The role of Ring1 and Rnf2 in skeletal muscle regeneration

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Abstract

The skeletal muscle environment is highly complex, with inputs from multiple cell types and environmental challenges in homeostatic and regenerative conditions. Satellite cells, a population of quiescent adult stem cells, maintain the regenerative potential of muscle well into adulthood, but the ability of the cells to maintain this is gradually lost as organisms age. In response to cues following injuries, or other insults to muscle integrity, satellite cells become activated. The activated satellite cells proliferate to provide nuclei to regenerate the damaged muscle, while also providing nuclei to return to the quiescent state for subsequent injuries. The changes between these states are tightly regulated and enabled through the flexible epigenetic modifications to the satellite cells. The Polycomb proteins have been shown to make significant contributions to cells' "memories of self", and in the regulation of stem cell fate transitions. How the Polycomb repressive complex 1 affects the regulation of the satellite cells has been poorly defined to date, but studies have shown that it is important for the regulation of myogenesis. In this study, we analyzed how the Polycomb repressive complex 1 affects the epigenetic regulation of satellite cells. To do this, we used shRNA's to Ring1 and Rnf2 to deplete the levels of the E3 ubiquitin ligases of the Polycomb repressive complex 1, and Prt4165 to determine the contribution of the enzymatic activity of the Polycomb repressive complex 1 complex without disrupting the non-enzymatic contributions of Ring1 and Rnf2. In our study, we have found *in vitro* that the Polycomb repressive complex 1 regulates the proliferation rate and differentiative capacity of C2C12 cells. We have also found that the enzymatic activity of the complex specifically regulates the ability of primary myocytes to fuse *in vitro,* potentially through regulating the motility of the cells. These results broaden our

knowledge of the role of the Polycomb repressive complex 1 in skeletal muscle myogenesis, and the distinct contributions of the Prc1 complexes subunits to its regulatory functions.

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Abbreviations

BGS – Bovine growth serum

BSA – Bovine serum albumin

CaPS – Chromatin and phase separation

CpG - 5'—C—phosphate—G—3'

cPrc1 – canonical Polycomb repressive complex 1

Cbx - Chromodomain proteins

ChIP-seq - chromatin immunoprecipitation-sequencing

CUT&Tag - Cleavage Under Targets and Tagmentation

DAPI - 4′,6-diamidino-2-phenylindole

DMD - Duchenne muscular dystrophy

DMEM - Dulbecco's Modified Eagle Medium

DTT - Dithiothreitol

EdU - 5-ethynyl-2′-deoxyuridine

ESC – Embryonic stem cell

HAT – Histone acetyltransferase

HDAC – Histone deacetylase

HEK – Human embryonic kidney

Hox - Homeobox

FIJI – FIGI Is Just Image-J

FSHD - FacioScapuloHumeral Muscular Dystrophy

KO - Knockout

MRF – Myogenic regulatory factor

MyHC – Myosin heavy-chain kinase

ncPrc1 – non-canonical Polycomb repressive complex 1

NEB – New England Biolabs

PBS – Phosphate buffered saline

PcG - Polycomb group

PCGF - Polycomb group RING finger protein

PCR - Polymerase chain reaction

PEI - Polyethylenimine

Phc – Polyhomeotic homologues

Prc1 – Polycomb repressive complex 1

Prc2 – Polycomb repressive complex 2

PR-DUB - Polycomb repressive deubiquitinase

qPCR - Quantitative polymerase chain reaction

SAM – Sterile alpha motif

SDS - Sodium dodecyl sulfate

SDS-PAGE - Sodium dodecyl sulfate–polyacrylamide gel electrophoresis

SEM – standard error of the mean

SET - Su(var)3-9, Enhancer-of-zeste and Trithorax

shRNA – short-hairpin RNA

TAD - Topologically associated domain

TBS – Tris buffered saline

TBST - Tris buffered saline-Tween

TrxG - Trithorax group

Chapter 1: Introduction

1.1 Regeneration in skeletal muscle tissues

1.1.1 Overview of myogenesis

Skeletal muscle tissues are highly regenerative tissues, capable of maintaining this capacity into adulthood(1). A population of adult stem cells, known as satellite cells discovered in 1961, ensures this regenerative capacity(2). In homeostatic conditions, satellite cells reside in a quiescent state between the muscle fiber sarcolemma and the basal lamina of the basement membrane(3). The basal lamina contains many extracellular matrix proteins often with post-translational modifications which modulate signal transduction, which plays key roles in satellite cells' quiescence and activation(4, 5). When muscle injuries occur, the satellite cells become activated and proliferate to expand the myogenic progenitor population in the muscle(6). At this stage, the myogenic progenitors are frequently referred to as myoblasts.

The myoblasts are faced with three key decisions, to self-renew regenerating the stem cell population(7–9), to continue proliferating to further expand the population, or terminal differentiation of the myoblasts, which then fuse into the damaged multi-nucleated myofibers (or fuse to form new myofibers) to repair the muscle(10). The ratio of myoblasts exiting towards self-renewal or towards terminal differentiation is essential for the long-term health of an organism’s muscles. Indeed, the perturbation of these processes contributes to the pathogenesis of muscle diseases(9, 11), and genetic changes that alter this ratio can have profound effects on the integrity of an organism's muscles following injury(12). As the myogenic progenitors change between these states, the differential expression of Pax7 (and

sometimes Pax3(9, 13)), Myf5, Mrf4, MyoD, and Myogenin in satellite cells govern many of the changes that occur within the myogenic program(14).

1.1.2 Paired box protein transcription factors in myogenesis

There are two paired box transcription factors that are relevant to myogenesis, Pax3, and Pax7. Pax3 is most closely associated with functions during the development of the organism *in utero* where it positively regulates the satellite cell's motility(15). When the Pax3 allele is replaced by Pax7 during development, the myogenic progenitors in mice fail to properly infiltrate the limb buds, causing defects in the limb muscle development(15). In most adult muscles, Pax7 is the predominant paired box transcription factor expressed (albeit with a few exceptions such as the diaphragm) where it contributes anti-apoptotic functions that Pax3 is incapable of fully rescuing(16). The anti-apoptotic functions of Pax7 are essential even in muscles such as the diaphragm where Pax3 is expressed at a higher level than Pax7, further underscoring the role of Pax7 in the maintenance of the satellite cell pool. Furthermore, Pax7 is indispensable for the expansion of the myogenic precursor population following injury where it prevents precocious differentiation of the proliferating myoblasts(17), and the ability of a subset of these myoblasts to return to quiescence to maintain the stem cell pool for subsequent injuries(18–20). During development, the $Pax3^{+}/Pax7^{+}$ myogenic progenitors expand their population alongside the developing muscle in the myotomes. The population of these cells which do not co-express later myogenic genes such as MyoD establish the population of satellite cells(21). In the absence of Pax7 expression, mice develop with minimal overt phenotypes to term, but the population of satellite cells fails to be maintained, and most mice die within 3 weeks of birth(22, 23).

1.1.3 The myogenic regulatory factors

Myf5, Mrf4, MyoD, and myogenin are all members of the basic helix-loop-helix family of transcription factors. These 4 proteins constitute the group of proteins known as the "myogenic regulatory factors (MRFs)", and have core functions in defining the myogenic population of cells and ensuring the productive progression of the myogenic program(24–28). Myf5, MyoD, and Mrf4 are all independently capable of specifying the muscle lineage during the development of the embryo(29), showing that the MRFs have overlapping functions. While Myf5, Mrf4, and MyoD do have significant functional overlap during embryogenesis, the viability of the organism postnatally can still be adversely affected due to defects during embryogenesis when the levels of one or more of these genes are reduced(30). Thus, while the myogenic regulatory factors have some redundant activities, these proteins do also have distinct functions that cannot be compensated for by other mechanisms.

Following birth, Myf5 has been shown to have a role in regulating the transient amplification of the satellite cells(31). When injuries occur to the muscles, the satellite cells become activated and the activated satellite cells can be categorized into two pools of cells based on Myf5 expression, one being $Pax7^{+}/Myf5^{-}$ and the other being $Pax7^{+}/Myf5^{+}$. The $Pax7^{+}/Myf5^{-}$ cells can divide asymmetrically, giving rise to one cell which will self-renew, and one cell that is $Pax7^{+}/Myf5^{+}$ which will continue to proliferate, and form the pool of myogenic cells that will differentiate into myotubes. The $Pax7^{+}/Myf5^{-}$ can also divide symmetrically, which will give rise to two $Pax7^{+}/Myf5^{-}$ stem cells, expanding the stem cell compartment(31).

MyoD has several functions in regulating adult myogenesis. At the protein level, MyoD is not translated in quiescent satellite cells, however, it is transcribed in quiescent cells. It is initially transcribed in these cells with a retained intron, allowing the rapid translation of the

MyoD protein once the molecular cues for activation are met(32). In mice lacking MyoD (it should be noted though that this occurred in a dystrophic context and not otherwise healthy cells) there is a vastly increased propensity for self-renewal of the satellite cells, disrupting the self-renewal/terminal differentiation balance(33). When the satellite cells divide asymmetrically, most $Pax7^{+}/Myf5^{+}$ cells will express MyoD committing these cells to the terminal differentiation lineage(34), whereas the $Pax7^{+}/Myf5^{-}$ cells will express Notch-3, which has been shown to have roles in the return to quiescence(35). MyoD has roles in promoting the proliferation of myoblasts by creating a permissive chromatin environment amenable to transcription(36). While MyoD binding has been shown to open chromatin during proliferation, it only does so at a subset of its target genes(36, 37), due to the differential binding of transcriptional activators and repressors at the MyoD targets(38). One example of this regulation can be shown during the proliferative phase, where a MyoD/Mef2D/KAP1 complex is bound to the myogenin locus. While MyoD is bound to the myogenin locus during proliferation, it is not expressed, and the locus is kept silent by the binding of HDAC1 and G9a to KAP1. During differentiation, KAP1 is phosphorylated, releasing HDAC1 and G9a, allowing the transcription of the genes targeted by this complex (including myogenin, a key regulator of terminal differentiation) to begin transcription(38).

Myogenin, a gene that is transcribed in response to the actions of MyoD, is a key regulator of late differentiation(39) and adult muscle homeostasis(40). Myogenin has been shown to promote the expression of an essential myogenic fusion gene, myomaker(40), as well as other genes essential for the myocytes to fuse into the damaged myofibers during regeneration(41). When myogenin levels are reduced *in utero,* the mice are not viable and die shortly after birth, likely due to defective diaphragm muscle function as the myofiber

formation has been severely impeded(42). Interestingly when the expression of myogenin is reduced 1-day post-birth, the mice remain viable albeit with significantly reduced muscle fiber size and reduced whole-body weight(43). In these post-natal mice with reduced myogenin levels, there was a significant number of dysregulated genes involved in differentiation, however, it would appear that the upregulation of the other MRFs can compensate for myogenin's function postnatally.

Mrf4 (commonly referred to in the literature as Myf6 as well) is the last of the MRF proteins to be expressed in healthy muscle tissues. Mrf4 is active in adult myogenesis during the very late stages of differentiation, mainly being expressed in myotubes(44). In the myotubes, Mrf4 has essential roles in regulating the secreted factors from the muscle fiber. These secreted factors influence the development and maintenance of the muscle niche and promote the maintenance of the quiescent state of the satellite cells(44). This function of Mrf4 is essential for the long-term health of muscle tissues. Without Mrf4 and the secreted factors whose expression it promotes, the stem cells could be depleted prematurely as the cells begin to be activated precociously.

1.1.4 Epigenetic regulation of satellite cell fates

The regeneration of skeletal muscle tissues is a highly complex process, with both intrinsic and extrinsic factors in satellite cells contributing. Skeletal muscle tissues are highly regenerative tissues, capable of maintaining this capacity into adulthood(1). While the regenerative capacity of skeletal muscle is maintained into old age, many elderly patients develop a disease known as sarcopenia, marked by a substantial decline in the skeletal muscle's regenerative capacity(45). Sarcopenia is a multifactorial disease, and the precise mechanism is unknown. Sarcopenia at the phenotypic level can be broadly characterized by

decreased satellite cell function (self-renewal, proliferation, and differentiation) and decreased total number of satellite cells per muscle fiber(46). Epigenetics have a profound effect on the pathology of muscle diseases(47, 48). While much is known about the epigenetic regulators for satellite cells and in particular factors such as the myogenic transcription factors, less is known about the contribution of histone modifications and the roles of the proteins which deposit these modifications or otherwise modify the chromatin landscape. Bridging these gaps in the knowledge of epigenetics with a basic research approach offers the potential for elucidating a greater understanding of diseases such as sarcopenia, and potential treatments for such diseases.

1.2 Role of Histone Modifications in transcriptional regulation

1.2.1 The histone core and the epigenetic code

Histones package the DNA around an octamer of 8 histone proteins(49, 50) (two each of H2A, H2B, H3, and H4), wrapping around 145-147 base pairs of DNA to form the nucleosome. These nucleosomes in large part form the basic structural organizing unit of the genome. These nucleosomes can be organized into tightly packed arrays essential for organizing the genome(51, 52). Outside of the core histones which form the nucleosome, there is also the linker histone H1 and its subtypes(53). H1 has important roles in stabilizing the nucleosome arrays when it is packed into higher order structures(54, 55). The function of the histone proteins in nucleosomes can be modified by post-translational modifications of their histone tails(56–58). Post-translational modifications of histone tails give these proteins roles beyond merely organizing the chromatin and allows them to affect the transcriptional output of the cells both positively and negatively. The roles of the nucleosomes in regulating transcriptional outputs can also be affected by the histone variants incorporated into the

histones(59), and by histone chaperone proteins needed to deposit the various histones and their variants(60, 61).

The combination of epigenetic modifications to the histone tails is frequently referred to as a "histone code"(62). Histone modifications are deposited by epigenetic enzymes, the "writers" of the epigenetic code. These writer enzymes deposit acetyl groups, methyl groups, ubiquitin, phosphoryl groups, and other small functional molecules onto the histone tails(57). However, writers with no audience cannot influence their environment. The epigenetic code elicits its effects through the "reader" proteins that recognize and bind to these histone modifications, and in some instances through modifying characteristics of the histone tails such as neutralizing their charged domains(63, 64). Most reader proteins do not directly change the histone modifications but can have roles in remodelling chromatin to increase or decrease the accessibility for transcription(65) and influencing the binding of the transcriptional machinery(66, 67). Most reader proteins are highly specific, reading both the modification and the flanking sequences on their histone targets(65). Some histone reading complexes will also bind specifically to a combination of histone modifications either on the same nucleosome or nearby nucleosomes(65). Lastly, as epigenetics entail reversible changes, there is another class of proteins in the epigenetics system to remove the histone modifications, known as "erasers". The erasers have essential functions in preventing the overaccumulation of specific modifications which can dilute the targeting of the reader proteins below functional thresholds(68). Histone erasers also provide a method for cells to integrate extracellular signals from their environment, allowing them to regulate their epigenome and subsequent gene expression in response to changing conditions(5). The combinations of writers, readers, and erasers are essential in regulating the genome, and in maintaining cells' "sense of self" within

tissues. As cells replicate, they require a method of remembering what cell type they are in a heritable manner, and epigenetics give them the mechanism(69).

1.2.2 Acetylation of histone tails

The acetylation of histones is catalyzed by histone acetyltransferases (HATs)(70). Acetylation has long been associated with more actively transcribed chromatin(56). Acetylation of the histone tails occurs on the positively charged lysine's neutralizing the charge on the histone, weakening its affinity for the DNA(63). This results in a sharp reduction in the histone tails affinity for binding the negatively charged DNA, opening up the chromatin in this region for the binding of transcription factors and the transcriptional machinery(64, 71). H3K27ac is strongly associated with actively transcribed genes(72). During myogenesis, MyoD as part of a complex with Kap1 recruits the acetyltransferases p300 to activate Myogenin(38, 73), and p300 has been shown to catalyze the acetylation of H3K27(74), although p300 acetylates a variety of other targets on histones H3 and H4 as well(75). When cells require transcription to be repressed, histone deacetylases (HDACs)(76) are frequently recruited (often alongside other transcriptional repressors)(77, 78). In the context of heterochromatin formation, it has been shown that deacetylation is essential for promoting H3K9 methylation, an important histone modification for heterochromatin(79–81). The acetylation of histone variant H3.3 has also been shown to be important for maintaining the sense of self of myogenic precursors during regeneration following a BaCl2 injection injury(60). In this context HIRA, a histone chaperone for H3.3 deposits this histone variants at myogenic genes, where it has essential roles in promoting the maintenance of H3K27ac to ensure these genes remain activated. When HIRA was depleted H3.3 was lost at these genes, and a broad spectrum increase in H3K4me3 deposited by Trithorax group complexes Mll1 and

Mll2 was found in the now “myogenic” cells which increases the expression of non-skeletal muscle related genes(60). It was unclear exactly how the ablation of H3.3 deposition and subsequent decrease in H3K27ac lead to the increased activity of Ml1 and Mll2 in satellite cells. It is clear however that the epigenome is dynamically regulated, and the coordination of disparate events can have far-reaching consequences in regulating transcription, which may not be initially obvious based solely on one histone modification in isolation.

