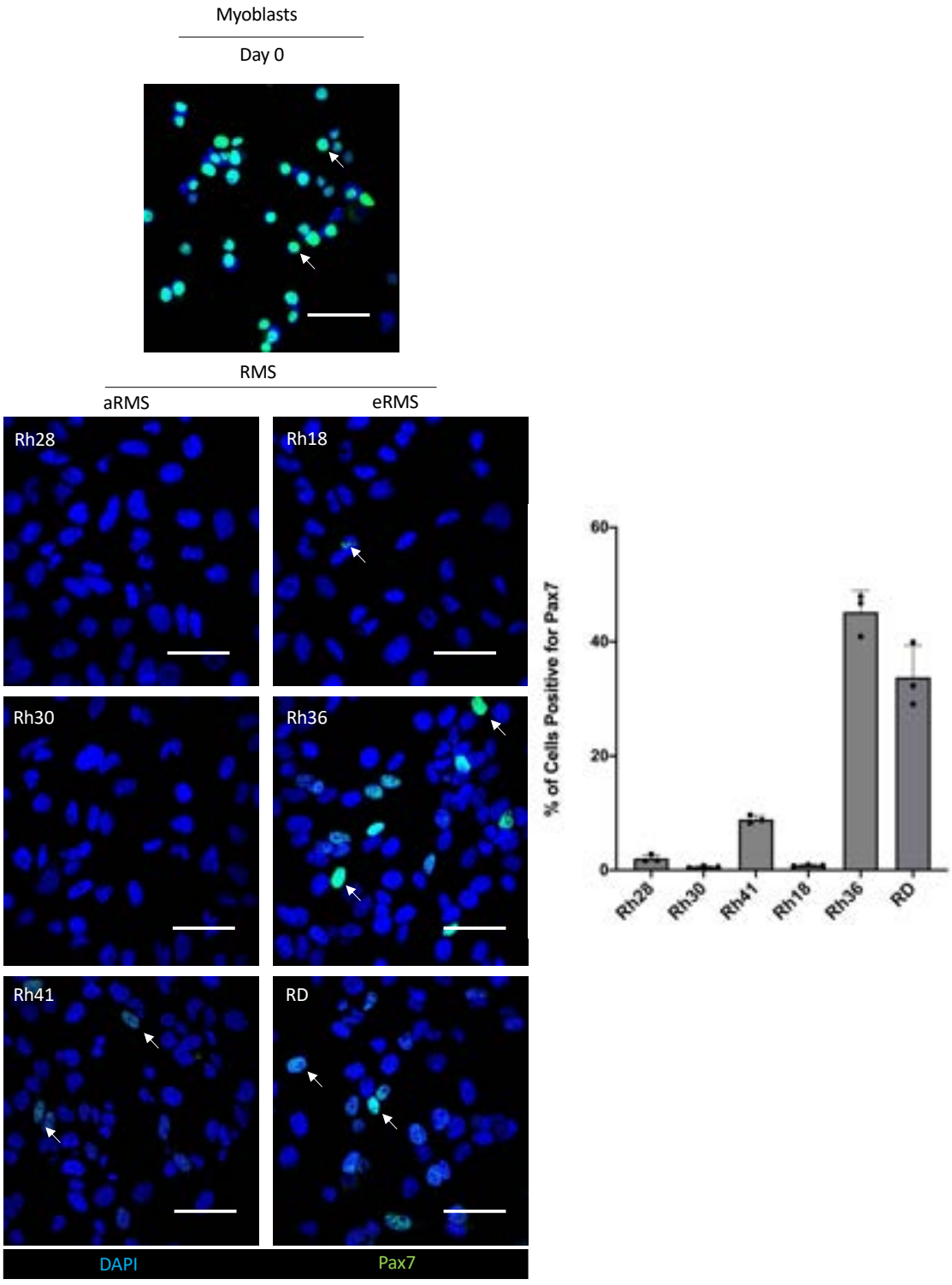

Figure 14: *Expression of PAX7 in patient-derived aRMS and eRMS cell lines.*
Cells were analyzed from three aRMS (Rh28, Rh30, and Rh41) and three eRMS (Rh18, Rh36, and RD) cell lines. Blue: DAPI; Green: PAX7; Bars: 30μm. Representative cells expressing PAX7 have been indicated by an arrow. Undifferentiated mouse primary myoblasts were used as the positive control. Results are expressed as mean ± sd (n=3).