1.2.3 Methylation of histone tails

The methylation of histones is a common histone modification and is associated with both transcriptional activation and repression. H3K4me3 is one of the most well-studied methylation sites associated with transcriptional activation(82). This modification can be deposited by the Mll complexes through their SET domain(83) containing subunits such as Ash2L(84, 85). During myogenic differentiation, a vast array of chromatin remodelling events are required to change the expression patterns of the cells. A part of this process is the deposition of H3K4me3 at specific promotors through the interaction of the Mll2 complexes and its methyl transferase Ash2L with Mef2D(86). This histone modification has recently been shown to also mediate transcriptional pause release, leading to the productive elongation and transcription of its target genes(87). Unlike acetylation where only a single group is added to the lysine, methyl groups can be added in a stepwise manner, up to trimethylation modifications. At H3K4, the monomethylation can be catalyzed by the Mll3 and Mll4 complexes(88). The monomethylation of H3K4 has roles in long-range chromatin associations and also serves as a docking site for Mll1 to catalyze the addition of more methyl groups, up to H3K4me3(89). H3K4me3 modifications are removed by KDM5C, which functions as a tumour suppressor by preventing the activation of bivalently marked genes. These genes when

expressed inappropriately result in the de-differentiation of myeloid progenitors and the oncogenic transformation into adult myeloid leukemia(90).

Another common methylation on histone tails is H3K9me3, commonly associated with heterochromatin and transcriptional repression(91–93). To elicit the formation of H3K9me3, H3K9 is first methylated by G9a, GLP(94), Prdm3, or Prdm16(91). This sets the stage for Suv39h to methylate the H3K9me1/2, to create H3K9me3 modifications(92). These modifications are essential in driving heterochromatin formation, where HP1 will bind to H3K9me3 on adjacent nucleosomes, an important function in creating higher-order compacted chromatin arrays(93, 95). H3K9me3 is removed by the JmjC domains of the KDM4 family of lysine demethylases. When overexpressed, these demethylases frequently lead to cancers, highlighting the importance of balancing the activities of the writers and erasers of histone modifications(96).

The last histone methylation to discuss is H3K27me3. This is deposited by EZH2 of the Prc2 complex(97) which deposits both H3K27me2 and H3K27me3 onto monomethylated H3K27. H3K27me3 is a repressive histone modification, serving as a docking site for other repressive complexes(98, 99), repressive transcription factors(100), and a contributing factor to chromatin condensation(101, 102).

As satellite cells exit quiescence and proceed forward into terminal differentiation, this requires a vast remodelling of the epigenome, coordinated in part by integrating signals from the satellite cell niche. It has been shown that Jmjd3 expressed in the satellite cells, a H3K27me3 demethylase, is essential for the transition from quiescence to activation. The targeted activity of Jmjd3 at select genes such as Has2 integrates the signals from the inflammatory environment in the early stages of regeneration following a cardiotoxin injury(5)

(cardiotoxin is a common myonecrotic agent used in animal studies to study the regeneration of the muscle tissue(103)). Has2 then produces hyaluronic acid chains which are then extruded from the satellite cells allowing the quiescent satellite cells to sense the change in the extracellular environment, which in turn activates the ERK and RAC signalling pathways(104). These pathways then contribute to the activation of the satellite cells, directing the satellite cells to exit quiescence(5, 105). Without the activity of the H3K27me3 demethylase Jmjd3, the satellite cells fail to be properly activated, and cannot remove the H3K27me3 which was keeping the satellite cells in the quiescent state(5).

As the cells proceed into proliferation, H3K27me3 deposition has been shown to regulate both positive and negative cell cycling genes, highlighting the importance of its targeted deposition to coordinate the proliferation rate of the myogenic precursors(106). Lastly, during terminal differentiation, the nucleosomes in the Pax7 locus are methylated by EZH2 to repress Pax7 for the entrance into terminal differentiation and fusion into the myotubes(107). UTX, another H3K27me3 demethylase is required for the expression of myogenin, and essential myogenic transcription factor for terminal differentiation(108). H3K27me3 is an excellent example of the dynamic regulation of histone modification and highlights the importance of the temporal regulation of the deposition of removal of these histone modifications.

The functional output of histone modifications can depend greatly on the combination of histone marks at a locus, with the modification of histones at bivalent promoters providing a great example. Bivalent promoters are promoters paradoxically marked with H3K4me3 and H3K27me3(109–113). There is no clear consensus on the exact role of bivalent promoters as embryonic stem cells (ESCs) mature from their totipotent state and begin to differentiate into

different lineages in the genome(110, 111, 113). It is clear that these bivalent promoters directly contribute to preventing the DNA methylation of these promoters, which contributes significantly to preventing their irreversible repression and maintaining their genomic plasticity(111). As ESCs differentiate, bivalent promoters are reorganized extensively to support new gene expression patterns(110). When reorganized, these promoters will lose their H3K4me3 signature and become further repressed(114), or lose H3K27me3 and begin transcription(115). Interestingly, the promoters who more stringently retain H3K27me3 will frequently be marked by H2AK119ub1(109), suggesting that the chromatin environment will greatly influence the direction that these bivalent promoters are resolved during differentiation. While ESCs have been the most studied cell type in the context of bivalent promoters, it has also been shown that these promoters are frequently present in adult stem cells and cancerous cells, and contribute to the regulation of differentiation in these cells as well(111).

1.2.4 Ubiquitinylation of histone tails

Ubiquitin is a small protein which is frequently a post-translational modification on proteins and a prevalent epigenetic modification for histone tails(116, 117). Ubiquitin is predominantly added to lysine residues and can be added to proteins alone, or in branched chains(117). The addition of ubiquitin to proteins is catalyzed by the actions of the E2-E3 ubiquitin ligase pairs where the E3 ubiquitin ligase is essential for recognizing the target substate(118, 119). Protein ubiquitinylation is frequently associated with protein turnover, where unprotected branched ubiquitin chains such as K48/K63 and K11/K48 are targeted to the proteosome(120). This can also occur on histone proteins. APC/C is targeted to the promoters of pluripotency-related genes in ESCs during mitosis, where it ubiquitinylates histones with K11/K48 branched ubiquitin chains(121). This allows the rapid re-expression of

pluripotency genes post-mitosis, as the histones will then be rapidly degraded after the cells have finished dividing.

Not all ubiquitylation events on histones lead to their degradation, however. The most well-characterized occurrence of this is in the DNA repair pathway. The monoubiquitinylation of H2A.X at K13 and K15 by Rnf168 and Rnf8 have been shown to recruit 53BP1, which leads to a subsequent cascade of protein binding and recruitment which results in the promotion DNA repair of non-homologous end joining(122, 123). Paradoxically, Rnf168 and Rnf8 have also been shown to promote homologous recombination through the same ubiquitinylation pathway, however, in this instance, PALB2 reads this histone modification(122, 124), which leads to the recruitment of BRCA1. It is unclear what dictates whether PALBP2 or 53BP1 will bind to the ubiquitinylated H2A.XK13/15 which is an outstanding question to be answered in the DNA repair response field.

Sites of DNA damage repair have also been shown to recruit the Prc1 and Prc2 complexes, where they catalyze the ubiquitinylation of H2A.XK119ub1, and H3K27me3 respectively(125–127). The precise function of this recruitment is still debated; however, non-mutually exclusive theories of their roles have been forwarded. One potential function they could have in this context is to repress transcription to prevent conflicts between the transcriptional machinery and the DNA damage response signalling by repressing the damaged gene(127, 128). Another function which could easily occur alongside the previous theory is that the chromatin compaction driven by the cPrc1 complexes helps organize the DNA damage response pathway and creates a local environment permissive to repair(125).

Histones are also monoubiquitylated outside of the DNA damage response pathways. H2A and H2B are both monoubiquitinylated with transcriptional effects independent of

histone turnover or DNA damage response signalling (5%-15% of H2A, and 1%-2% of H2B are estimated to be monoubiquitinylated in vertebrate cells)(116). Histone H2BK120 ubiquitinylation (this is the site in mammals, in budding yeast this occurs at K123) catalyzed by Rnf20 is involved in the positive regulation of transcription by contributing to the relaxation of the chromatin(129). This paper also showed a reduction in the acetylation at H3K14 when Rnf20 levels were reduced, which could be showing that the H2B ubiquitinylation serves as a docking site to mediate the cross-talk between different histone-modifying proteins. Other systems have also shown the role in H2BK120ub mediated crosstalk between H3K4me3 and H3K79me3 deposition(130). While the above examples of the function of the mark are both integrated with other histone modifications, H2BK120ub1 has also been shown to have roles independent of H3 methylation(131).

While H2BK120ub1 is predominantly associated with transcriptional activation, H2AK119ub1 is predominantly a repressive mark, although recently there have been found some instances where it appears to stimulate transcriptional activation(132). H2K119ub1 is deposited by Ring1 or Rnf2 of the Prc1 complexes(133). H2K119ub1 has been shown to serve as a docking site for the Prc2 complex, and this enzymatic mark thus frequently co-occurs with H3K27me3(134). While these enzymatic modifications are frequently found together and co-regulate their targets, approximately 40% of H2AK119ub1 is found outside of H3K27me3 domains. Thus a non-insignificant portion of the role of H2AK119ub1 deposition occurs outside of the influence of H3K27me3 related activity, which has often been underappreciated in the literature(135). Interestingly, another complex for which H2AK119ub1 serves as a docking site is the Prc1 complex itself, which provides a major mechanism for spreading Polycomb domains(136, 137). The spread of these domains is checked by the activity of BAP1

of the PR-DUB complex, a deubiquitinase for H2AK119ub1(68). Paradoxically, when BAP1 levels are reduced, the targets of the Prc1 complex are upregulated, despite the increase in the generally repressive histone modification H2AK119ub1. What likely occurs is that the binding of the Prc1 and Prc2 complexes to H2AK119ub1 becomes overly diluted at their normal target genes as these complexes are dispersed below repressive thresholds(68). Another potential mechanism which constrains the spreading of H2AK119ub1 domains is H3K36me3 deposition(138). In mouse pre-implantation embryos it was found through ChIP-seq experiments that H2AK119ub1 domains would not spread past H3K36me3 marked regions(138), demonstrating that H3K36me3 can constrain the spread of Polycomb domains.

The role of H2AK119ub1 in conjunction with the Prc2 complex has been well studied(99, 134, 139, 140), however, approximately 40% of H2AK119ub1 is found outside of H3K27me3 domains. Clearly, there are more functions to this histone modification than recruiting the Prc2 complex(135). The functions of H2AK119ub1 independent of H3K27me3 are less well characterized, in large part due to a lack of tools to specifically study the role of H2AK119ub1 deposition independently from other activities of the Prc1 complex. In the last years, small molecular inhibitors such as Prt4165(141), and catalytically dead variants of Rnf2 such as the I53A mutant have been developed(142, 143). This opens the door to studying the deposition of H2AK119ub1 in adult stem cell populations. H2AK119ub1 in conjunction with the Prc2 complex has been shown to have roles in maintaining the potential to repress target genes upon the induction of the satellite cells differentiation in some contexts(109). In other contexts, H2AK119ub1 has been shown to seed new Polycomb domains upon differentiation of embryonic stem cells(144). Whether the seeding of new Polycomb domains would occur in a myogenic system upon induction of differentiation has not been documented.

1.3 Polycomb complexes and their regulation of myogenic cell fates

1.3.1 Epigenetics beyond histone tail modifications

Histone modifications form an important core of epigenetic regulation, however, the proteins that modify histones can be differentially regulated in different cell types. This is a major contributor to providing each unique cell lineage with its unique histone code, which dictates the expression patterns that ensure that the cells maintain their lineage even during periods of rapid proliferation. Furthermore, epigenetic modifiers are not solely restricted to eliciting their effects through the post-translational modification of histone proteins. Heterochromatin formation requires the large-scale organization of chromatin in tightly packed nucleosomal arrays(54). HP1α and HP1γ are both instrumental in this organization, driving this effect through liquid-liquid phase separation effects mediated by the hinge domain of HP1α and HP1γ(145). HP1β however, opposes this liquid-liquid phase separation and de-compacts these arrays(145), and is overexpressed in cancers(146). In these cancers, there is aberrant upregulation of genes normally locked away in heterochromatin domains(146). These effects are certainly epigenetic in nature but are driven through mechanisms independent to direct histone tail modifications.

1.3.2 The Polycomb complexes

The Prc1 and Prc2 complexes are well known to modify histone tails, being the drivers of H2AK119ub1, and H3K27me3 deposition respectively(136, 147, 148), but the Prc1 complex also mediates the compaction of chromatin through liquid-liquid phase separation effects driven by the chromodomain proteins (Cbx)(98, 149) and the polyhomeotic

homologues (Phc)(150) of the Prc1 complex. The Prc1 and Prc2 complex are both multi-subunit complexes, and with the various accessory co-factors they can bind, there is a significant amount of cell-to-cell variability in the composition of Polycomb complexes(147, 151), an important driver of the cell-to-cell variability in the regulation of the epigenome. This variability is essential for regulating cell fate transitions in myogenesis, as the myogenic transcription factors need precise regulation to ensure that myogenesis proceeds productively and sustainably, and the Polycomb complexes are integral for this activity(152, 153).

The changes to the epigenetic landscape require chromatin-modifying proteins to fine-tune the expression of genes across myogenic state changes and are essential for controlling the temporal expression of myogenic genes(154). Two key families of chromatin modifiers are the Polycomb group (PcG) proteins(155), and the Trithorax group (TrxG) proteins(156). These two families form an antagonistic pair where the PcG proteins repress gene expression during development until the TrxG proteins re-activate the expression of their target genes(157). These families have essential roles in preserving cellular memory and specifying the cellular fate of differentiating stem cells(158, 159), which remains true in satellite cells(86, 153).

The Prc1 complex has essential roles in preserving the cellular identity of both embryonic and adult stem cells(77, 160, 161). The Polycomb complexes are also important during the transition between embryonic stem cell populations into adult stem cell populations(162, 163). The Prc1 complex is essential for maintaining adult stem cell populations by repressing non-lineage transcription factors(160) and can be targeted to cell-specific genes through its protein-protein interactions. For example in rod photoreceptor cells, Samd7 binds to the Polyhomeotic subunit of the cPrc1 complex through the polymerization of

the SAM domains of Samd7 and the Polyhomeotic subunit of the Prc1 complex(164). This interaction targets the cPrc1 complexes to repress genes such as S-Opsin which must be repressed to preserve the rod cell's function. It is unclear if similar interactions occur within satellite cells, however. Given the upregulation of non-lineage genes when Trithorax group proteins activities are aberrantly stimulated(60), it is reasonable to expect that there are mechanisms targeting the Polycomb group proteins to these same genes as in the rod photoreceptor cells(164).

PcG proteins are divided into two major families which have well characterized repressive functions. Their most well-known function is in the transcriptional repression of Hox genes, where the PcG proteins are essential for maintaining the segmentation patterns in Drosophila(165). The first Polycomb protein was initially found in 1947, when a mutation in Drosophila caused a posterior to anterior transition, and the development of extra sex-comb "bristles" on the fly's second and third legs(166). These families are the Polycomb repressive complex 1 (Prc1) (Fig. 1A) and the Polycomb repressive complex 2 (Prc2). While the Prc2 complex has been extensively studied, the Prc1 complex has not. It was originally thought that the binding of the Prc1 complex was entirely dependent on H3K27me3 deposited by the Prc2 complex, however, this was later disproven(99). While the binding of the Prc1 complex is not entirely dependent on the Prc2 complex, there are a significant number of genes at which it acts redundantly with the Prc2 complex, which often encompasses key transcription factors for different lineages(135) (Fig 1B). Furthermore, evidence has been accumulating that the Prc1 complex has underappreciated roles in transcriptional activation, however, is only well demonstrated in some instances(132, 167, 168).

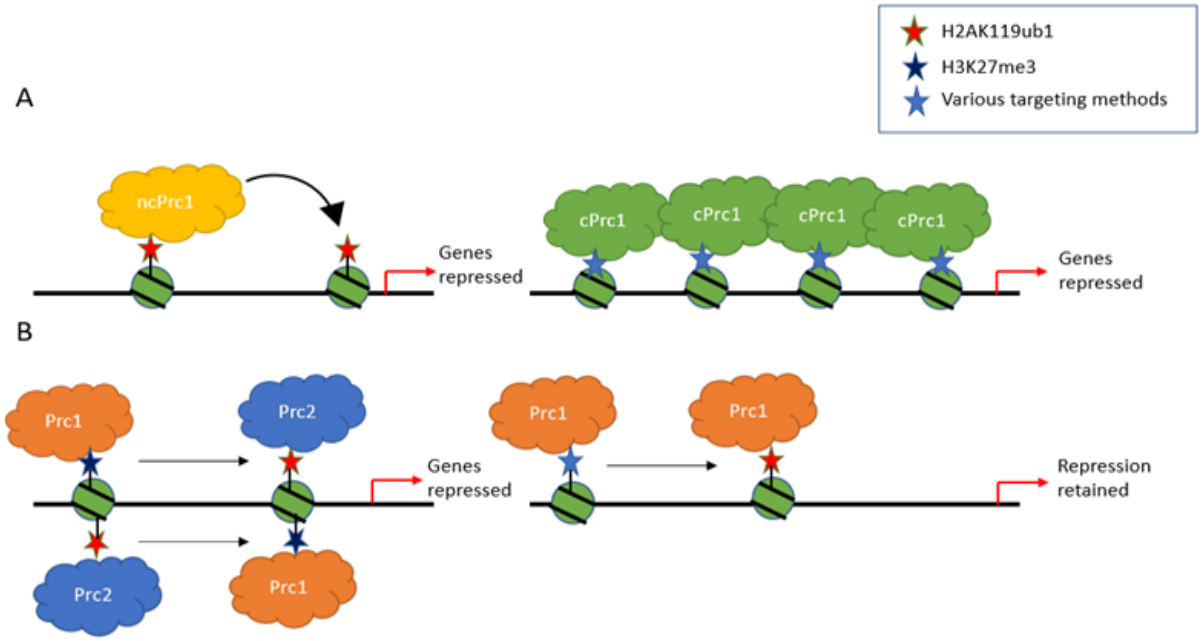

Figure 1: Modes of Prc1 mediated repression.

A) The Prc1 complex can be targeted to the genome by binding to H2K119ub1 through the ncPrc1 complexes leading to the spread of H2AK119ub1 histone modifications. The cPrc1 complexes can be targeted to the genome through binding to some instances of DNA sequences or co-factors which target the complex but are predominantly targeted to the genome through binding to methylated histones such as K3K27me3. **B)** The Prc1 complex co-represses genes with the Prc2 complex. In these instances, the absence of one of the two complexes can be compensated for by the other Polycomb complex leading to the repression being retained in the face of genomic instability.

1.3.3 Prc1 complex composition

All Prc1 complexes contain an E3 ubiquitin ligase (Ring1 or Rnf2) and a Pcgf protein (Pcgf's 1, 3, 5, 6, Mel18, and Bmi1). Some Prc1 complexes will also contain Chromobox proteins (Cbx2, 4, 6-8), Polyhomeotic homologues (Phc1-3), and a variety of accessory proteins(147). The Prc1 complexes are divided into subtypes according to which Pcgf protein is contained within the complex, as the Pcgf proteins are mutually exclusive. These are further split into two types of Prc1 complex, the canonical Prc1.2 and 1.4 (containing Mel18 or Bmi1 respectively) and non-canonical complexes Prc1.1, 1.3, 1.5, and 1.6 containing the Pcgf's 1, 3, 5, or 6(147). The ncPrc1 complexes are the main complexes responsible for H2AK119 ubiquitylation as the enzymatic activity of the Rnf2 and the Ring1 E3 ubiquitin ligases are

stimulated by the binding of either Rybp or Yaf2(137). The cPrc1 complexes do not bind to Rybp or Yaf2 but instead bind to one of the Cbx and one of the Phc proteins, which drive the cPrc1 complex's roles in nucleosome compaction(169). While the cPrc1 complexes have a lowered enzymatic activity, it is not completely absent(170). The bonds between Bmi1 and Rnf2 (presumably Mel18 and Ring1a and other combinations of these proteins would be similar, although this has not been directly tested) form salt bridges, which decreases the efficiency of ubiquitin transfer to the histones. However, the studies also showed that, in particular DNA conformations, the nucleosomes are positioned in a manner which enhances the enzymatic activity of the cPrc1 complexes for specific loci(170). So, while the ncPrc1 complexes broadly have a more active enzymatic activity which is essential for spreading Polycomb domains(133), the cPrc1 complexes do still have some limited but highly specific enzymatic activity to some loci.

1.3.4 Targeting the Prc1 complexes to the genome

The most well-documented mechanism of Prc1 complex deposition is the primary method of the cPrc1 complexes deposition, the binding of the Cbx proteins to H3K27me3 which had been deposited by the Prc2 complex(99, 109, 157). This method of targeting the cPrc1 complexes facilitates the co-repression of genes with the Prc2 complex which is observed at Hox genes(134, 171) among many other targets of the Polycomb complexes(138). The ncPrc1 complexes cannot bind to H3K27me3 and are recruited through other means. One mechanism is through the binding of KDM2B to the ncPrc1.1 complexes which targets these complexes to unmethylated CpG islands(172). This prevents the methylation of these loci while maintaining their reversible repression until molecular cues trigger the activation of these genes. Another major mechanism of ncPrc1 complex binding is through its interaction

with Rybp(136). Rybp (or Yaf2 which shares the C-terminal binding motif with Rybp) targets the ncPrc1 complexes to H2aK119ub1 which the ncPrc1 complexes themselves deposit(136). L3mbtl2 has been shown to recruit ncPrc1.6 alongside Max, MGA, G9a and HDAC1which weakly recruits the ncPrc1.6 to some E-box motifs, and strongly to germ cell lines genes in ESCs(78). The ncPrc1.3/5 can both be recruited to some of their target genes through Usf1 and Usf2(173), and through Tex10(174). These mechanisms of binding the Prc1 complexes to the genome can explain some of its targeting, but there remain many questions as to the factors which can bind to, and target the Prc1 complex to its target loci in different cell types, and as cells differentiate from ESCs into distinct tissues.

1.3.5 Genome compartmentalization, and phase-separated domains

Transcriptional activity can also be coordinated by compartmentalization in the genome. Stretches of chromosomes can be grouped into two broad compartments, A (predominantly active) and B (predominantly inactive) which typically do not interact(175). These compartments fold into topologically associated domains (TADs) which organize chromosomal compartments into self-interacting regulatory pockets to coordinate gene expression within these TADs(176). Nuclear compartmentalization is a complicated process driven by many forces, including liquid-liquid phase separation(177), and the organization it imparts is essential for maintaining lineage identities(178, 179). Phase separation results in the formation of nuclear bodies concentrating transcription factors within these droplets, some of which are formed by the Prc1 complex(180).

Components of the canonical Prc1 complexes such as Cbx2(98) (and likely Cbx4 and Cbx8 as the phase separation activities of Cbx2 are mediated by a shared domain with these 3 proteins(181)) are capable of forming liquid-liquid phase separated droplets through their

highly charged Compaction and Phase Separation (CaPS) domain, which is essential for the transcriptional repression driven by these complexes(182). Studies done in ESCs have shown that Cbx2 and Cbx7 containing Prc1 complexes form nuclear condensates, (indirectly indicative of phase separation in this context), however, Cbx6 does not(183) (ESCs do not express Cbx4 and Cbx8 and unfortunately could not be tested in this system). Cbx2 and Cbx7 both formed nuclear condensates, however, Cbx2 formed a significantly higher quantity of these. They also showed that the stability of these proteins binding to chromatin correlated to the number of condensates formed, with Cbx6 having the least stable binding time(183). What is likely the case is that Cbx2's increased stability on chromatin is a function of its phase separation activity. Cbx6 having the least stable binding time could be indicative of a function similar to HP1β(145), as the decrease in binding stability could be due to the disruption of phase separation when Cbx6-containing complexes bind to Prc1 complexes that were already phase-separated. Cbx7's binding time was found to be in between Cbx6, and Cbx2. Cbx7 does not contain the CaPS domain(181) and cannot drive phase separation itself, but based on these results likely does not oppose phase separation. While Cbx7 does not drive phase separation, it could potentially be binding to other cPrc1 complexes which are phase separated through the polymerization of the Phc domains within these complexes, or other accessory proteins bound to the Cbx7 containing complexes themselves could be driving the phase separation observed in Cbx7 containing complexes.

The most likely mechanism for the Cbx7-containing complex's phase separation is through the Phc subunit of these complexes. The Phc proteins have also been implicated in the phase-separation functions of the Prc1 complex. A truncated version containing the SAM domain of the Phc proteins can drive phase separation, which stimulates the ubiquitination

activity of the Prc1 complexes through an unknown mechanism(184). Phc1 was also found in a screen of prion-like proteins in humans and likely also is capable of phase-separating(185) suggesting that Phc1 has a secondary mechanism to elicit phase separation. In Phc1-KO ESCs (encompasses all cPrc1 complexes in ESCs), the genomic organization imparted by the Prc1 complex was lost(169). While this study does not look at the ability of these complexes to drive phase separation for this effect, it would be interesting to determine if this were the case.

1.3.6 The current knowledge of the Prc1 complex in myogenesis, and muscle-related diseases

As organisms age, they frequently develop a disease of muscle wasting known as sarcopenia(45, 46). This is a complicated disease with no clear single cause (and likely there is no single causative factor), however, the upregulation of the p16ink4a locus has been suggested to contribute to the disease(186). The expression of p16ink4a from the Cdkn2a locus leads to the downstream activation of the Rb/E2F transcription factors, which caused cell-intrinsic senescence which could not be rescued by transplanting aged mouse myoblasts into young mice. While transplantation could not rescue these defects, silencing the Cdkn2a locus reversed senescence in these cells(186). The silencing of this locus is dependent on many factors, including the Polycomb complexes(187, 188). When cells accumulate an excess of stress such as telomeres shortening below critical thresholds, EZH2 is downregulated, which was shown to then displace Bmi1 (and thus the cPrc1 complexes) from p16ink4a(188). These Bmi1-containing complexes were later shown to be complexes including Cbx7 or Cbx8(187). Interestingly reducing the levels of either of these Cbx proteins abrogated the binding of the other, suggesting that the binding of adjacent Prc1 complexes is essential for the stability of Polycomb repression at the p16ink4a locus.

The Prc1 complex has been shown to repress the Dux4 locus, which when transcribed is a causative factor for FacioScapuloHumeral Muscular Dystrophy (FSHD)(189). The Dux4 proximal promoter contains a CpG-dense microsatellite repeat which in FSHD is significantly reduced. This creates a situation in which an insufficient quantity of Prc1 complexes can be recruited to the Dux4 locus, and they are insufficient to drive the proper repression of Dux4. Dux4 is a double-homeobox transcription factor which has functions in the testis but has been inserted in a secondary location by a retro transposon(190). Most cells will have the proper repression of this gene, however, in multinucleated cells such as the myotubes one cell which improperly expresses the Dux4 mRNA can spread the mRNA to other nuclei within the same fiber. Many genes are deregulated by Dux4, but a major contributor to the muscle atrophy seen in Dux4 is the expression of a Dux4-related protein which induces cell death through the apoptotic pathway(191). As myotubes have many nuclei within them, the probability of these cells being killed due to the disease is raised significantly, contributing to the pathogenesis. Due to this, maintaining its repression at all times is essential for the tissue integrity of skeletal muscle, and the maintenance of a sufficient inhibitory concentration of the Prc1 complexes is essential for the regulation of Dux4 in somatic tissues.

In Duchenne muscular dystrophy (DMD), it has been shown that there is an increased quantity of DNA damage through NO-synthase-induced oxidative stress(192). Bmi1 of the canonical Prc1 complexes when “mildly” overexpressed is protective against the accumulation of DNA damage when healthy human myoblasts with mild Bmi1 overexpression were transplanted into a severely dystrophic mouse model(193). In mice with a whole-body knockout of Bmi1, it has been shown that the myoblasts isolated from these mice have a reduced proliferative rate *in vitro* and that there is a decrease in the number of satellite cells *in*

vivo suggesting that Bmi1 (and therefore the cPrc1 complexes) has a role in regulating proliferation, and potentially the return to quiescence(153). One weakness of the latter point however is that Bmi1 was removed from all cell types, and it cannot be ruled out that this depletion of satellite cells was due to a cell extrinsic mechanism. What could be shown though outside of the proliferative effects *in vitro* was that Bmi1 was expressed in both the $Pax7^{+}/Myf5^{+}$ and $Pax7^{+}/Myf5^{-}$ cell pools, and that its expression levels were decreased in myofibers, suggesting that Bmi1 is gradually downregulated as myogenesis progresses(153). Another study on Bmi1 in C2C12's (an immortalized mouse myoblast cell line) has shown through ChIP-seq experiments that the Bmi1 containing Prc1 complexes co-segregate with H3K27me3 on genes which remain permanently repressed as C2C12 cells differentiate(152). When Bmi1 was removed in these cells their differentiative capacities were significantly abrogated(152). So, while Bmi1 is downregulated as myogenesis progresses, it remains important for regulating the myogenic transitions.

Interestingly, the ncPrc1 complexes may have an anti-differentiative effect as when Rybp levels are reduced, differentiation is severely affected(194). When Rybp levels in mice are reduced *in vivo,* it has been shown to increase myogenin expression and provide a one-time increase in the regenerative potential of muscle tissues after cardiotoxin injury(194). Part of this effect is due to the increase in the expression of Mir-29, a micro-RNA which has been shown to decrease proliferation and increase terminal differentiation in myoblasts(195). What was found was that there was a decrease in H3K27me3 at Mir-29 when Rybp was depleted, however, they did not test the levels of H2AK119ub1. While it was beneficial to the muscle during a single injury, there was also a significant decrease in the $Pax7^{+}$ cell number. The $Pax7^{+}$ cells in this model would be the residual stem cell pool, which could indicate that the

increase in myogenic potential came at the cost of the self-renewal of the stem cells. However, this possibility was not tested(194).

1.4 Rationale and Hypothesis

The Prc1 complexes have been shown to regulate myogenesis through the regulation of proliferation(153) and differentiation(152, 194). However, previous experiments on the differentiation of muscle stem cells during differentiation have shown contradictory results(152, 194). These experiments reduced the expression of Rnf2 or Bmi1 and found that differentiation was inhibited(152), or reduced the expression of Rybp and found that differentiation was enhanced(194). These results were then extrapolated to encompass a role for the entire complex, which stretches the role of these proteins beyond what is reasonable(147). While these studies showed interesting results, not all Prc1 complexes contain Bmi1 or Rnf2(147), Rnf2 has both enzymatic and non-enzymatic roles(133, 143), and Rybp can have effects outside of its roles in the Prc1 complex(196, 197). Misregulation of the Prc1 complexes activity has been shown to contribute to skeletal muscle diseases and regulation highlighting the importance of understanding the functions of the Prc1 complex(68, 90, 114, 153, 189), but the specific role of the enzymatic ubiquitin ligase activity has not been determined in myogenesis. The studies to date leave the total scope of the Prc1 complex's contributions to myogenesis, and the specific roles of the different Prc1 complex components and regulatory functions unanswered. The Prc1 complex is a large, multi-subunit complex, whose composition can impart different activities based on which Prc1 complex components are included(147). Core proteins such as Ring1 and Rnf2 have roles in the monoubiquitinylation H2AK119(133, 198) but are also core components of the predominantly non-enzymatically active canonical complexes where Ring1 and Rnf2 are essential for

recruiting the Cbx proteins to the cPrc1 complexes(199). Alone, an Rnf2 knockout in skeletal muscle stem cells cannot differentiate between the enzymatic and non-enzymatic roles of Rnf2, and Ring1 would be present in these cells potentially compensating for some of Rnf2's activity. As stem cells differentiate, the monoubiquitinylation of H2AK119 by the Prc1 complexes is essential for both maintaining Polycomb domains, and seeding new Polycomb domains for genes which need to be repressed as the cellular environment changes during the differentiation process(133, 136, 138, 200, 201)·(109).