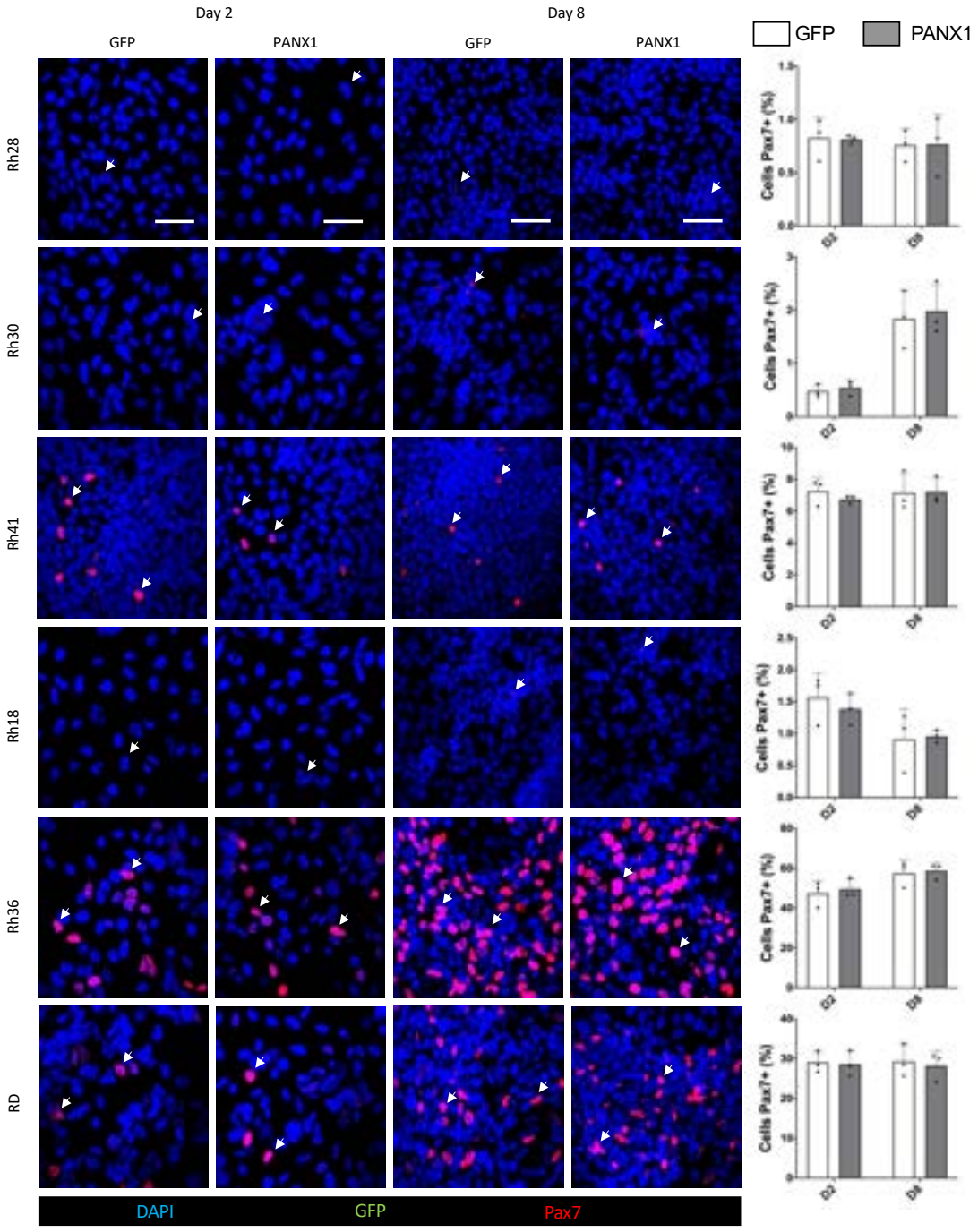

Figure 15: *PAX7 levels are not Regulated by PANX1 in Rh28, Rh30, Rh41, Rh18, Rh36 and RD RMS cell lines upon induction of PANX1 based on Immunofluorescence Analysis*
Inducible stable cell lines over-expressing PANX1 or the control vector GFP after treatment with cumate over ten days in culture have been labeled for PAX7 (red). Representative cells expressing MHC have been identified with an arrow. Blue = nuclei, bars 30µm. Results are expressed as mean ± sd.

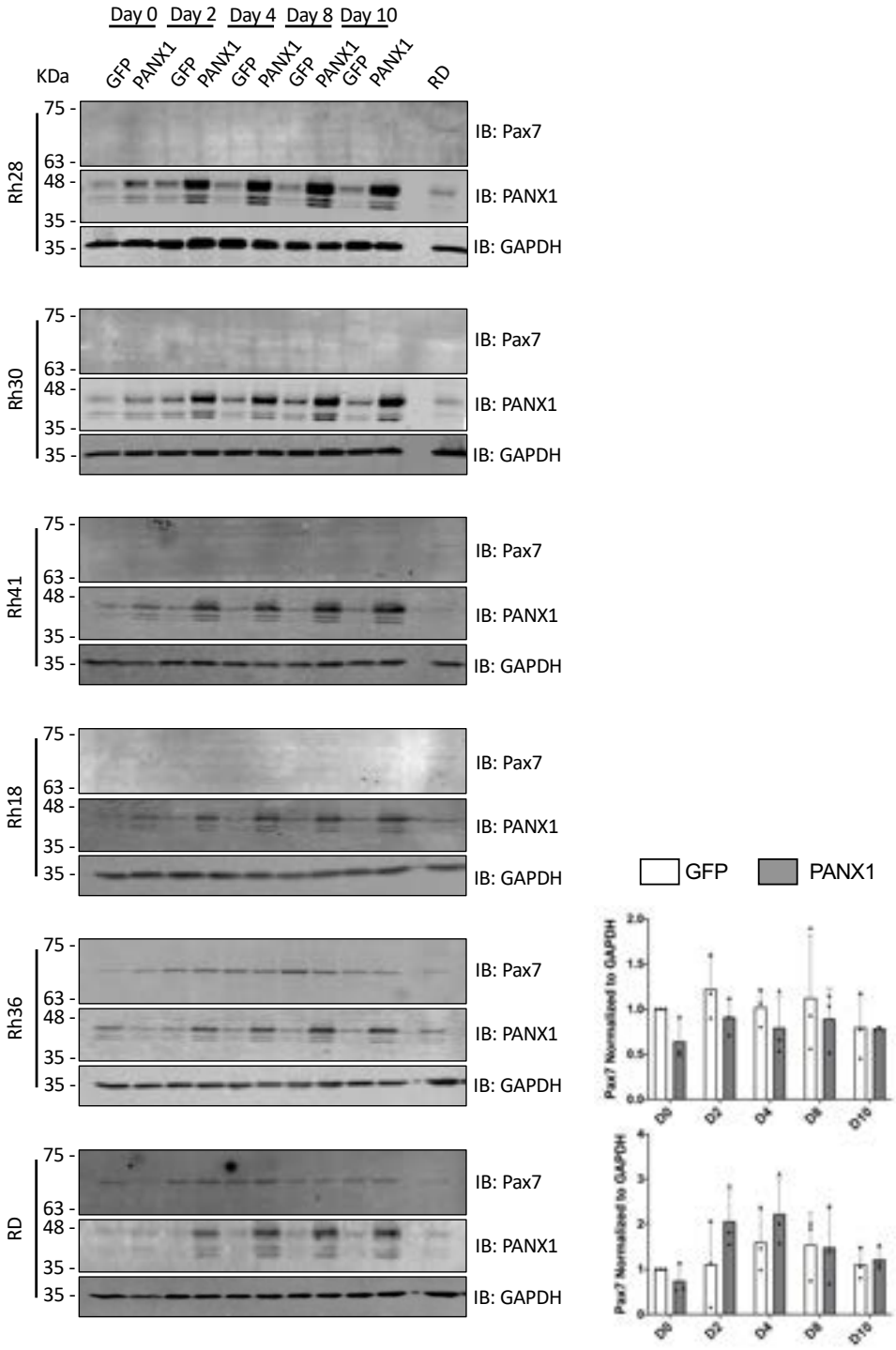

Figure 16: *PAX7 levels are not Regulated by PANX1 in Rh28, Rh30, Rh41, Rh18, Rh36 and RD RMS cell lines upon induction of PANX1 based on Western Blot Analysis*

Protein lysates were collected from the inducible stable cell lines over-expressing PANX1 or the control vector GFP after treatment with cumate over ten days in culture. Results are expressed as mean ± sd. GAPDH was used as a loading control. Wild-type RD cells was used as a positive control.

3.8 Generation of a Cumate Inducible PANX1 Overexpressing Rh30 Cell Line that Constitutively Overexpresses APOBEC2

As PANX1 overexpression downregulates APOBEC2 levels, we used our stable Rh30 cell line in which PANX1 can be overexpressed using the cumate switch system and transduce these cells with a lentivector to constitutively overexpress APOBEC2 or its empty control vector. These cells were infected at various multiplicity of infections (MOIs) and APOBEC2 levels were compared to ensure adequate/significant induction of overexpression and physiological relevance. In Figure 17A we found that MOI values of 0.1 to 5 were not enough to induce the expression of APOBEC2. Although MOI values from 25 to 200 did show expression of APOBEC2, it was decided that the expression was too low at an MOI of 25 or 50 and that the MOI of 200 may be too high (Figure 17A). Based on this, an MOI of 100 was chosen to see whether it was a physiologically relevant APOBEC2 expression, by comparing it to that of differentiating HSMM (Figure 17B). Here, when comparing the overexpression of APOBEC2 to that of differentiating HSMM we found that they were very similar and thus this MOI was appropriate. We then wanted to ensure that the APOBEC2 expression remained steady upon the overexpression of PANX1 and that it was consistent over several days. As shown in Figure 17B, these stable cell lines constitutively overexpressed APOBEC2, and this expression is consistent and physiologically relevant based on its endogenous levels in differentiating HSMM.