Considering this I hypothesized that the Prc1 complex is essential for regulating the progression of myogenesis through its ability to ubiquitinylate histones. In this thesis, I used shRNA's to the Ring1 and Rnf2 subunits of the Prc1 complex to deplete their levels in myoblasts before assessing the differentiative and proliferative capabilities of these cells. The role of the enzymatic activity of Prc1 was examined using Prt4165, an inhibitor for ubiquitin ligase activity of the Prc1 complexes(141). Analyzing the experiments in this thesis allowed us to begin understanding the specific roles of deposition of H2AK119ub1 by the Prc1 complexes in an adult stem cell population, and differentiate the enzymatic and non-enzymatic contributions of Ring1 and Rnf2. It is hoped that the insight into the specific functions of the enzymatic activity of the Prc1 complex will contribute to the development of therapeutic approaches that take advantage of small molecule inhibitors such as Prt4165 to inhibit the activity of the complex in patients(141).

Chapter 2: Materials and Methods

2.1 Methods

2.1.1 Primary myoblast isolation from mouse arm and leg muscles

Mice were euthanized by CO_2 asphyxiation before cervical dislocation was performed on the now-dead mice. The mouse carcasses were sprayed with 70% EtOH before the tibialis anterior, extensor digitorum longus, gastrocnemius, quadriceps, bicep posterior, hamstring, and triceps muscles were removed from the mouse limbs. Non-skeletal muscle pieces were removed, then the tissues were minced into small pieces (less than 1 mm^3). Minced muscle tissue was washed with PBS twice and then centrifuged at 200 x g for two minutes after each wash. The minced muscle tissue was then digested to liberate single myoblasts from the muscle tissue using digestion media (1.8 U/mL dispase and 1 mg/mL Collagenase in DMEM) for 90 minutes, rotating at 37°C. The digested muscle tissue was strained through a 100 µM filter, followed by a 40 µM filter to remove large chunks of muscle tissue debris. The strained muscle was washed in PBS, then centrifuged at 350 x g for 7 minutes. The cells were resuspended in primary growth media (20% BGS (v/v), 1 % penicillin/streptomycin (v/v), and 2.5µg/L of recombinant bFGF in Ham's F-10 media) and pre-plated on an uncoated cell culture dish. After pre-plating, the cells were transferred to a cell culture dish coated with 0.01% collagen. After 36 hours the media was replaced, and subsequently replaced every 24 hours thereafter.

2.1.2 Collagen-coated plates

Collagen was diluted to 0.01% (w/v) in 0.2% (w/v) acetic acid. Tissue culture treated plates were then covered in the diluted collagen for 40 minutes, before being air-dried overnight before use.

2.1.3 Cloning of shRNA vectors

To clone the shRNA's into pLKO.1_puro, the first shRNAs targeting Ring1 from a publication from the Wang lab were used(202), and shRNAs to Rnf2 were designed using the Broad Institute Genetic Perturbation Platform. These designs can be found in the "sense" orientation in Table 1 in section 2.2: Materials. The oligonucleotides were designed as followed to create approximately 60 base pair long oligonucleotides.

Forward oligonucleotide:

5' CCGG—21bp sense—CTCGAG—21bp antisense—TTTTTG 3'

Reverse oligonucleotide:

5' AATTCAAAAA—21bp sense—CTCGAG—21bp antisense 3'

Once designed, 20 µM working stocks of the oligonucleotides, and 5 µL of each oligonucleotide were annealed in 1X NEB buffer 2 (in a total volume of 50 uL) in an Applied Biosystems SimpliAmp Thermal Cycler. The conditions used to anneal the oligonucleotides were an initial incubation at 95°C for 4 minutes, a second incubation at 70°C for 10 minutes, then a colling and annealing phase where the temperature was decreased by 1°C per 2 minutes to 20° before holding the samples at 4°C.

To prepare the pLKO.1_puro plasmids for ligation, 6 µg of plasmid was digested with 20U Age1-HF in a 50 µL reaction in 1X NEB buffer 1 for 2 hours at 37°C. The buffer was exchanged using a QiAquick PCR cleanup kit, and the pLKO.1 vector was digested again, this time using 20U EcoRI in a 50 µL reaction in 1X NEB EcoRI buffer for 2 hours at 37°C. The samples were then heat killed at 65°C for 20 minutes to eliminate residual enzymatic activity, before purifying the desired band (an approximately 7 kb band) using a QiAquick gel

extraction kit. To ligate the annealed shRNAs into the digested pLKO.1 vector, 2 µLs of annealed oligonucleotides were incubated with 20 ng of digested vector and 4 uL's NEB T4 DNA ligase in 1X T4 DNA ligase buffer in a 20 µL final volume for 2 hours at room temperature.

To generate the neomycin-resistant vectors, we excised the neomycin resistance gene from 5 µg of pLKO.1_neo, and the puromycin resistance gene from 5 µg of pLKO.1_puro_shRnf2_1 and 2_3 using the restriction enzymes Bbs1 and BamH1 in NEB buffer 2.1 in a 20 µL volume. The neomycin resistance fragment, and the pLKO.1 vector with no resistance marker was purified using a QiAquick gel extraction kit. These purified fragments were then ligated, using a 5:1 ratio of the neomycin fragment to the pLKO.1 fragment in a 10 µL volume in NEB T4 DNA ligase buffer with 2 µL's T4 DNA ligase. The ligation was performed at room temperature for 2 hours.

2.1.4 Western blots and SDS-page

To estimate protein concentrations before loading, 5 µLs of each sample was added to 795 µL's H_2O, and 200 µL's Protein Assay Dye Reagent Concentrate (Bio-Rad) and allowed to incubate for 10 minutes, before reading the optical density in a biophotometer d30 (Eppendorf). These samples were then mixed into 1 X SDS-PAGE loading dye (50 mM Tris base pH 6.8, 2% SDS, 10% glycerol (v/v), 100 mM DTT, 0.05% bromophenol blue (w/v), and boiled at 95°C for 5 minutes to denature the proteins. The denatured proteins were briefly spun down to remove liquid from the cap, then loaded into a 10% SDS-PAGE gel. The gel was electrophoresed at 60V in the stacking portion of the gel, and 120V for the running portion of the gel in running buffer (25 mM Tris base pH 8.3, 0.192 mM glycine, 1% (w/v) SDS).

For the Western blots, the SDS-PAGE gel was moved into the sandwich and transferred onto a nitrocellulose membrane. The samples were transferred at 120V for 70 minutes in 1X transfer buffer (25 mM Tris base pH 8.3 and 0.192 mM glycine). After the transfer, the membrane was blocked in a blocking buffer (3% (w/v) BSA) for 1 hour at room temperature. After blocking, the blocking buffer was removed, and the antibodies were added to the sample (antibody dilutions used are listed in materials) and incubated overnight. The following morning the antibodies were removed from the membrane, and the membrane was washed twice for 5 minutes in 1X TBST, and twice more in 1X TBS. After the final wash, secondary antibodies were diluted 1:10 000 in 1X TBST (1:5 blocking buffer) and the membrane was incubated for 45 minutes with the secondary antibodies. Following this incubation, the membrane was washed twice in 1X TBST for 5 minutes, and twice more in 1X TBS. Images were then taken in a Chemidoc MP imaging system (Bio-Rad). The images were then processed in FIJI(203) to determine the relative expression of the proteins. The samples were normalized to a control gene by dividing the signal measured from the gene of interest divided by the signal measured from the control gene as calculated by the fluorescence intensity in FIJI.

2.1.5 Cell culture for C2C12 cells and Hek293T cells

C2C12 cells were grown in growth media (10% BGS (v/v) and 1 % penicillin/streptomycin (v/v) in DMEM). These cells were grown on tissue culture-treated plates with no additional coatings in an incubator at 37°C and 5% CO_2.

HEK293T cells were grown in growth media (10% BGS (v/v) and 1 % penicillin/streptomycin (v/v) in DMEM). These cells were grown on tissue culture-treated plates with no additional coatings in an incubator at 37°C and 5% CO_2.

2.1.6 Viral Transfections

Viral transfections were prepared using a PEI transfection protocol. The DNA mix for the transfection (10 µg pLKO.1 vector, 9 µg psPax2, and 1 µg pMD2.G made to 900 µL by adding Opti-MEM and balanced to 150 mM NaCl by adding 1.5M NaCl as needed) and PEI mix (319 µL's PEI (1 mg/mL in 150 mM NaCl at pH 7.2) and 581 µL's Opti-MEM) were shaken vigorously for 15 seconds, before incubating for 5 minutes. After this period the DNA mix and PEI mix were combined, shaken vigorously for 15 seconds, then incubated for 30 minutes.

HEK293T cells were grown in growth media to 70% confluence. After the final incubation, the media for the HEK293T cells was changed, and the prepared viruses were added slowly to the HEK293T cell media while gently swirling the media in the plate. After 15 hours, the viral-laden media was removed, and fresh media was added to the HEK293T cells. Viral particles were allowed to accumulate in the media for 72 hours before the media was collected and centrifuged at 3 000 x g for 10 minutes at 4°C. The supernatant was transferred to a new Falcon tube and mixed 1:4 with sucrose buffer (50 mM Tris-HCl pH 7.4, 100 mM NaCl, 0.5 mM EDTA, and 10% (w/v) sucrose). The tubes were balanced <0.05 g variance, then centrifuged in an Avanti J-26 XPI high-speed centrifuge at 10 000 x g for 4 hours at 4°C using the fast-start and slow-stop settings(204).

After centrifugation, the supernatant from the viral preparations was removed, and the pellets were resuspended softly in growth media overnight, to allow the pellet to resuspend overnight. The following morning, the prepared viral media was added slowly with gentle swirling to 50 000 C2C12 cells and incubated for 36 hours at 37°C at 5% CO_2. After 36 hours, the viral media was removed, and fresh growth media was added with selection markers (2

μg/mL puromycin or 400 μg/mL G418 matching the plasmids resistance mark) until the cells within a control plate (no infections thus no resistance) were all deceased. After selection, the cells were cultured in growth media, with 1/10 the selection concentration of the antibiotic to help ensure the maintenance of the inserted genes.

2.1.7 Cell counting for growth experiments

To count the cells during proliferation phases, the cells were grown in tissue culture treated plates (with 0.01% collagen for primary cultures), then harvest by using gentle trypsinization (0.05% trypsin) for 5 minutes incubated at 37°C at 5% CO_2. The cells were resuspended in growth media, then 50 μLs of the resuspended cells were transferred to fresh centrifuge tubes and subsequently mixed with 50 μLs of Trypan blue. Cells were then counted using a Countess™ hemocytometer in triplicate. The counts were then graphed in GraphPad Prism v6.0 with the results of a student's two-tailed t-test also performed in GraphPad Prism v6.0 overlaid upon it in Microsoft PowerPoint.

2.1.8 Differentiation protocol for primary myoblasts and C2C12 cells

To differentiate C2C12 cells, the cells were grown in C2C12 growth media to 70% confluence. The growth media was removed, and C2C12 differentiation media (2% horse serum, 0.1% insulin, 1% penicillin/streptomycin) was added. The cells were differentiated for 3 days, changing the media every 24 hours.

To differentiate primary myoblasts, the cells were grown in primary growth media to 70% confluence on collagen-coated 6-well plates. The growth media was removed, and primary differentiation media (0.5% BGS, 0.5 % BSA (w/v), 0.1% (v/v) insulin (10 mg/mL), and 1% penicillin/streptomycin in DMEM, and filter sterilized) was added. To these well either

10 µM Prt4165 was added, or an equivalent volume of DMSO. These cells were differentiated for 2 days, refreshing the media and drugs after 24 hours.

2.1.9 Immunofluorescence and calculating differentiation and fusion indexes

To begin immunofluorescence, the growth media was removed from the cells in a 6-well plate, and 1 mL of PBS was added to prevent the cells from drying out. To this, 1 mL of 2% formalin was added, to make a 1% formalin solution, and the cells were fixed at room temperature for 30 minutes. After fixation, the cells were washed with PBS 3 times, shaking gently for 5 minutes. The cells were then permeabilized in 1 mL of permeabilization buffer (2% Triton X-100 and 0.1M glycine in PBS) for 5 minutes. The cells were then blocked in 500 µLs blocking buffer (5% goat serum, 2% (w/v) BSA in PBS, with 1:30 M.O.M. blocking reagent added for primary cell samples) for 1 hour at room temperature. Blocking media was then removed, and 250 µLs of antibody buffer (1% goat serum, 0.4% (w/v) BSA in PBS with antibodies diluted in as noted in the materials section) was added to each well, and these were incubated overnight.

The following morning, the cells were washed three times for 5 minutes in PBS. Secondary antibodies were diluted 1:750 in antibody buffer (1% goat serum, 0.4% (w/v) BSA in PBS) and 750 µL were added to each well and incubated for 1 hour. After incubation with the secondary antibodies, each well was washed 3 times with PBS for 5 minutes, then 1 µg/mL DAPI was added, and incubated for 10 minutes. After incubating with 1X DAPI, the cells were washed 3 times in PBS, and images were taken on a Zeiss AxioScan Observer Z1. For each well, 5 images were taken, and the final counts of nuclei and target proteins were averaged from these counts. The nuclei outside and within myofibers were counted using the FIJI plugin

ViaFuse and its protocols(205) with the mask setting set to “Huang(206)” instead of “default”. Errors were calculated using standard error of the means (SEM).

To calculate the differentiation index, the total number of nuclei was counted, and the number of nuclei expressing Myosin heavy chain kinases was counted. The formula used to calculate the index was:

(Nuclei expressing Myosin heavy chain kinases / total nuclei) * 100 = differentiation index.

To calculate the fusion index, the total number of nuclei was counted, and the number of nuclei in myotubes with at least 3 nuclei were counted. The formula used to calculate the index was:

(Nuclei in myotubes with at least 3 nuclei / total nuclei) * 100 = fusion index.

2.1.10 RNA isolation and qPCRs

Cells for RNA isolation were grown in 3.5 cm tissue culture treated plates (with 0.01% collagen coating for primary cell cultures) and differentiated as above when noted. Before isolating the RNAs, the media was removed from the plates, and the plates were quickly transferred to a fume hood. RNA was isolated from the cells using RNA STAT-60 reagent to the manufacturer’s protocol (1 mL of RNA STAT-60 for 3.5 cm plates pipetted several times over the plate) and incubated for 5 minutes at room temperature. After the incubation, 200 µL of chloroform was added (200 µL’s / 1 mL RNA STAT-60), and the plates were shaken vigorously for 15 seconds before incubating the mixture for 3 minutes.

After this incubation, the lysate was transferred to a centrifuge tube, and centrifuged at 4°C for 15 minutes at 12 000 x g. The 400 µLs of the aqueous phase was transferred after the centrifugation to a fresh centrifugation tube, and 500 µLs of isopropanol was added. The mixture was incubated for 10 minutes at room temperature, then centrifuged at 4°C for 10

minutes at 12 000 x g. Following the centrifugation, the pellet was vortexed in 1 mL of 75% ethanol, then centrifuged once more at 4°C for 5 minutes at 7 500 x g. The RNA pellet was briefly allowed to dry (care was taken to ensure it did not completely dry out, as it would impede resuspension), then resuspended in 100 µLs nuclease from H_2O.

RNA concentrations were estimated using Thermo Scientific™ NanoDrop™ One Microvolume UV-Vis Spectrophotometer. For each sample, 2 µg of RNA was added to a fresh centrifuge tube, made to 20 µLs with nuclease-free H_2O, 2 µLs random primers (0.25 µg/µL), and 1 µL RNaseout. This mixture was incubated at 70°C for 10 minutes, then chilled on ice for 2 minutes, and briefly spun down to remove liquid from the cap. To this, 3.5 µls 10X M-MuLV Reverse Transcriptase Reaction Buffer, 4 µls 0.1 M DTT, 2 µls dNTP (10mM), 1 µl RNaseout, 1ul M-MuLV Reverse Transcriptase, 0.5 µls H_2O were added before incubating this mixture at 42°C for 50 minutes. After this incubation, the enzyme was heat killed at 70°C for 15 minutes and the samples were briefly spun down before storage at -20°C.