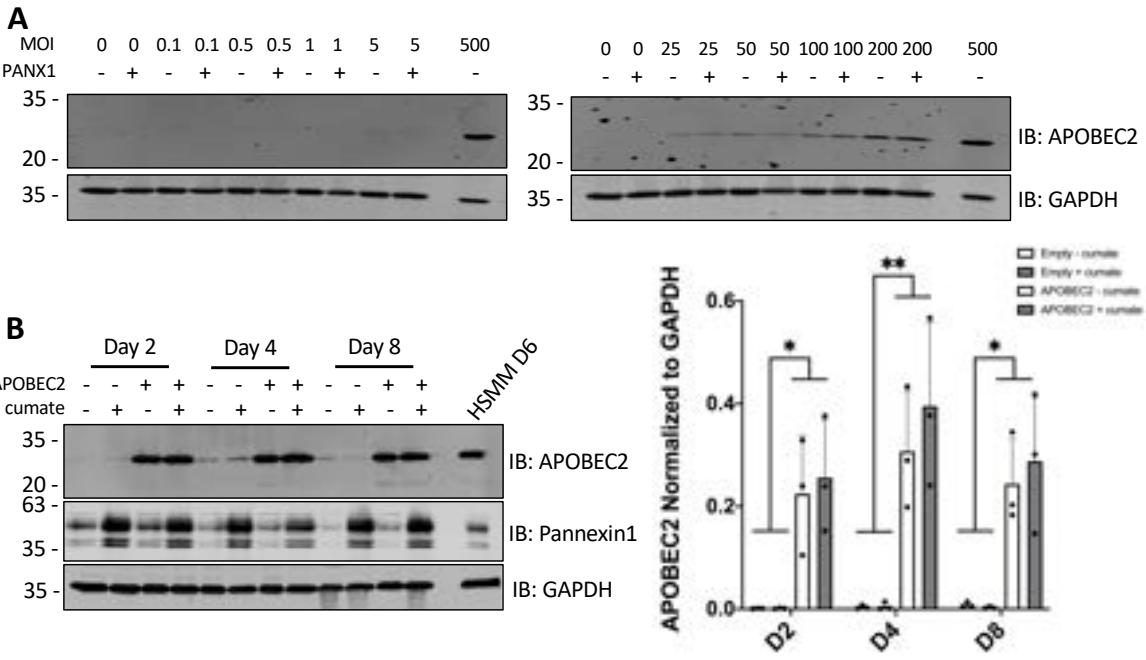

Figure 17: *Generation of a cumate inducible PANX1 overexpressing Rh30 cell line that constitutively overexpresses APOBEC2*

A. APOBEC2 expression levels upon infection at different MOIs of transfection in both the Rh30 stable cells with and without cumate (which is used to induce PANX1 overexpression). Rh30 cells infected with the APOBEC2 lentivirus at MOI of 500 was used as an APOBEC2 positive control. B. Validation of cumate inducible PANX1 overexpressing Rh30 stable cell line that constitutively overexpresses APOBEC2. GAPDH was used as a loading control, and HSMM differentiating for six days was used as an APOBEC2 positive control. * $P < 0.05$, ** $P < 0.01$ compared to GFP with cumate as determined by ANOVA. Results are expressed as mean ± sd (n=3).

3.9 APOBEC2 May Not play a Role in the PANX1-mediated Inhibition of Rh30 Cell Proliferation or Decrease in Cell Viability

Our laboratory previously demonstrated that the overexpression of PANX1 resulted in an approximately fifty percent reduction in proliferation and a significant reduction in viability in the Rh30 cell line *in vitro* compared to control cells expressing GFP[87]. Now that the stable cell lines have been established, we wanted to assess whether the overexpression of APOBEC2 could reverse the PANX1-mediated inhibition of RMS malignant properties. Cell proliferation was first examined using a BrdU incorporation assay, in which we compared our stable cell lines expressing APOBEC2 or an empty vector, with or without the presence of cumate (PANX1 expression in these cells is under a cumate switch system) (Figure 18). As expected, based on our published data[87], PANX1 overexpression reduced the proliferation rate of the Rh30 cells, which is observed due to a decrease in spectrophotometric absorbance. However, we found that APOBEC2 overexpression in these cells was not able to reverse the PANX1-mediated reduction in cell proliferation after two days (Figure 18A). As the regulation of APOBEC2 levels appears to be time-dependent, the cells were then kept in culture for a further two days (a total of four days) prior to performing the BrdU incorporation assay (Figure 18B). Despite the cells remaining in culture for longer, the previous results remained consistent. Finally, this experiment was repeated with a lower dose of cumate shown to be sufficient to overexpress PANX1 (0.06 μg/mL) (Xiang, X., unpublished results) in case PANX1 overexpression overrides that of APOBEC2 or that cumate affects cell proliferation. However, similar results were obtained with the two cumate concentrations (0.06 μg/mL vs. 30 μg/mL) to induce PANX1 overexpression (Figure 18C). Altogether, these results suggest that APOBEC2 may not play a role in the PANX1-mediated inhibition of RMS cell proliferation.

Previously our laboratory had also examined the effect of the overexpression of PANX1 on cell viability, finding that PANX1 overexpression cells tend to have a reduction in cell viability compared to that of control cells[87]. Thus, we wanted to continue to examine whether the overexpression of APOBEC2 could mitigate the PANX1-mediated reduction in cell viability. When examining the Trypan Blue assay to assess cell viability, as expected based on our published data[87], the overexpression of PANX1 on its own resulted in a reduction in cell viability after two days in culture (Figure 18D). However, when additionally overexpressing APOBEC2 within these cell lines, we were unable to reverse this reduction in cell viability (Figure 18D). To verify these results, we then subsequently completed an Alamar Blue assay. However, the previously states results were confirmed in which we found that the overexpression of PANX1 on its own resulted in a reduction in cell viability, and overexpressing APOBEC2 was unable to reverse the PANX1-mediated reduction in cell viability (Figure 18E). One other finding to note in the Alamar Blue assay was that there did appear to be a slight increase (however, not statistically significant) in cell viability when comparing the overexpression of APOBEC2 with the empty vector in the no cumate conditions. Thus, together the overexpression of APOBEC2 may be unable to reverse the PANX1 mediated reduction in cell viability.