The qPCR experiments for all expression assays except for HoxA2 were performed using Taq-Man probes to manufacturers protocols in an Applied Biosystems™ QuantStudio™ 3 Real-Time PCR System, 96-well, 0.2 mL. Reads were normalized to GAPDH using the delta-delta CT method. The probes used are listed in the materials section, The statistical significance was calculated using a normal One-Way Anova statistical assay without multiple comparisons correction in GraphPad PRISM.

The qPCR experiment for HoxA2 was performed using PowerUp SYBR Green Master Mix to the manufacturer's protocols in a Corbett Research RG-6000, using the Rotor-Gene Q Series Software 1.7 (Build 94) to process the data and run. Reads were normalized to GAPDH using the delta-delta CT method. The Hox primers were designed using IDTDNA's

PrimerQuest™ Tool with default settings selected. The sequence of the forward primer was 5'-TCC CTG GAT GAA GGA GAA GAA-3', and the reverse primer was 5'-CCG CTG CCA TCA GCT ATT T-3'.The statistical significance was calculated using a normal One-Way ANOVA statistical assay without multiple comparisons correction in GraphPad PRISM.

2.1.10 Cell counting for growth experiments

To count the cells during proliferation phases, the cells were grown in tissue culture treated plates (with 0.01% collagen for primary cultures), then harvest by using gentle trypsinization (0.05% trypsin) for 5 minutes incubated at 37°C at 5% CO_2. The cells were resuspended in growth media, then 50 µLs of the re-suspended cells were transferred to a fresh centrifuge tube and subsequently mixed with 50 µLs of Trypan blue. Cells were then counted using a Countess™ hemocytometer in triplicate. The counts were then graphed in GraphPad Prism v6.0 with the results of a student's two-tailed t-test also performed in GraphPad Prism v6.0 overlaid upon it.

2.2 Materials

Table 1: shRNA sequences used (sense strand).

Target	**shRNA sequence**
shRing1-1	GTGGGAACTGAGTCTGTATGA
shRing1-2	CACTGACCTTGGAGCTTGTAA
shRnf2-1	CCATGACTACAAAGGAGTGTT
shRnf2-3	ATGACTACAAAGGAGTGTTTA

Table 2: Purchased plasmids list.

Plasmids	**Addgene number**
pLKO.1 puro	8453
pLKO.1 neo	14525
HOXA1 (murine) HIS-tag pET	8556
HOXA2 (murine) HIS-tag pET	8557

MISSION® pLKO.1-puro Luciferase shRNA Control Plasmid DNA	SHC007

Table 3: Antibodies used for Western blots and dilutions.

Antibody	**Dilution and conditions**
Rnf2 (PA5-96361)	1:1000 at 4°C Overnight
Ring1 (2820S)	1:1000 at 4°C Overnight
H2AK119ub1 (8240S)	1:2500 at 4°C Overnight
H4 (ab9051)	1:2500 at RT for 1 hour
TFIIH p89 (sc 377301)	1:1000 at 4°C Overnight
IRDye® 680RD Goat anti-Mouse IgG Secondary Antibody (LIC-926-68070)	1:10000 at room temperature for 45 minutes
IRDye® 680RD Goat anti-Rabbit IgG Secondary Antibody (LIC-926-68073)	1:10000 at room temperature for 45 minutes
IRDye® 800RD Goat anti-Mouse IgG Secondary Antibody (LIC-926-32210)	1:10000 at room temperature for 45 minutes
IRDye® 800RD Goat anti-Rabbit IgG Secondary Antibody (LIC-926-32213)	1:10000 at room temperature for 45 minutes

Table 4: Antibodies used for immunofluorescence and dilutions.

Antibody	**Dilution and conditions**
MF-20	1:2 at 4°C Overnight
Ki-67 (ab15580)	1:200 at 4°C Overnight
Goat anti-Rabbit IgG Alexa Fluor 647 (A-21244)	1:750 at 4°C at room temperature for 1 hour
Goat anti-Mouse IgG (H+L) Secondary Antibody, Alexa Fluor® 488 conjugate (A11001)	1:750 at 4°C at room temperature for 1 hour

Table 5: Reagents used.

Reagent name	**Providing Company**	**Catalogue Number**
2-Propanol	Fisher Scientific	A4164-4
4′,6-Diamidino-2-phenylindole dihydrochloride (DAPI)	Sigma	D9542
Acetic acid	Fisher Scientific	A38-212
Acetic acid, glacial	Fisher Scientific	A38s-212
Agarose	Fisher Bioreagents	BP160-500
Ammonium persulfate	Sigma-Aldrich	A3678

bFGF	Millipore/Cedarlane	GF003AF
Bovine serum albumin	Sigma-Aldrich	A9647-100G
Bromophenol Blue	Sigma-Aldrich	B8026
Chloroform	Fisher Scientific	C298
Collagenase from Clostridium histolyticum	Sigma	C0130-1G
Collagen solution type I, from rat tail	Corning/Fisher	C354236/CB40236
Complete EDTA-free Protease inhibitor	Roche/Sigma	11873580001
Disodium phosphate	Sigma-Aldrich	S0876
Dithiothreitol	Sigma-Aldrich	D0632
Dynabeads Protein A	Life Technologies	10001D
Ethanol	Chaptec	0G95-6151
Ethylenediamine Tetraacetic Acid, Disodium Salt Dihydrate	Fisher Chemical	S311-100
Formaldehyde, 37%	Fisher Scientific	BP531
Formalin	Fisher Scientific Company	23-245-684
G418 Sulfate	Wisent Biocenter	400-130-IG
Glycerol	Fisher Chemical	G33-1
Glycine	Fisher Scientific	G48
Goat serum	Thermo Scientific/Invitrogen	31872
HAM's F-10 media	Wisent Biocenter	318-050-CL
HEPES	Fisher Scientific	BP310
Horse serum	Sigma-Aldrich	H1138
HyClone DMEM/high glucose	Fisher Scientific	SH3024301
Hydrochloric acid	Fisher Scientific	351283
Hygromycin B	Wisent Biocenter	450-141-XL
Insulin solution from bovine pancreas (10 mg/mL)	Sigma-Aldrich	I0516
Invitrogen™ Nuclease-Free Water (not DEPC-Treated)	Fisher Scientific	AM9938
Opti-MEM reduced serum medium,500ml	Life Technologies	31985-070

Ovalbumin	Sigma-Aldrich	O1641
Magnesium Chloride	Sigma	M8266
Milk powder, skim	Loblaws (no name)	20119655_EA
M-MuLV Reverse Transcriptase	NEB	M0253S
M-MuLV Reverse Transcriptase Reaction Buffer	NEB	B0253S
Mouse on Mouse (M.O.M.™) Blocking Reagent	Vector Laboratories/Cedarlane	VECTMKB22131
NP-40	Sigma-Aldrich	74385
Penicillin Streptomycin 100X	Wisent Biocenter	450-201-EL
Phenylmethanesulfonyl fluoride	Sigma Aldrich	P7626
Polyethylenimine (PEI) Linear, MW 25,000	Polysciences,Inc, /Cedarlane	23966-1
Potassium chloride	Sigma-Aldrich	P9541
Potassium phosphate, dibasic	Sigma-Aldrich	P3786
Potassium Phosphate, monobasic	Sigma-Aldrich	P9791
PowerUp SYBR Green Master Mix	Thermo Fisher/Life Technologies	A25777
Protein Assay Dye Reagent Concentrate	Bio-Rad	5000006
Puromycin	Wisent Biocenter	400-160-EM
Random Primer,100ul ,3ug/uL	Life Technologies	48190011
Ring1 Taqman Primers	Thermo Fisher	Mm01278940_m1
RNaseOut Recombinant Ribonuclease Inhibitor	Life Technologies	10777-019
RNA STAT-60	TEL-TEST, INC	CS-111
Rnf2 Taqman Primers	Thermo Fisher	Mm00803321_m1
Silver nitrate	Sigma-Aldrich	S6506
Sodium carbonate	Sigma-Aldrich	S7765
Sodium chloride	Sigma-Aldrich	S9888
Sodium deoxycholate	Fisher Scientific	S285
Sodium dodecyl sulphate	Sigma-Aldrich	L4390/L3771

Sodium hydroxide	Fisher Chemical	S318500
Sodium orthovanadate	Sigma	S6508
Sodium pyrophosphate tetrabasic	Sigma-Aldrich	P8010
Sodium thiosulfate	Sigma-Aldrich	217263
Sucrose, 99%	Thermo Scientific	A15583.36
TaqMan™ Fast Advanced Master Mix	Applied Biosystems/Life Technology	4444963
TC bovine growth serum	Fisher Scientific	SH3054103
TEMED	Sigma-Aldrich	T7024
Tris base	Fisher Bioreagents	BP152
Tris hydrochloride	Fisher Bioreagents	BP153
Triton X-100	Sigma-Aldrich	T8787
Trypan Blue solution	Sigma-Aldrich	T8154
Trypsin .05% 1X Liquid	Fisher Scientific	SH3023601
Tween-20	Sigma-Aldrich	P7949

Chapter 3: Role of the E3 ubiquitin ligase of the Prc1 complex in myogenesis

3.1: Depletion of Ring1 and Rnf2 in C2C12 myoblasts

To study the Prc1 complex, we first sought to establish an effective system for studying the biology of the Prc1 complex in a cell culture model. For studying biology in muscle systems, C2C12 cells, an immortalized myoblasts cell line, offer a robust system to study the biology of myogenesis between proliferation and differentiation(207). To begin our experiments probing the role of the Prc1 complex within myogenesis, we first transduced the C2C12 cells with either Ring1 or Rnf2 shRNA lentiviral particles, before adding puromycin to the media to select for the cells that had taken up the shRNAs. After selecting cells that had stably integrated the plasmid, we then measured the mRNA levels of either Ring1 (Fig. 2A)

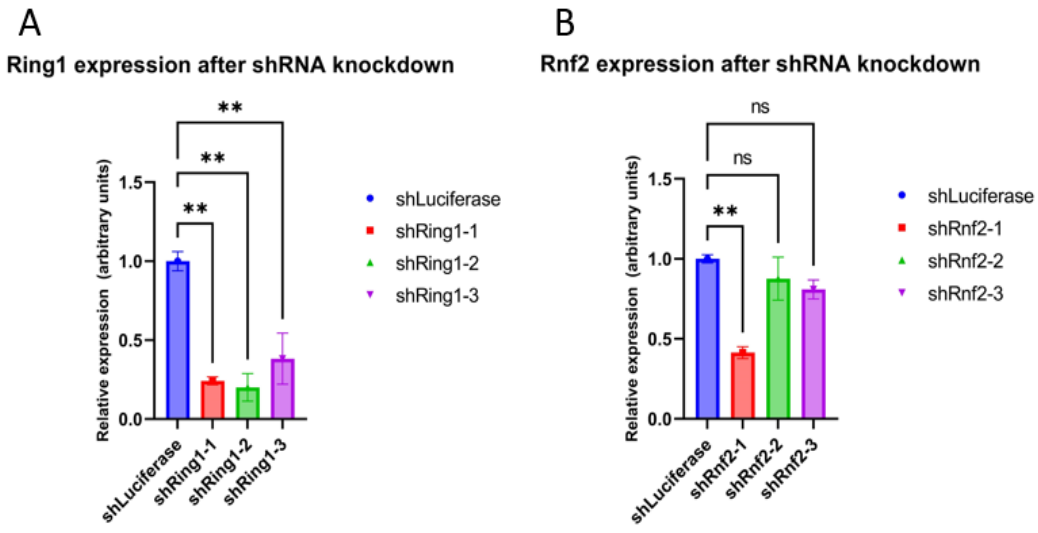

Figure 2: Depletion of Ring1 and Rnf2 in C2C12 cell lines.

A and B) The mRNAs for the qPCR expression assays were extracted using stat-60 and then probed with TaqMan probes Mm01278940_m1 for Ring1 and Mm00803321_m1 for Rnf2 with 3 replicates of each, normalized to GAPDH (Mm99999915_g1). The statistical significance was calculated using a normal One-Way Anova statistical assay without multiple comparisons correction in GraphPad PRISM.

or Rnf2 (Fig. 2B). These experiments showed an excellent depletion of Ring1 in all samples tested. The Rnf2 samples were not depleted at the mRNA level as efficiently, however, with only one sample meeting statistical significance.

The next step in constructing the tools to study the role of the Prc1 complex in myogenesis was to generate a compound knockdown that depleted both ubiquitin ligases that can contribute to the complex. To do this we re-transduced the shRnf2 C2C12 cells with shRing1 lentiviral particles. We then confirmed at the RNA level that Ring1 had been depleted in these cells (Fig. 3 A-D, each figure representing one C2C12 construct) and that the Rnf2 depletion had been maintained (Fig. 3 A-D, each figure representing one C2C12 construct) (in fact the Rnf2 mRNAs had even been further depleted). In the Ring1 and Rnf2 depleted cells, Ring1 was not depleted as significantly as was the case in the shRing1 cells. This is likely because there was not a second selection marker that could be used to select for Ring1 in all cells.

We also examined the protein levels using Western blots to fully confirm that we had depleted the levels of Rnf2. From this we found that Rnf2 was depleted in our shRNA containing cell lines (Fig. 3E). Unfortunately, Ring1 was unable to be tested this way as the different antibodies tested did not show sufficient specificity to generate reliable results. Our cells lines saw an Rnf2 depletion of 22% for shRnf2-3, 29% for shRing1-1_Rnf2-3, 53% for shRing1-2_Rnf2-1, and 32% for shRing1-2_Rnf2-3.

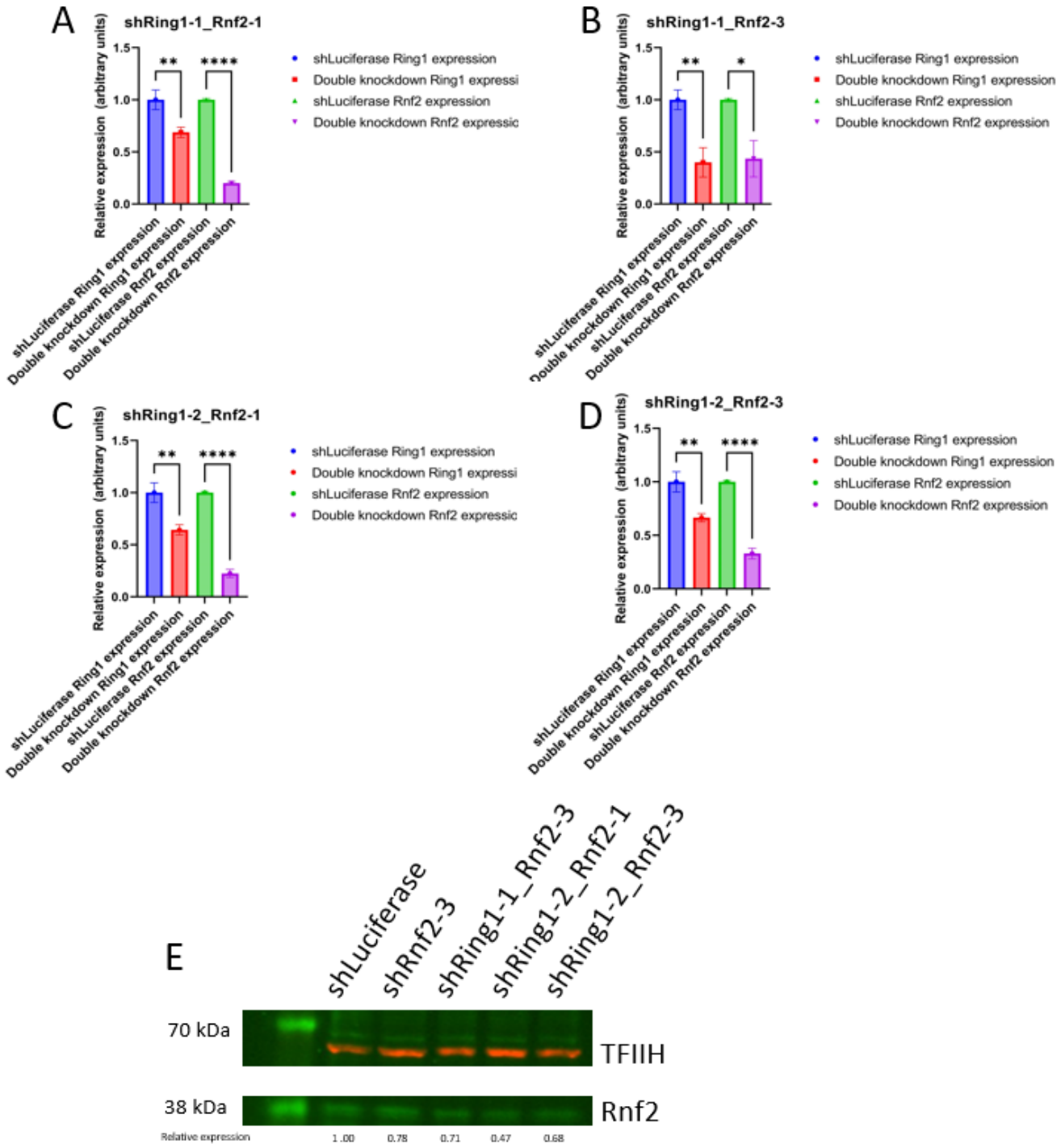

Figure 3: Depletion of Ring1 and Rnf2 at the mRNA and protein level in Ring1 and Rnf2 depleted C2C12 cell.