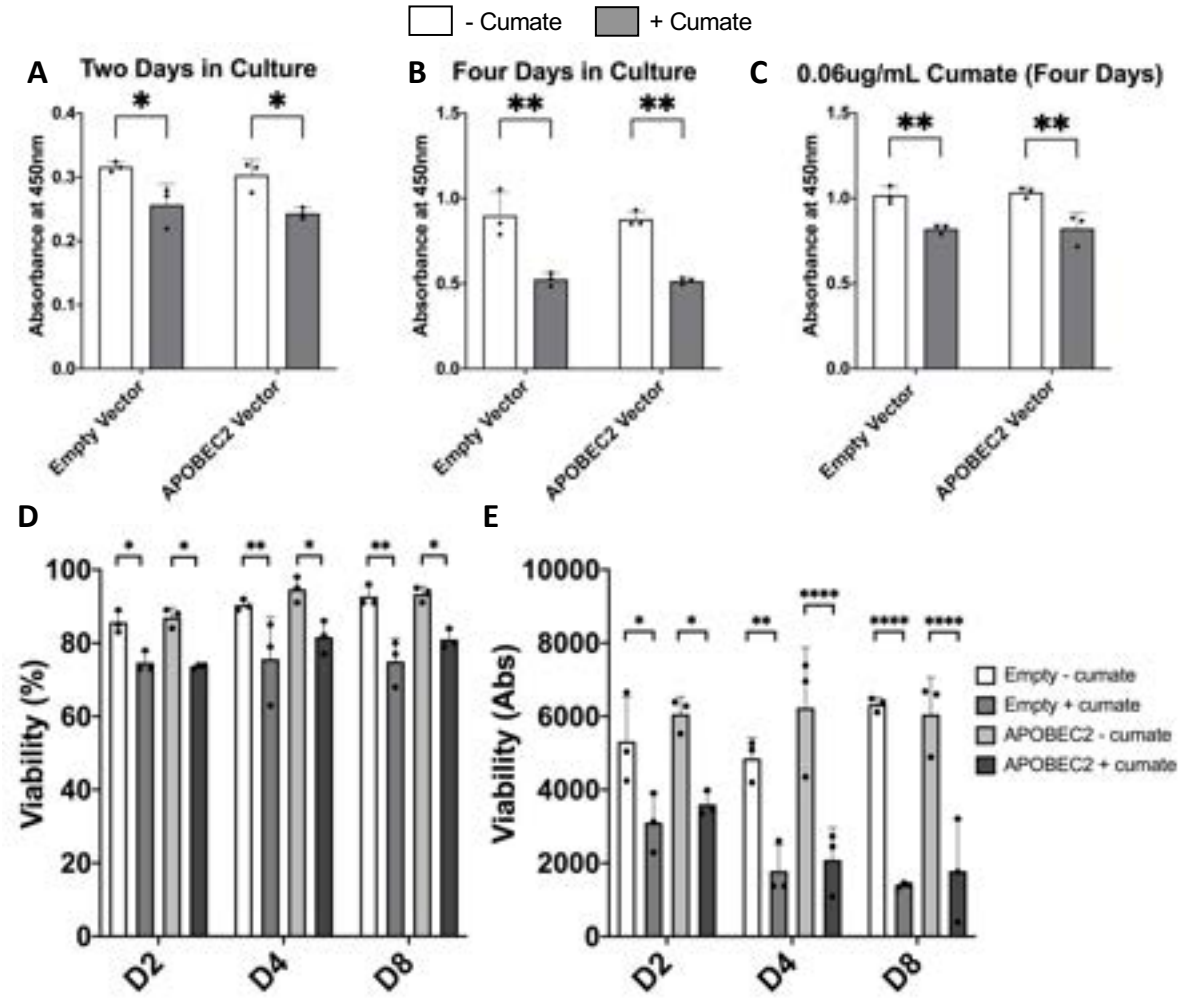

***Figure 18**: APOBEC2 May Not Play a Role in the PANX1-mediated Inhibition of Rh30 Cell Proliferation or Decrease in Cell Viability*

Cumate inducible PANX1 overexpressing Rh30 stable cells that constitutively overexpress APOBEC2, or an empty vector were analyzed for their incorporation of BrdU as a measure of cell proliferation, or Trypan/Alamar Blue as a measure of cell viability. A. Cells were analyzed for proliferation after two days in culture, in the presence or absence of 30μg/mL of cumate to induce the overexpression of PANX1. B. Cells were analyzed for proliferation after four days in culture in the presence or absence of 30μg/mL of cumate. C. Cells were analyzed after four days in culture, in the presence or absence of 0.06μg/mL of cumate. D. Viability (%) via Trypan Blue Assay of Rh30 stable cells overexpressing APOBEC2 or an empty vector after between two to eight days in culture, in the presence or absence of 30μg/mL of cumate to induce the overexpression of PANX1. E. The previous experiment was repeated but with an Alamar Blue Assay. Results are expressed as mean ± sd as determined by ANOVA. *P< 0.05, **P<0.01, ***P<0.001, ****P<0.0001 (n=3).

3.10 The Overexpression of APOBEC2 Reversed the PANX1-mediated Induction of Rh30 Cell Fusion

As our data suggests that the downregulation of APOBEC2 by PANX1 may be linked to the ability of PANX1 to trigger cell fusion, we wanted to evaluate whether APOBEC2 overexpression would reverse the PANX1-mediated induction of Rh30 cell fusion (Figure 19). As expected, based on our previous findings, PANX1 overexpression on its own led to a significant increase in multinucleation (approximately 17%) of Rh30 cells. However, when APOBEC2 was overexpressed, the majority of the cells remain mononucleated (approximately only 5% multinucleation). Thus, despite overexpression of both APOBEC2 and PANX1, Rh30 cells still remain mononucleated (similar to that of the control vectors). These findings suggest that ectopic PANX1 mediates cell fusion by downregulating APOBEC2 levels.

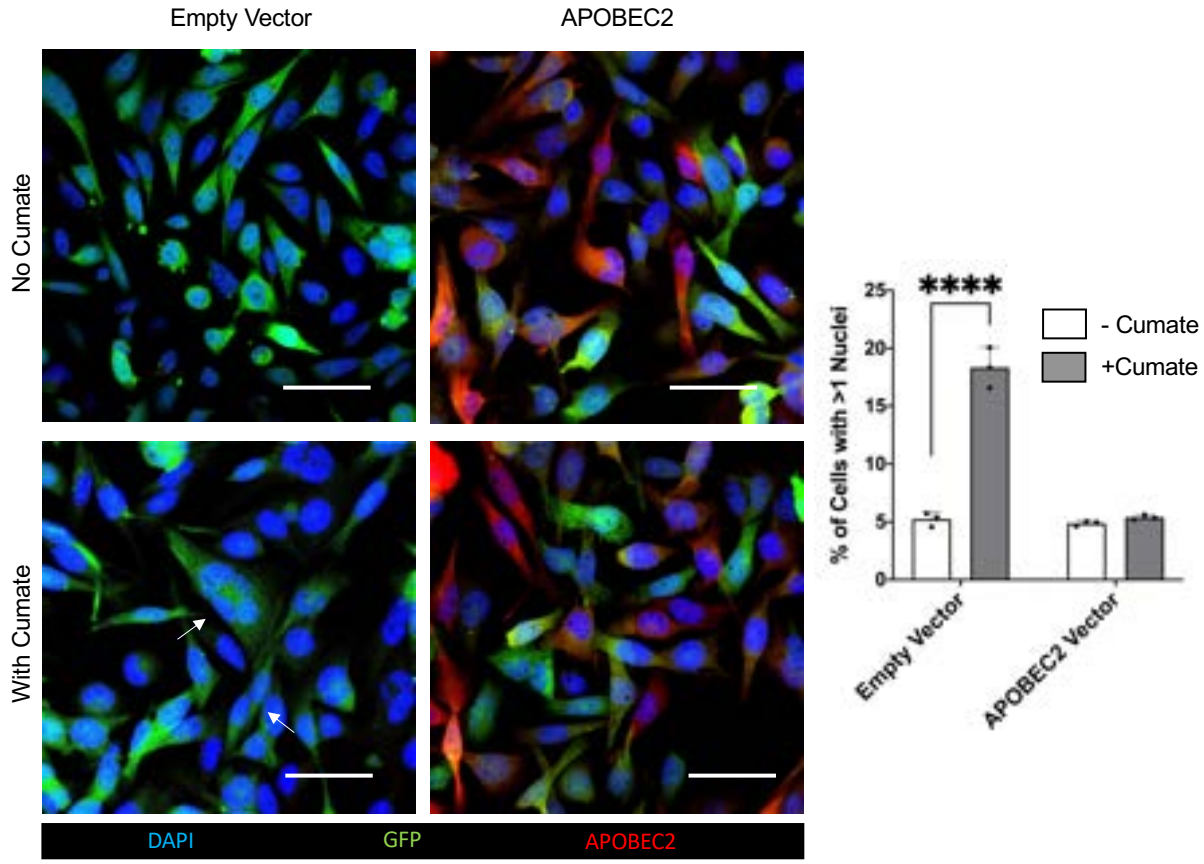

Figure 19: *APOBEC2 May Play a Role in the PANX1-Mediated Induction of Rh30 Cell Fusion*
Cumate inducible PANX1 overexpressing Rh30 stable cells that constitutively overexpress APOBEC2, or an empty vector were analyzed in the presence of absence of 30μg/mL of cumate to induce PANX1 expression. (Blue: DAPI (nuclei); Green: PANX1; Red: APOBEC2; Bars: 30µm) In the empty vector cells overexpressing PANX1 there was a subset of cells that were multinucleated (arrows). Results are expressed as mean ± sd ****P< 0.0001 compared to GFP as determined by ANOVA (n=3).

3.11 APOBEC2 May Not Play a Role in the PANX1-mediated Inhibition of RMS Spheroid Formation and Regression.

Finally, we wanted to evaluate the potential role that APOBEC2 may play in tumour formation and growth using tumour spheroid cultures, a model that is typically used for its ability to mimic the growth of *in vivo* tumours[199]. As our laboratory has previously shown[87], PANX1 overexpression prevents the formation of spheroids (Figure 20), while also inducing the regression of established spheroids upon administration of cumate (Figure 21). It is important to note that this cellular aggregation begins to appear after forty-eight hours in the absence of cumate and continues to grow and aggregate further until the endpoint of the assay (ten days). APOBEC2 overexpression did not reverse the PANX1-mediated effect on the spheroid formation (Figure 20A). Although APOBEC2 overexpression appears to delay spheroid regression, this effect was not statistically significant (Figure 20A). For reasons stated earlier, we have also tested a lower dose of cumate (0.06 μg/mL) and obtained similar results for both spheroid formation (Figure 20B) and regression (Figure 21B). This suggests that the downregulation of APOBEC2 by PANX1 and subsequent cell fusion by itself is not sufficient to alter spheroid formation and growth. However, in the absence of cumate when comparing the APOBEC2 overexpression cells to that of the empty vector there was a significant increase in spheroid growth observed upon the overexpression of APOBEC2 (Figure 20 and Figure 21).

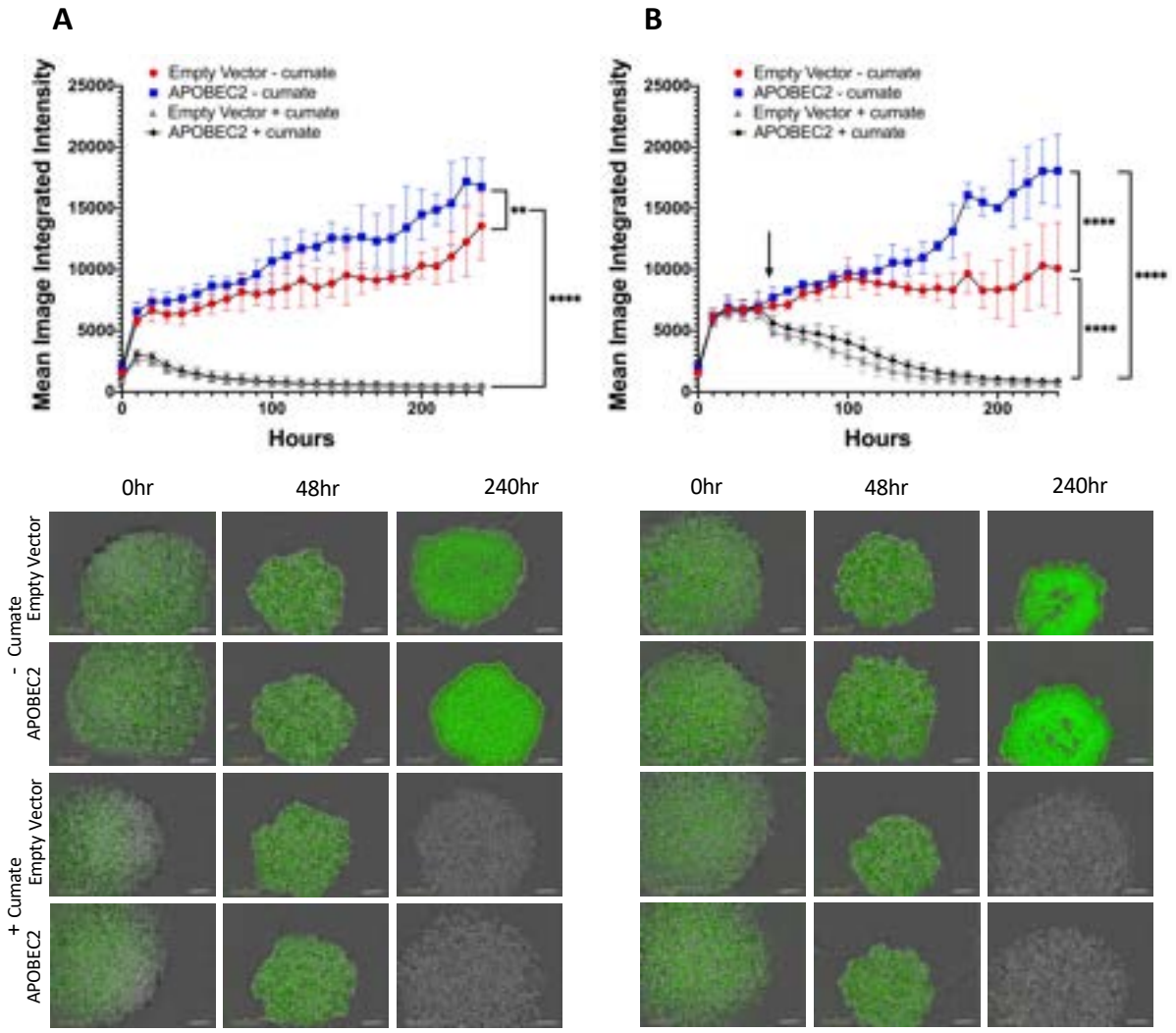

Figure 20: *APOBEC2 May Not Play a Role in the PANX1-mediated Inhibition of Rh30 Cell Spheroid Formation*

A. Spheroid formation and growth as quantified by the Mean Image Integrated Intensity, of Rh30 cells overexpressing APOBEC2 or an empty vector were quantified over two hundred and forty hours. These cells were cultured in the presence or absence of 30μg/mL of cumate to induce PANX1 expression. Representative images were taken at 0hr, 48hr and 240hr. B. The same experiment was performed but using 0.06μg/mL of cumate instead of 30μg/mL. Bars = 1000μm. Results are expressed as mean ± sd **P< 0.01, ****P<0.0001 compared to GFP as determined by ANOVA (n=3).