A-D) The mRNAs for the qPCR expression assays were extracted using stat-60 and then probed with TaqMan probes Mm01278940_m1 for Ring1 and Mm00803321_m1 for Rnf2 with 3 replicates of each, normalized to GAPDH (Mm99999915_g1). The statistical significance was calculated using a normal One-Way Anova statistical assay without multiple comparisons correction in GraphPad PRISM. **E)** Western blots were performed on proteins isolated from C2C12 cells using a nuclear extraction. These extracts were then stained for Rnf2 (PA5-96361) at a dilution of 1:1000, then normalized to TFIIH p89 (sc 377301) at a dilution of 1:2000.

We later put a neomycin resistance marker into the shRnf2 plasmid and re-transduced Ring1 cells (Fig. 4), however, this did not generate significantly different rates of depletion compared to the previous experiments shown in Figures 2 and 3. Still, these depletions were effective and these two C2C12 cell lines were used in section 3.2's experiments on ubiquitination levels, and section 3.3's growth assays. As the depletion level is in line with previous results, they are still readily comparable to the results from the other stable C2C12 lines with the E3 ubiquitin ligase of the Prc1 complex depleted.

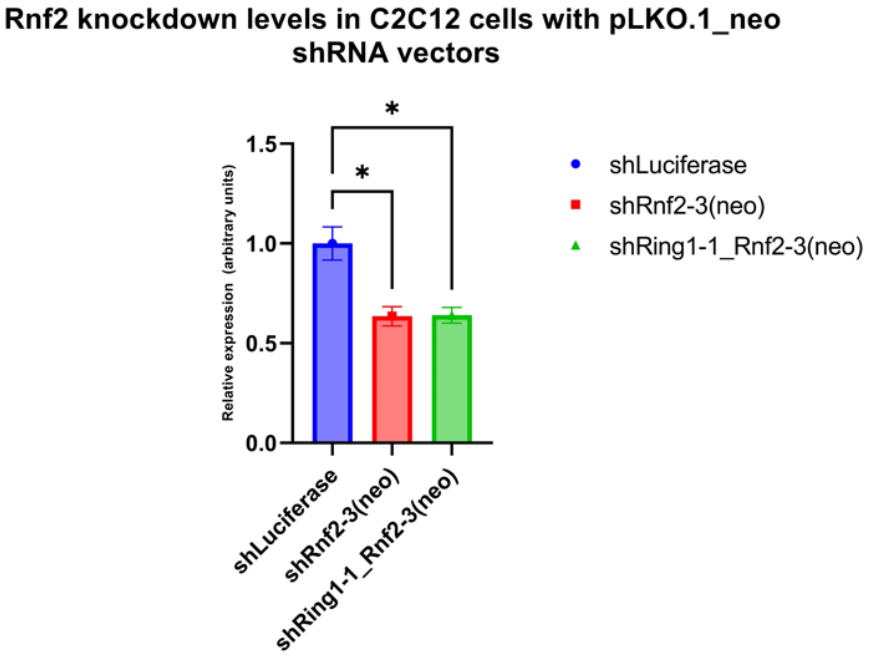

Figure 4: Depletion of Rnf2 in C2C12 cells

A) The mRNAs for the qPCR expression assays were extracted using stat-60 and then probed with TaqMan probes Mm00803321_m1 for Rnf2 with 3 replicates of each, normalized to GAPDH (Mm99999915_g1). The statistical significance was calculated using a normal One-Way ANOVA statistical assay without multiple comparisons correction in GraphPad PRISM.

3.2: Validating the functional effects of depleting the E3 ubiquitin ligase of the Prc1 complex in C2C12 cells

Before we proceeded into studying the role of the Prc1 complex in our C2C12 cells using this system, we wanted to verify that these shRNAs elicited an effect on the known roles of the Prc1 complex. The first validation we sought to perform was to assess the level of H2AK119ub1 after depleting the E3 ubiquitin of the Prc1 complexes. To do this we performed Western blots on shRing1 (Fig. 5A), shRnf2-3 (Fig. 5B), and shRing1-1_Rnf2-3 (Fig. 5C) C2C12 cells. These assays showed a 36% decrease in H2AK119ub1 levels for shRing1-1, 58% decrease for shRing1-2, 41% decrease for shRnf2-3, and a 66% decrease for shRing1-2_Rnf2-3 C2C12 cell lines.

The second validation we performed was to assess the expression level of HoxA2, a Hox gene which is not expressed in skeletal muscle tissues but is expressed in cardiac muscle and some brain tissues(208, 209), and the Hox genes are well-known targets of the Prc1 complex making them an excellent target to validate the functionality of the shRNAs(210). What we saw in these experiments was that depleting only one of Ring1 or Rnf2 did not affect the expression of HoxA2 in a significant manner, however depleting both caused HoxA2 to be re-expressed (Fig. 6). This could indicate that both Ring1 and Rnf2 can be targeted to HoxA2 interchangeably in C2C12 cells, which would explain why depleting only a single of these proteins elicited no effect on the expression of HoxA2.

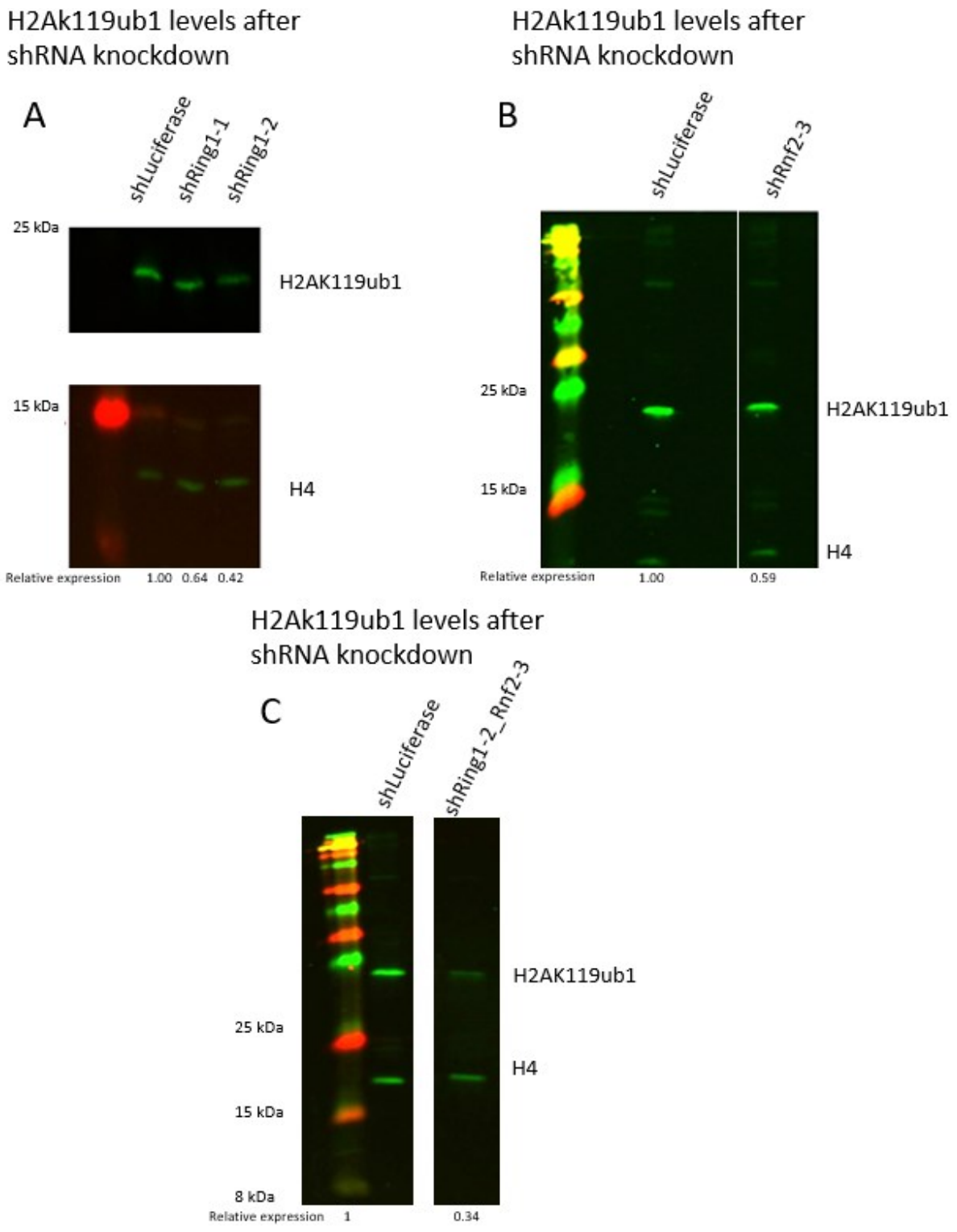

Figure 5: Reduced levels of the E3 ubiquitin ligase of the Prc1 complex decrease the levels of H2AK119ub1.

A, B, and C) Western blots were performed on histone proteins isolated from C2C12 cells using a histone acid extraction. These extracts were then stained for H2AK119ub1 (8240S) at a dilution of 1:2500, then normalized to H4 (ab9051) at a dilution of 1:2500.

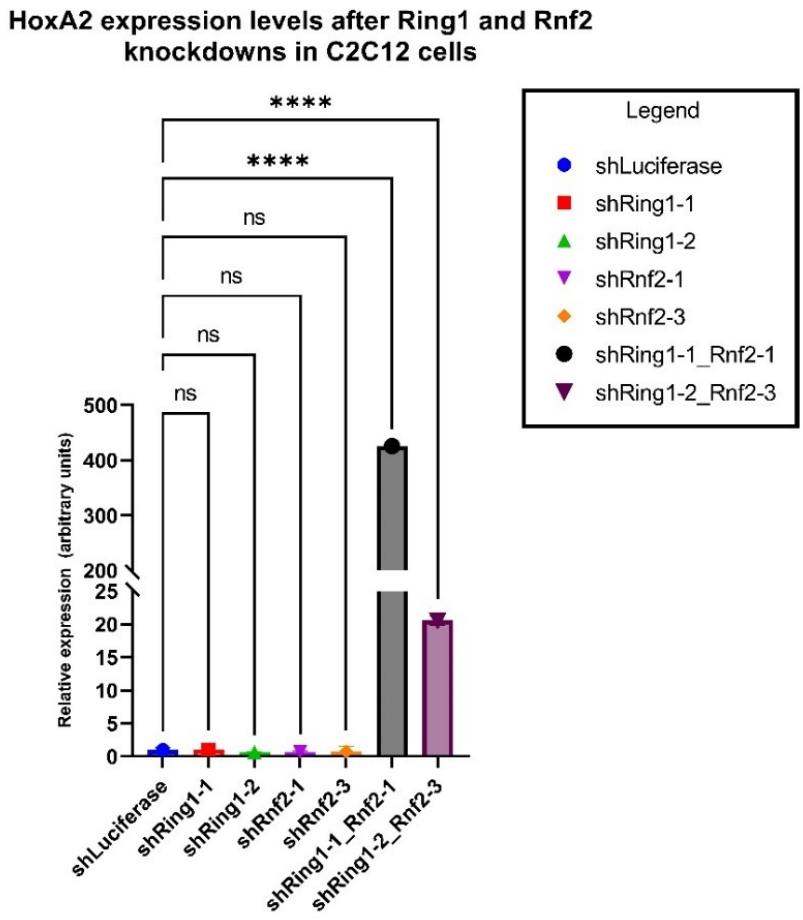

Figure 6: Single depletions of Ring1 and Rnf2 do not affect the transcription of HoxA2, however depleting both proteins does results in HoxA2's re-expression.

A) The mRNAs for the qPCR expression assays were extracted from proliferating C2C12 cells containing Ring1 and Rnf2 shRNAs using stat-60 and then probed with the HoxA2 primers (Methods section 2.10) and using the SYBR green chemistry to assess the fluorescence. Each experiment was performed in triplicate, normalized to GAPDH. The statistical significance was calculated using a normal One-Way Anova statistical assay without multiple comparisons correction in GraphPad PRISM.

3.3: Depletion of the E3 ubiquitin ligases of the Prc1 complex diminishes the growth rate of C2C12 cells

To study Prc1 function in myogenesis we first performed an analysis of their growth rates. When comparing the growth rates of shRing1-1, shRing1-2, shRing1-2_Rnf2-3, and shRnf2-3 cell lines to the growth rate of the shLuciferase cell line, there was a stark difference in their growth rate (Fig. 7). The expansion of the shLuciferase cell line after 5 days of

proliferation was significantly higher than all of the shRNA cell lines and it is reasonable to conclude that the Prc1 complex positively regulates the growth rate of C2C12 cells.

During proliferation, our cell lines had varying levels of the E3 ubiquitin ligases of the Prc1 complex (Fig. 2 and 4) allowing us to make some inferences into the levels of these proteins required to regulate myogenesis. We had two cell lines with an Rnf2 depletion at roughly the same level (Fig. 4), one was depleted on its own, and one with Ring1 depleted as well. The Rnf2-depleted cell lines' proliferation rates were nearly identical (Fig 7A), and it is clear that the decrease in the amount of the E3 ubiquitin ligases of the Prc1 complex in the already Rnf2-depleted cell lines did not create a more pronounced defect in its growth rate compared to the cell lines with Rnf2 depleted on its own. The shRing1-2 cell line grew marginally faster than the two Rnf2 cell lines, but it is only a slight difference, and not statistically significant (P=0.2764). Interestingly between shRing1-1 and shRing1-2, there is a statically significant difference in their growth rates. Unfortunately, we could not assess the protein levels of these shRNAs, as that may be able to explain the discrepancy, as their mRNA depletions were nearly identical (Fig. 2A). As we saw in the Rnf2 depleted cells (Fig. 2 B and 3E) the efficiency of the shRNA's to deplete the proteins can vary, which could explain why the different Ring1 depleted cell lines with similar RNA depletions produced different results. Because of this, we are unable to make any inferences on the dosage effect of the two Ring1-depleted cell lines. What we did see though was that further decreasing the levels of the E3 ubiquitin ligases after depleting the Rnf2 levels did not elicit a further effect (Fig 7A).