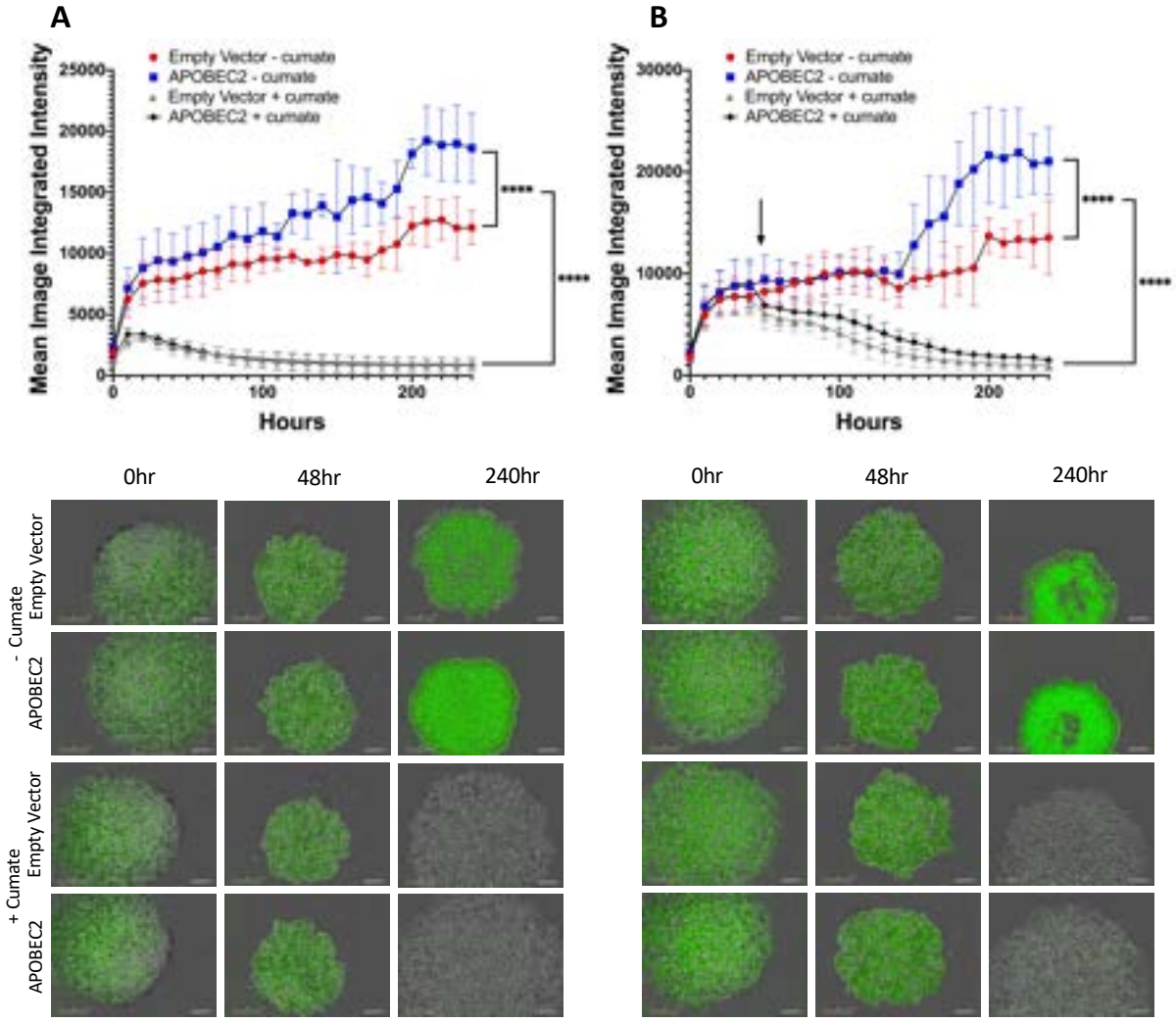

Figure 21: *APOBEC2 May Not Play a Role in the PANX1-mediated Inhibition of Rh30 Cell Spheroid Regression*

A. Spheroid regression as quantified by the Mean Image Integrated Intensity, of Rh30 cells overexpressing APOBEC2 or an empty vector were quantified over two hundred and forty hours. The arrow on the graph indicates the time point in which 30μg/mL of cumate was added and kept within the experiments to induce PANX1 expression (48hr). Representative images were taken at 0hr, 48hr and 240hr. B. The same experiment was performed but using 0.06μg/mL of cumate instead of 30μg/mL. Bars = 1000μm. Results are expressed as mean ± sd ****P<0.0001 compared to GFP as determined by ANOVA (n=3).

4.0 Discussion

Previously, our laboratory had discovered that PANX1 is down-regulated in RMS and that its overexpression both *in vitro* and *in vivo* resulted in a significant reduction in RMS malignant properties, including the inhibition of cell proliferation and migration, and the induction of partial differentiation[87]. While the molecular mechanisms involved remained to be deciphered, the lack of ectopic PANX1 channel activity in RMS cells, the absence of effect of channel blockers on the PANX1-mediated inhibition of RMS malignant properties, as well as the ability of PANX1 mutants devoid of channel activity to trigger these processes similar to wild-type PANX1 suggested that the PANX1-mediated reduction of RMS malignancy was independent of its canonical channel function[87]. To gain some insights into the mechanism by which PANX1 mediates these processes, the genes that are upregulated and downregulated following PANX1 overexpression were identified[129]. Amongst this analysis, it was found that PANX1 overexpression led to a reduction of *APOBEC2* expression in Rh30 cells[129].

Based on the expression of APOBEC2 in the skeletal muscle, and its role as a negative regulator of myogenesis, we wanted to investigate the role of its downregulation by PANX1 in the regulation of RMS malignancy[26,129,178,200]. Here, we demonstrated, for the first time that APOBEC2 expression is downregulated in all of the eRMS and aRMS cell lines compared to that of differentiated HSMM. Since APOBEC2 levels are low within undifferentiated myoblasts compared to that of myoblasts within the early stages of differentiation, it was not surprising that RMS cells would express low levels of APOBEC2 due to their undifferentiated proliferative state[4,26,45]. Our results obtained after ten days in culture indicate that APOBEC2 levels increase as the cells proliferate. Interestingly, it has been shown that APOBEC2 augments during early myoblast differentiation, but its expression then decreases as differentiation proceeds[26]. Thus, the

expression profile of proliferating RMS cells is in accordance with the increase of APOBEC2 seen during the early differentiation phase, and may not go down as the cells were unable to proceed to terminal differentiation[26]. As very little is known about the endogenous expression of APOBEC2 and its dysregulation in the context of diseases, our findings expand the current knowledge of APOBEC2 expression patterns and its regulation.

Our data indicated that the overexpression of APOBEC2 did not affect Rh30 cell viability and cell proliferation in 2D culture. However, its overexpression in Rh30 cells significantly increased spheroid growth. To further examine whether an effect on cell viability and/or cell proliferation can be involved in the inhibition of growth seen in 3D culture, these assays could be done in the presence of fluorescent reagents such as Annexin V or Nuclight red reagents and measured using the Incucyte Imaging System. Interestingly, while the role of APOBEC2 on cell viability, cell proliferation, and cancer remains largely unknown, Okuyama, *et al* in 2012 demonstrated that the constitutive ectopic expression of APOBEC2 increased liver and lung tumorigenesis[179]. Interestingly, this effect was not directly linked to an increase in proliferation or viability, rather it was due to an accumulation of mRNA mutations, which was correlated to the development of liver and lung tumors in APOBEC2 transgenic mice[179]. These liver and lung tumours were specifically characterized by their enlarger and hyperchromatic nuclei, along with cellular atypia and atypical lymphocytes[179]. While not statistically significant, APOBEC2 overexpression within these hepatoma-derived cell lines led to an increased number of mRNA mutations in common tumor-suppressor genes (*Pten,* and *Tp53)*[179]. It may thus be of interest to investigate the total RNA from the spheroids to determine if this is the same cause for the increase in spheroid formation in Rh30 cells upon the overexpression of APOBEC2. It would be of interest to investigate the genes in which the study found to have significant nucleotide alterations which

included the common tumor-suppressor genes including *Pten,* and *Tp53*, as well as commonly mutated AID/APOBEC1 genes *Bcl6* and *Eif4g2*[179]. Altogether, our findings suggest that APOBEC2 regulates tumour growth *in vitro* and that an increase in APOBEC2 expression promotes RMS malignancy.

Upon the overexpression of PANX1, we found that APOBEC2 was downregulated in three (Rh28, Rh30, and RD) out of our six patient-derived RMS cell lines. The regulation of APOBEC2 by PANX1 that was observed was expected due to the role of APOBEC2 as a negative regulator of differentiation[26]. When observing APOBEC2 knockout (*APOBEC2*$^{-/-}$) myoblasts, they showed more myotubes detected after forty-eight hours post-differentiation (a marker of accelerated differentiation), and a marked increase in protein expression of myogenic regulatory factors including myosin heavy chain and myogenin[26]. Since this regulation pattern mimics what we have observed upon the overexpression of PANX1, we can extrapolate that the downregulation of APOBEC2 may be contributing to the induction of partial differentiation that has been previously identified[26,87]. Additionally, it is important to note that the induction of differentiation through a variety of methods that result in the upregulation of myogenic factors in RMS has been shown to reduce its malignant properties including cell migration and invasion[201,202]. This suggests that the tight regulation of APOBEC2 may play a role in this alleviation of RMS malignant properties upon the overexpression of PANX1[87]. There was no correlation however between the cell lines in which APOBEC2 expression is regulated by PANX1 and the endogenous expression of MyoD, myogenin, or MHC. Therefore, the expression of APOBEC2 is not correlated and does not directly affect the expression of these select myogenic regulatory factors. Accordingly, APOBEC2 has been previously found to lack a role during cellular proliferation as its expression does not appear to increase until early differentiation stages, and when examining MyoD expression, no difference

in MyoD expression nor cells positive for MyoD was found in a study comparing APOBEC2$^{-/-}$ to wild-type myoblasts[26]. Based on our findings on the role of APOBEC2 in PANX1-mediated increase in multinucleation, it may be of interest to examine markers of fusion, such as myomaker, or myomerger as a potential correlation between the cell lines in which APOBEC2 is, or is not, regulated by PANX1[24].

Indeed, one commonality among the cell lines where APOBEC2 levels are regulated by PANX1 was the induction of multinucleation upon the overexpression of PANX1[87]. A recent study demonstrated a role for PANX1 in myoblast fusion and skeletal muscle regeneration[46,118,124]. Per our previous study, it was found that PANX1 expression appears to peak during the early regeneration and muscle inflammation phase after injury[46,118,124]. Moreover, myoblasts isolated from *Panx1* Knockout (Panx1$^{-/-}$) mice show fusion deficits[118]. The release of molecules such as ATP through Panx1 channels led to the activation of P2 receptors and myoblast fusion through a novel pathway of bleb-driven amoeboid movement within a lipid signaling cascade[118]. This signaling cascade involved Phospholipase D and A_2, both of which may be of interest to investigate in the future to determine if these are also signaling lipids involved in the APOBEC2/PANX1-mediated induction of cell fusion[118]. To identify any further connections, a literature search was conducted on the cell-cell and cell-matrix genes identified within this study that are significantly altered within *Panx1*$^{-/-}$ mice by microarray analysis[118]. Unfortunately, due to a lack of research on APOBEC2, there was nothing known in the literature connecting any of the nine significantly regulated genes to APOBEC2[118]. However, three of the genes (Cdh2, Col6a1, and Tgfbi) all were respective hits within the RMS transcriptome[118,129]. When considering the roles of these proteins, Cadherin-2 (Cdh2) is a transmembrane protein that plays a role in cell-cell adhesion, Collagen VI (Col6a1) is a subtype of collagen within the extracellular matrix that stabilizes the membrane, and

transforming growth factor beta-induced gene which is found within the extracellular matrix and is involved in cell-matrix interactions[118]. Cdh2 and Col6a1 were significantly downregulated and Tgfbi was significantly upregulated upon the overexpression of PANX1[118,129], whereas within the microarray analysis of the *Panx1*$^{-/-}$ mice Cdh2 and Col6a1 were both found to be upregulated whereas, Tgfbi was downregulated[118]. As these genes are all involved in adhesion and/or the extracellular matrix, they provide an alternative pathway worth examining these proteins in the future, to determine whether they could provide a mechanistic link between PANX1, APOBEC2, and RMS cell fusion. Our research is further supported by previous findings showing a relationship between APOBEC2 expression and myoblast fusion[26,170,180]. It was found that while *APOBEC2*$^{-/-}$ myoblasts not only had a higher fusion index (comparing the percentage of nuclei within myotubes staining positive for myosin heavy chain) but that they also had an accelerated rate of fusion (comparing the number of fusion-positive myotubes at twenty-four and forty-eight hours post-differentiation)[26]. Therefore, although it has been previously observed that there is a relationship between the downregulation of APOBEC2 and cell fusion, this link has never previously been made to PANX1 nor seen within RMS[26,170,180].

We have shown that the downregulation of APOBEC2 by PANX1 plays a role in the subsequent induction of RMS cell fusion, however, our findings suggest that it is not involved in the PANX1 regulation of cell proliferation, viability, spheroid formation, and/or regression. Based on the known benefit of increasing differentiation to halt RMS progression as RMS tends to present itself similar to that of undifferentiated myoblasts, we had expected to see at least an effect on spheroid growth[4,165,200]. However, we have previously shown that PANX1 overexpression led to an increase in both early and late apoptotic events within the speroids[87]. This does illustrate the benefits of 3D cultures, as they can mimic both the physical and biochemical environment

including cell-cell interactions, cellular morphology, and invasion processes more effectively than 2D cultures[199]. It is thus possible that the effect of PANX1 on apoptosis has happened before the induction of cell fusion or may override this process in spheroids. Therefore, the overexpression of APOBEC2 is unable to overcome the effects observed because of the overexpression of PANX1. To provide a better understanding of this potentially confounding variable and the effects of APOBEC2 overexpression, it may be of interest to grow these cells into xenografts *in vivo* and then look at markers of differentiation and/or apoptosis.

Due to a lack of mechanistic information regarding the role of APOBEC2 in skeletal and cardiac muscle, there were only a few known potential pathways which we examined to gain a better understanding of the mechanism by which PANX1 regulated APOBEC2 expression. The first that we investigated was the previously known interaction between AHNAK and PANX1, in which it was found that the downregulation of AHNAK was capable of abrogating the PANX1-mediated reduction in RMS malignancy[129]. Although the downregulation of AHNAK led to an increase in APOBEC2 levels, this result was independent of PANX1 overexpression. However, to rule out the role of AHNAK, we may need to also compare RMS cells in which PANX1 has been knocked out as opposed to cells that express low and high levels of PANX1.