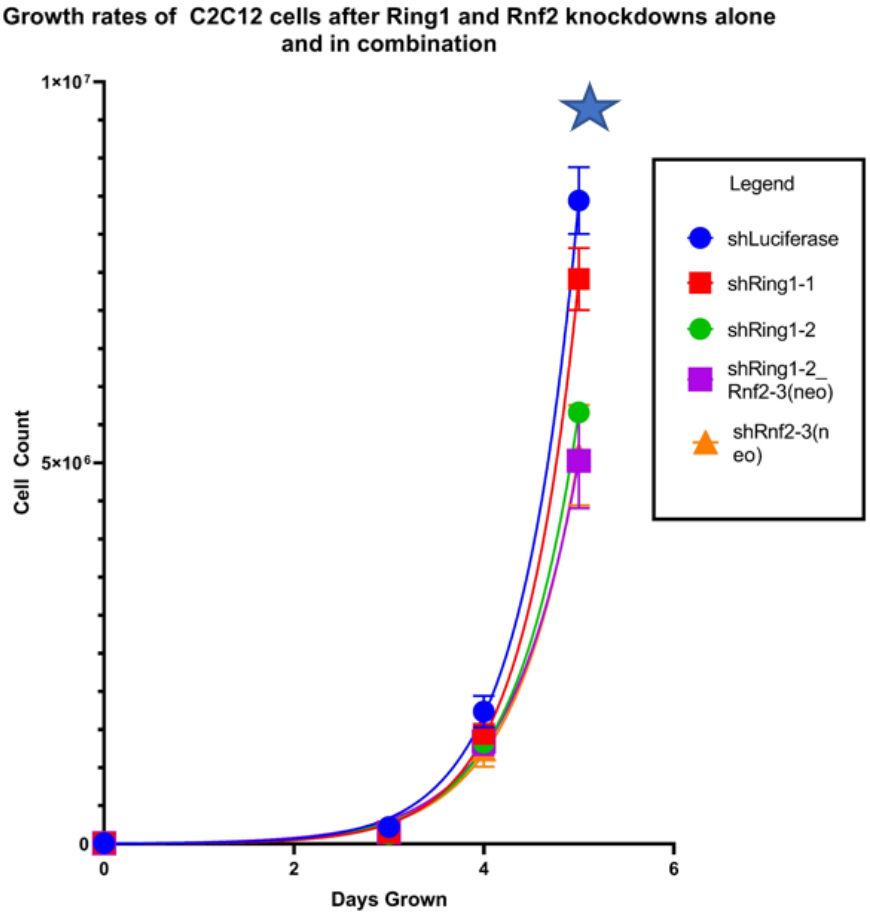

Figure 7: C2C12 growth rates are reduced when the E3 ubiquitin ligase of the Prc1 complex is depleted.

A) Ring1, Rnf2, and Ring1/Rnf2 depleted C2C12 cells were grown in growth media, before seeding 10,000 cells into a 10cm plate. These cells were allowed to grow for three days, before trypsinizing the plates at either 3,4, or 5 days. Cell counts were assessed using a hemocytometer and dead cells were excluded using trypan blue. Each data point in the average count of 3 independent plates for each sample (N=3). Statistical significance was calculated using independent Welch's t-tests with each shRNA cell line compared to the shLuciferase cell line.

Bmi1 has previously been shown to inhibit the ability of the cells to proliferate properly(153). Given that it can form complexes to elicit this effect with both Ring1 and Rnf2 and that depleting Rnf2 on its own decreased the growth rate of the cells by nearly 50%, it would appear the dosage of Prc1 complexes to properly regulate proliferation is highly sensitive to abnormal alterations. In the context of myogenesis, this makes sense for it to be as sensitive as it is, as the proliferative state needs to be readily reversible to ensure the cells can

either return to quiescence or properly differentiate into the myotubes. So based on our results, both Ring1 and Rnf2 are necessary for the regulation of proliferation. However, we cannot tell whether they are completely interchangeable at their targets, or if they are regulating distinct sets of proliferation-associated genes.

3.4: Depletion of the E3 ubiquitin ligase of the Prc1 complex affects the differentiation capacity of C2C12 cells

The next step in determining the function of the E3 ubiquitin ligases of the Prc1 complex in myogenesis was to assess its ability to regulate the differentiation and fusion of C2C12 cells. To do this we differentiated Ring1, Rnf2, and Ring1/Rnf2 depleted C2C12 cells in differentiation media for 3 days, changing the media each day. First, the ability of C2C12's with Rnf2 depleted within them was assessed for their ability to properly undergo differentiation and fusion (Fig. 8A). These results showed a statistically significant decrease in both the differentiation index (Fig. 8B) and the fusion index (Fig. 8C).

We next assessed the differentiative and fusion capabilities in our Ring1-depleted cell lines (Fig 9A). These results showed there was a mixed change in the differentiation index with the Ring1 shRNA where only shRing1-2 produced statistically significant results (Fig 9B), but both shRNAs produced a statistically significant decrease in the fusion index of these C2C12 cells (Fig. 9C).

The last assay we performed was to differentiate cells with Ring1 and Rnf2 co-depleted within them (Fig. 10). These cells showed a decrease in the differentiation index (Fig. 10B), and a profound decrease in their fusion indexes, particularly for the shRing1-2 containing C2C12 cells (Fig. 10C).

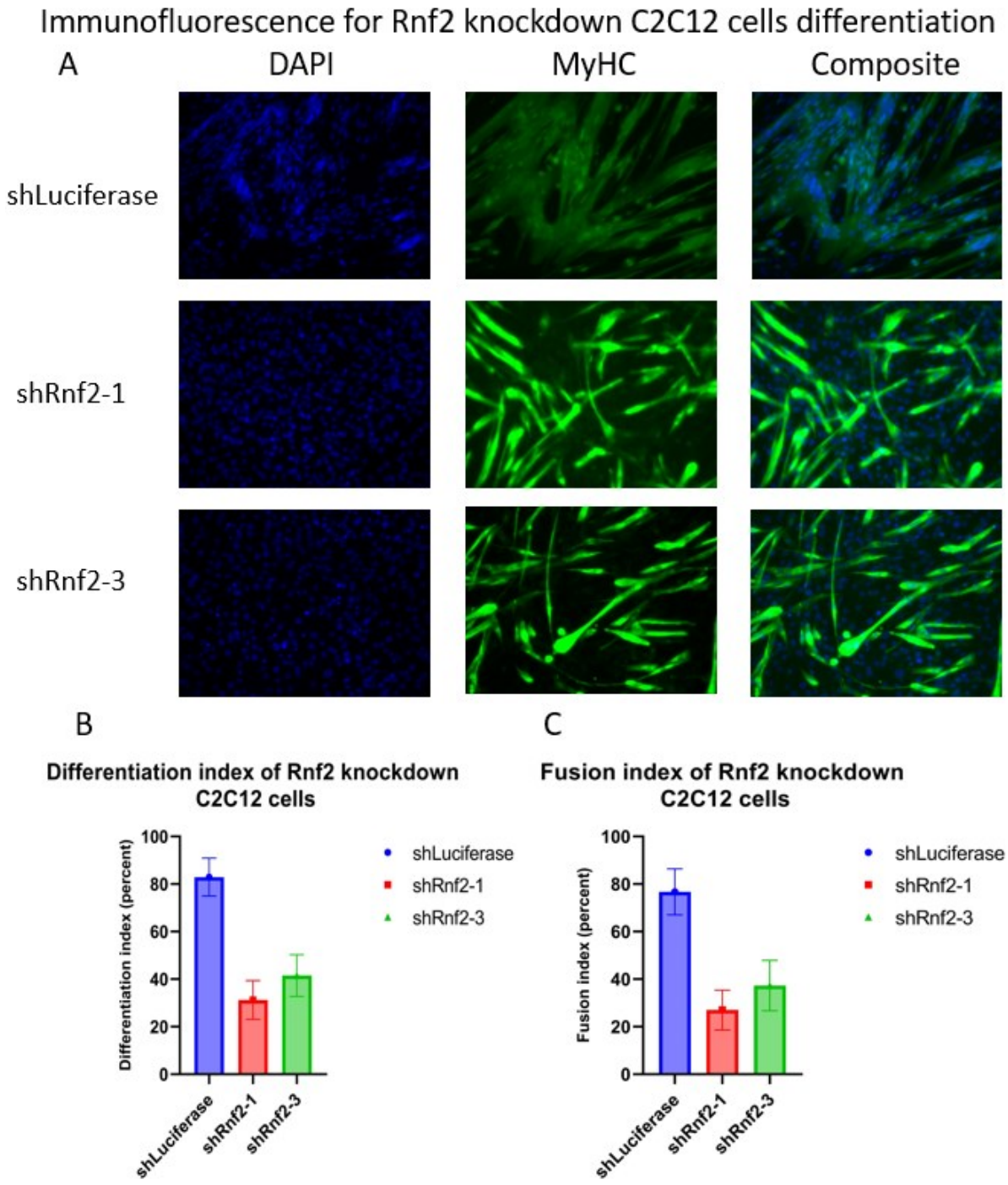

Figure 8: Depletion of Rnf2 decreases C2C12 cells differentiation and fusion ability.

A) Rnf2-depleted C2C12 cells were differentiated for 3 days (N=2). Cells were fixed in 1% formalin for 30 minutes, then permeabilized in 2% Triton X-100. Nuclei were detected using 1X DAPI (D9542) and pan-MyHC proteins were detected using a 1:2 dilution of an MF20 hybridoma created in our lab. The images were taken on a Zeiss Axiovision Observer Z1 microscope. **B)** Differentiation index was calculated as the number of MyHC+ nuclei over the total number of nuclei. **C)** The fusion index was calculated as the number of nuclei in myofibers with more than 3 nuclei over the total number of nuclei. The calculated values were derived from the average of 5 images from each sample. As N=2, statistical analyses were not possible on these samples.

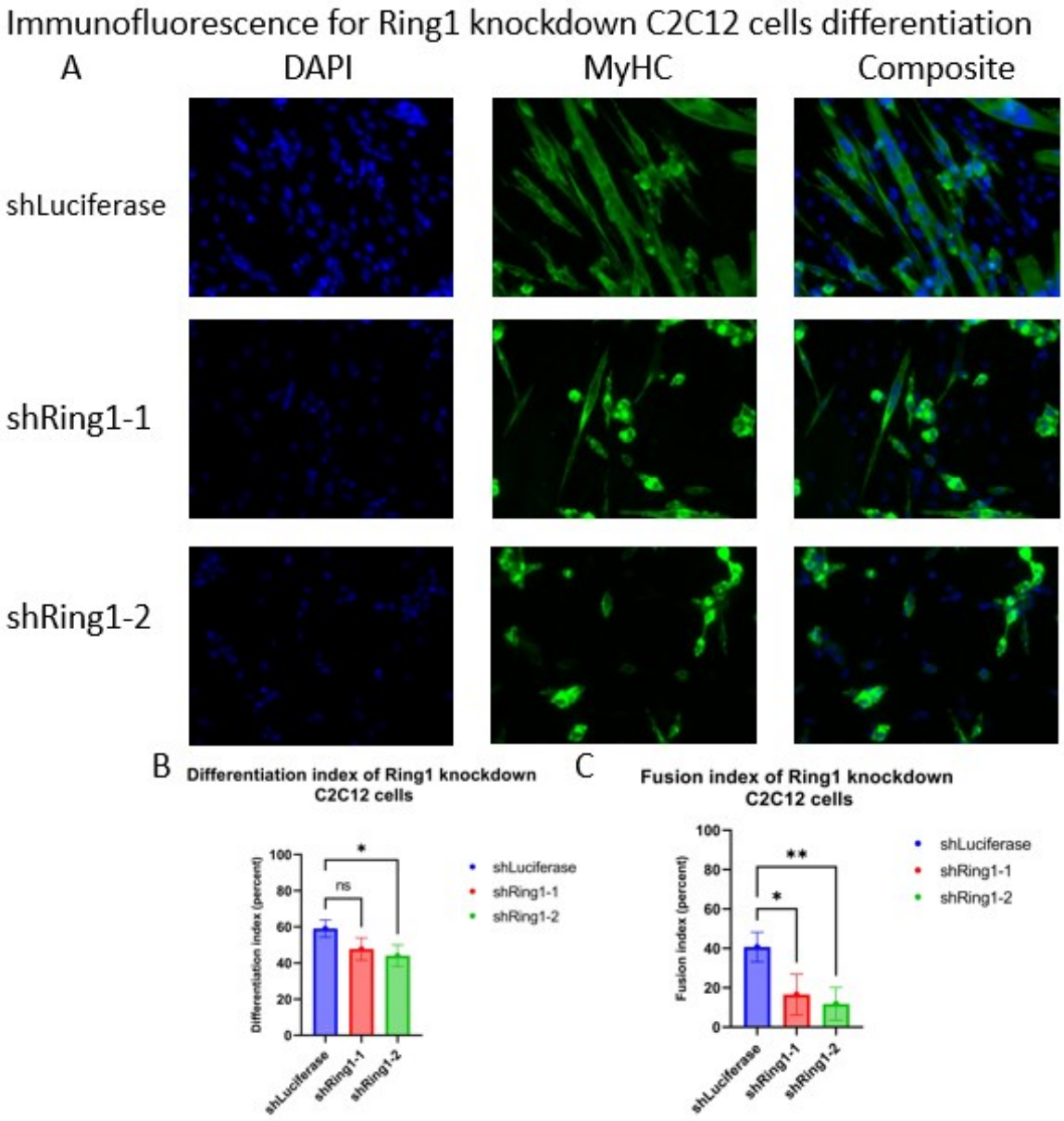

Figure 9: Depletion of Ring1 decreases C2C12 cells differentiation and fusion ability. **A)** Ring1 depletion C2C12 cells were differentiated for 3 days (N=3). Cells were fixed in 1% formalin for 30 minutes, then permeabilized in 2% Triton X-100. Nuclei were detected using 1X DAPI (D9542) and pan-MyHC proteins were detected using a 1:2 dilution of an MF20 hybridoma created in our lab. The images were taken on a Zeiss Axiovision Observer Z1 microscope. **B)** Differentiation index was calculated as the number of MyHC+ nuclei over the total number of nuclei. **C)** The fusion index was calculated as the number of nuclei in myofibers with more than 3 nuclei over the total number of nuclei. The calculated values were derived from the average of 5 images from each sample. The statistical significance was calculated using a normal One-Way Anova statistical assay without multiple comparisons correction in GraphPad PRISM.

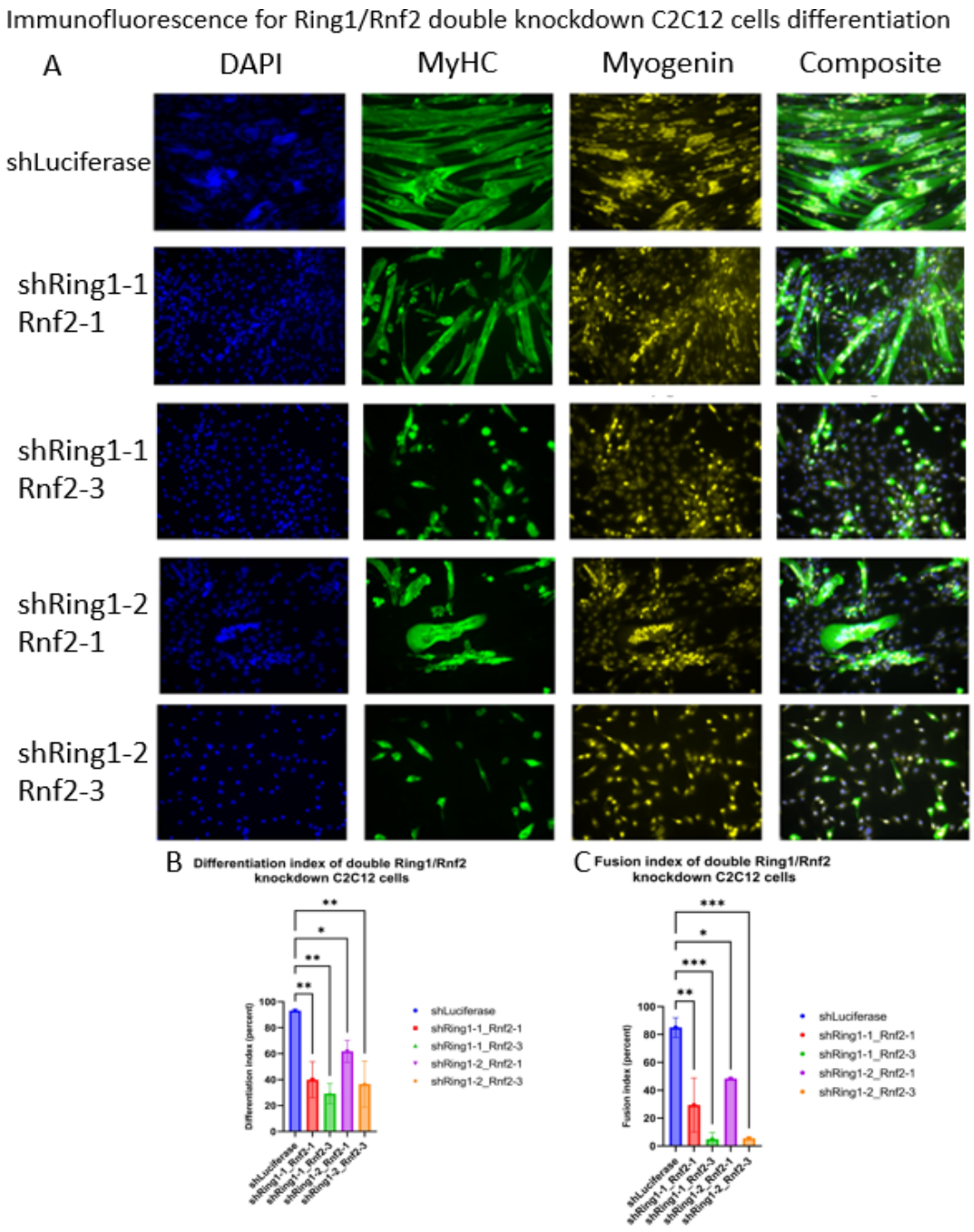

Figure 10: Ring1/Rnf2 co-depletion has a severe effect on C2C12 cell differentiation and fusion ability.