The other potential mechanistic link between APOBEC2 and PANX1 that was investigated here was Pax7 based on a study that examined PAX7 Knockout (*PAX7*$^{-/-}$) teratomas, in which there was a resultant downregulation of APOBEC2 expression[136]. The reverse has also been found to be true, with the observations that in *APOBEC2*$^{-/-}$ mice compared to WT skeletal muscle tissue, there are significantly fewer cells that stain positive for PAX7[203]. Although the initial expression of PAX7 closely resembled that of endogenous APOBEC2 expression within the RMS cell lines, there was no direct regulation of PAX7 levels upon the overexpression of PANX1. Direct

regulation of PAX7 levels by PANX1 is thus not likely playing a role in its regulation of APOBEC2. Additionally, our previous RNA-sequencing analysis in Rh30 cells showed a significant downregulation in the PAXBP1 gene in the PANX1-expressing cells compared to their control[129]. PAXBP1 has also recently been observed to play many similar roles to that of APOBEC2 including that of DNA methylation and the transcriptional regulation of myogenesis[129,191,204]. PAXBP1 has been suggested to recruit PAX3 and PAX7, along with the histone methylation machinery (histone methyltransferase complex), to chromatin to regulate transcription involved in myogenesis[180,204]. Similarly, APOBEC2s role in the transcriptional control of myogenesis stems from the binding of its promoter region to chromatin, allowing for an interaction with the HDAC transcriptional corepressor complexes[180]. The purpose of this binding is to facilitate the entry of satellite cells into the cell cycle upon muscle regeneration following injury[204–206]. Additional research has found that within PAXBP1 Knockout (*PAXBP1*$^{-/-}$) mice, there is a resultant activation of p53 contributing to apoptosis and cell-cycle arrest[204–206]. This links to our research as the overexpression of PANX1 within RMS also led to apoptosis[87,129]. The resultant increase in p53 activity and apoptosis has been proposed to be linked to an increase in reactive oxygen species generation (which was assumed to be due to a downregulation of antioxidant genes, as well as reduced NADPH production)[204]. This also links as well to the regulation of APOBEC2, as the production of reactive oxygen species was a characteristic of APOBEC2$^{-/-}$ mice, due to an increase in mitochondrial defects including enlarged mitochondria and depolarized mitochondria[135]. Therefore, based on the similar role of PAXBP1 and APOBEC2 in DNA methylation, the transcriptional control of myogenesis and the production of reactive oxygen species, as well as the known regulation of PAXBP1 by PANX1 in RMS, this gene may be of further investigative interest[180,191,204].

Other pathways that could be of alternative interest include that of either TGF-β or NF-kB signalling[177,178]. Due to the lack of published literature on APOBEC2 regulation, these two pathways are the only currently known signaling mechanisms by which the regulation of APOBEC2 levels has been shown[177,178,207]. These two pathways have additionally been shown to regulate PANX1 levels as well, especially during inflammation[177,178,207]. For example, APOBEC2 has been found to have an NF-kB activation sequence within its promoter region, and TNF-α and IL-1β have been specifically identified as being able to stimulate APOBEC2 expression[173,178]. The potential concern, however, is that previously it has been found that both these signaling pathways result in both the overexpression of APOBEC2 (in hepatocytes, and *Xenopus* and zebrafish embryos) and the overexpression of PANX1 (in a variety of cell types including endothelial cells), and therefore may not show the regulation pattern which is expected (the downregulation of APOBEC2 upon the overexpression of PANX1)[100,177,178,207]. However, the effects of these signaling pathways on the regulation of APOBEC2 and PANX1 expression have never been examined within RMS, and thus this presents itself for future potential interest.

4.1 Future Directions

Our results established a novel relationship between APOBEC2 and AHNAK, that may or may not be dependent on PANX1. This is an important area of future study, as in several cancer models including RMS, a downregulation of AHNAK is predictive of increased cell migration and metastasis[132,133]. In addition, the overexpression of APOBEC2 has also been indicative of increased cellular mutations resulting in an accumulation during tumourigenesis[178,179]. For one, this relationship should also be investigated for a longer period of time in culture. Our research only looked at twenty-four hours post cumate induction, however, we are aware that the

relationship between PANX1 and APOBEC2 is time-dependent. Hence, a longer incubation with cumate (between four to ten days), may provide additional insight into a potential mediating relationship between AHNAK, APOBEC2, and PANX1. However, it is also of note that PANX1 remains expressed at lower levels within the control. Therefore, to have a clear answer regarding the involvement of PANX1 within this relationship, we would need to use a PANX1$^{-/-}$ Rh30 cell line as well. In this case, we would compare PANX1 KO to the overexpression of PANX1 both with the downregulation of AHNAK, to determine the dependence of PANX1 within this relationship. Together this research would provide insight to the involvement of AHNAK in the PANX1-mediated regulation of APOBEC2 expression.

Additionally, as our studies on PANX1 in RMS demonstrate that increasing PANX1 levels may be of therapeutic benefit, we have identified for the first time a clinic-ready drug that can increase PANX1 levels in RMS cells, namely quercetin[117]. We have shown that treatment with quercetin triggered the partial differentiation of RMS cells in a PANX1-dependent manner and inhibited RMS growth[26,117]. This would be interesting to assess whether quercetin also regulates APOBEC2 levels and/or its function. As DNA methylation has previously been linked as an epigenetic mechanism to regulate myogenesis, APOBEC2s role in myogenesis may be tied to its DNA demethylation ability[134,155,173,174]. For example, this could include a role for APOBEC2 in the demethylation of myogenic inhibitory transcription factors (such as for myogenin), as the deficiency of APOBEC2 increases (in some model systems) the transcription of such myogenic regulatory factors[26,173,174]. However, this has not been studied previously, especially not within the context of RMS and therefore it may be of interest in the future to examine whether APOBEC2 demethylation is playing a role in the PANX1-mediated reduction of RMS malignant properties.

4.2 Concluding remarks

For the first time, we were able to demonstrate the downregulation of APOBEC2 levels in RMS compared to that of differentiating HSMM. However, we additionally found that APOBEC2 expression increases as the cells proliferate. When considering the role of APOBEC2 on its own, we found that the overexpression of APOBEC2 did not affect Rh30 cell viability or proliferation, however, it did result in a significant accumulation of spheroid growth. This suggests that APOBEC2 expression regulates tumour growth in vitro and that its overexpression promotes RMS malignancy. Upon the overexpression of PANX1, there was a resultant downregulation of APOBEC2 expression in some RMS patient-derived cell lines (Rh28, Rh30, and RD). Despite a lack of correlation in the regulated cell lines in terms of endogenous expression of MyoD, myogenin, or MHC, it was found that only in the cell lines in which APOBEC2 levels are regulated by PANX1 was there a resultant induction of multinucleation upon the overexpression of PANX1. This was further confirmed by discovering that the overexpression of APOBEC2 was able to reverse the PANX1-mediated induction of multinucleation, suggesting that the regulation of APOBEC2 by PANX1 may be dependent on the ability of PANX1 to induce cell fusion. Further analysis is still required to evaluate the molecular mechanisms that are regulating the relationship between APOBEC2 and PANX1, as the two current inquiries into AHNAK and PAX7 were unsuccessful in further deciphering this mechanism. Overall, this study provided more insight into the PANX1 mediated inhibition of RMS malignancy, and further aids in understanding the mechanism by which PANX1 may act as a novel therapeutic target for RMS.

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