A) Ring1/Rnf2 depleted C2C12 cells were differentiated for 3 days (N=2). Cells were fixed in 1% formalin for 30 minutes, then permeabilized in 2% Triton X-100. Nuclei were detected using 1X DAPI (D9542) and pan-MyHC proteins were detected using a 1:2 dilution of an MF20 hybridoma created in our lab. The images were taken on a Zeiss Axiovision Observer Z1 microscope. **B)** Differentiation index was calculated as the number of MyHC+ nuclei over the total number of nuclei. **C)** The fusion index was calculated as the number of nuclei in myofibers with more than 3 nuclei over the total number of nuclei. The calculated values were derived from the average of 5 images from each sample. The statistical significance was calculated using a normal One-Way Anova statistical assay without multiple comparisons correction in GraphPad PRISM.

During differentiation, we showed that the shRNA cell lines almost all had decreased differentiation indexes. The exception to this was shRing1-1 containing C2C12 cells, which did not have a statistically significant decrease in its differentiation index (Fig 8-10). Comparing the results from Figures 8B and 9B, the change in the differentiation index (looking only at shRing1-2 in those samples as it was statistically significant) in the Rnf2 depleted C2C12 cells was much greater than in the Ring1 depleted cells. So, while both can regulate the entrance into differentiation, it would appear from these results that Rnf2 containing Prc1 complexes have a much greater role in this activity. In agreement with this, in the double Ring1 and Rnf2 depleted cell lines when comparing the differentiation index between the Rnf2 depletion in Figure 8B and the Ring1 and Rnf2 depletion in Figure 10B, there would not appear to be significant changes, and the extra reduction in the E3 ubiquitin ligases of the Prc1 complexes does not appear to further deregulate differentiation. However, the results are again limited by the fact that the relative decrease of Ring1 at the protein level cannot be calculated. However, with this limitation in mind, our data shows that the Rnf2-containing Prc1 complexes are more instructive in regulating the transition into differentiation, and the Ring1-containing complexes are more dispensable during the entrance into differentiation.

During fusion, all our C2C12 lines saw a significant decrease in their fusion scores. For Rnf2 when comparing its fusion index to its differentiation index, the two values are remarkably similar (Figure 8A and B). From this, it would appear that the decrease in the fusion index in the Rnf2-depleted cell lines may be primarily driven by the poor differentiation in these cells. In contrast for the Ring1 depleted cell lines, differentiation was only mildly affected at best, but its fusion index was significantly decreased in both cell lines. In the Ring1 and Rnf2 C2C12 cells, when adding the depleting Ring1 in the cell which already had had

Rnf2 depleted, there was a massive decrease in the fusion index relative to the control C2C12 cells, above what was seen in any of the single E3 ubiquitin ligase depleted cells. This leads to several possible interpretations of this.

The first interpretation is that Ring1 can compensate for some (if not most) of the Rnf2 targets during fusion, which would explain why Rnf2 saw a minimal change in the fusion vs. differentiation indexes. The Ring1-depleted cell lines had a significant change in the fusion vs. differentiation index, but it was not as drastic as seen in the double Ring1 and Rnf2-depleted cell lines (Fig 9 and 10 B/C).

Potentially this could indicate is that Rnf2 has some, but incomplete compensation for Ring1 during fusion. This would explain the further decrease in the fusion index in the Ring1 and Rnf2 depleted, as neither Ring1 nor Rnf2 were present at levels at which they could compensate for the other. A related hypothesis is that both proteins independently regulate some of the genes required for fusion. In the Ring1 and Rnf2 depleted cell lines the decrease in fusion relative to the single E3 ubiquitin ligase depleted cell lines would then be reflective of the Ring1 and Rnf2 targets being simultaneously dysregulated further disrupting productive myotube formation.

The second interpretation is that Rnf2 does not contribute to fusion directly at all. In this case, the further decrease in the fusion index of the Ring1 and Rnf2 depleted cell lines would be attributable to the decrease in the differentiation index of these cells. With reduced cells differentiating, and the (hypothetical) more direct effect on the ability of the cells to fuse through the Ring1 depletion, it would be logical that there would be massive defects in the fusion of the myocytes to the myotubes. In tentative support of this hypothesis, RNA-seq data from our lab in primary myocytes and myoblasts has shown that the ratio of Ring1 to Rnf2

increases during differentiation. If Ring1 is promoting fusion either directly or indirectly, the functional consequence of the changing ratio would make sense. This would need to be assessed more rigorously, however. Using Western blots to confirm the change in the levels of these two proteins over the course of differentiation, as the proteins are what would be driving the functional effects of the genes. While we could not directly confirm these theories, based on the data we have derived, I would expect that this second theory is the more likely case.

3.5 Summary of the results for Chapter 3

In this chapter, we have analyzed how the regulation of myogenesis is affected by the depletion of Ring1 and or Rnf2 levels. The first goal of our study was to determine what the function of the Prc1 complexes are during myogenesis when Ring1 and Rnf2 are removed from our cells (and by extension, also testing the effect of Rybp/Yaf2 and Cbx proteins binding to Prc1 complexes being disrupted, as they are incorporated into the Prc1 complexes by binding to the C-terminal domains of Ring1 or Rnf2(199, 211)). It is important to note here that it is unclear whether Ring1/Rnf2 depletion would disrupt the formation of the remaining components of the complex. In ESCs when Ring1 and Rnf2 were depleted, the results from the study suggest that PCGF1 and PCGF2 complexes were incapable of forming, but PCGF3 and PCGF6 complexes retained some of their functionality and binding to accessory proteins(173).

For our studies in the role of Ring1 and Rnf2 on the regulation of myogenesis, we assessed the ability of C2C12 cells to proliferate, differentiate, ubiquitinylate H2AK119ub1, and repress HoxA2 (a known target of the Prc1 complex(208–210)). With data compiled from

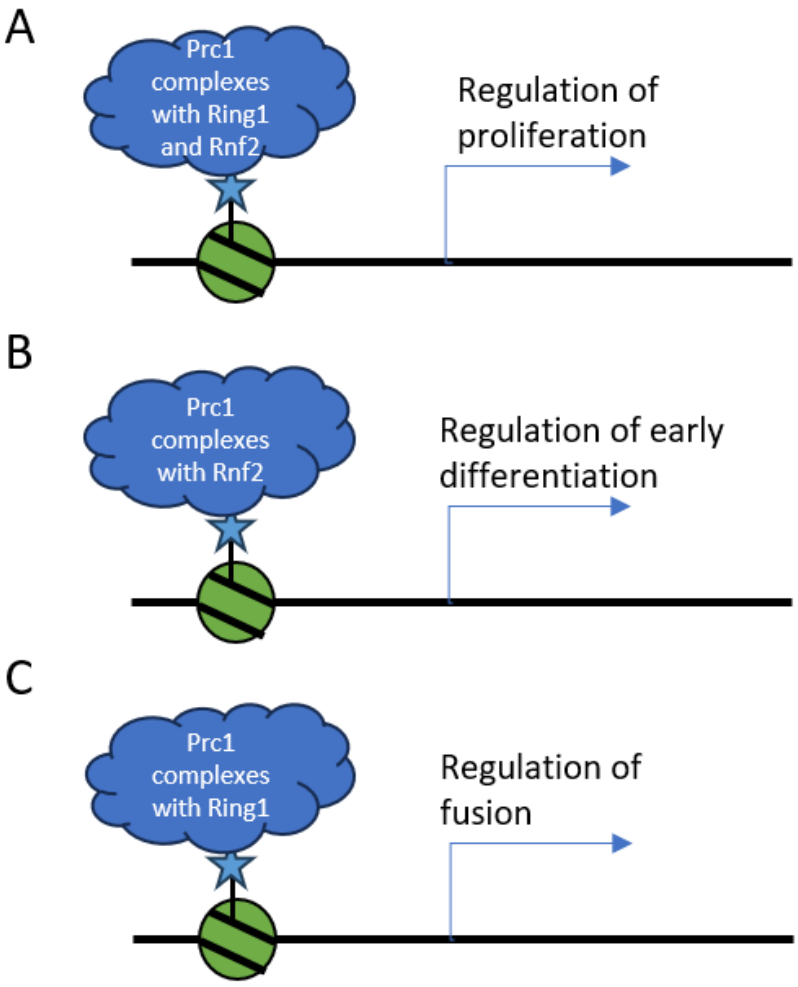

Figure 11: Proposed hierarchy of Ring1 and Rnf2 containing Prc1 complexes within myogenesis in C2C12 cells.

A) Based on our results, during the proliferative phase in C2C12 cells, both the Ring1 and Rnf2 containing Prc1 complexes have roles in regulating proliferation. **B)** The Prc1 complexes containing Rnf2 are essential for the entrance into differentiation, whereas the Prc1 complexes are mostly dispensable for this time. **C)** During fusion, the Prc1 complexes containing Ring1 are essential for mediating the productive fusion of myotubes, however, Rnf2 would seem to be largely dispensable for this stage of myogenesis.

cell lines with the expression of only one of the E3 ubiquitin ligases attenuated, and cell lines with the expression of both E3 ubiquitin ligases attenuated, it is possible to make tentative inferences on the effect of the dosage of the E3 ubiquitin ligases of the Prc1 complexes required to have their regulatory effects. What we found was that for the regulation of HoxA2, Ring1 and Rnf2 appear to be interchangeable, and both needed to be depleted from our cells to affect the expression of HoxA2. During proliferation, we found that Ring1 and Rnf2 are

both necessary for the expansion of the cells, but we cannot tell whether they are completely interchangeable at their targets, or if they are regulating distinct sets of proliferation-associated genes. During differentiation the Rnf2 depletion had a greater effect than Ring1 depletion, leading us to conclude that Rnf2 is more important than Ring1 during this period. During fusion, however, Ring1 has a distinct effect on fusion, far above its differentiation defect, leading us to conclude that it is more instructive than Rnf2 in the regulation of fusion. Our data lead us to a model in which Ring1 and Rnf2 are equivalent during proliferation, Rnf2 is the primary regulator during the early periods of differentiation, and Ring1 is the primary form during the fusion (late differentiation) phase (Fig. 11).

Chapter 4: Role of the enzymatic activity of the ncPrc1 complexes in myogenesis

4.1: Confirmation that Prt4165 reduces the level of H2AK119ub1 in primary myoblasts

While this model in Figure 11 can explain the individual contribution of Ring1 and Rnf2, it does not explain whether this occurs through the enzymatic or other functions of these proteins. To rectify this, we switched to an enzymatic inhibition model to determine the specific functions of Ring1 and Rnf2 during myogenesis. The Prc1 complex can elicit its effects through chromatin compaction and phase separation through the canonical complexes(98, 149, 181, 184), through the deposition of H2AK119ub1 at its target locations(133, 137, 198), or through the recruitment of various cofactors bound to the complex(78, 132). The experiments in Chapter 3 could not discriminate between these functions but did broadly show the role of the complex in myogenesis. Due to this, we sought to resolve this situation and directly test our hypothesis. Using Prt4165, a potent and selective enzymatic inhibitor for the Prc1 complexes(141), we could block the enzymatic activity of the Prc1 complexes (an activity mainly performed by the ncPrc1 complexes(212)). This would allow us to specifically test the functions of the ncPrc1 complexes' enzymatic activity, and use this information to determine what functions of the complex are being performed by it, and by exclusion, what functions are being performed predominantly by the cPrc1 complexes. For these experiments, we also opted to transition to a primary myoblast model as primary myoblasts are a more translatable model to human genetics than C2C12 cells. The shRNAs using a double transfection and selection protocols could have made it difficult to properly

select and expand the population of cells with the shRNAs inserted during the finite period in which primary myoblasts remain viable (approximately 2 weeks), which is not a concern we would have using a transient enzymatic inhibitor.

To begin these experiments, we first needed to ensure that the results from the primary myoblasts can be attributed to the depletion of H2AK119ub1 from the Prt4165 treatment. We performed Western blots for the level of H2AK119ub1 after 8 hours of Prt4165 treatment *in vitro* (Fig. 12). These results showed that there was a 74% reduction in the level of H2AK119ub1 after 8 hours of Prt4165 treatment in primary myoblasts, and confirms that the drug is indeed inhibiting the enzymatic activity of the Prc1 complex.

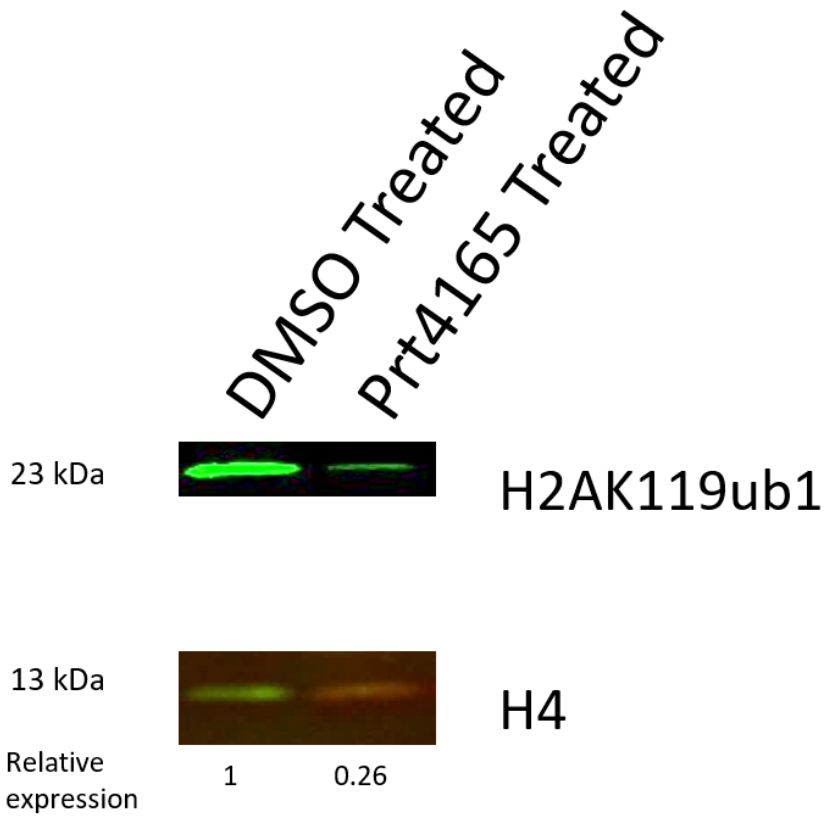

Figure 12: Prt4165 treatment in primary myoblasts *in vitro* reduces the level of H2AK119ub1.

A) Western blots were performed on histone proteins isolated from primary myoblasts using a histone acid extraction. These extracts were then stained for H2AK119ub1 (8240S) at a dilution of 1:2500, then normalized to H4 (ab9051) at a dilution of 1:2500.

4.2: Enzymatic activity of the Prc1 complex does not affect the proliferation rate of primary myoblasts *in vitro*

With the knowledge that Prt4165 was reducing the levels of H2AK119ub1 in our primary myoblast, we could begin testing the roles of the enzymatic activity of the Prc1 complex *in vitro.* The first experiments to this end we performed were growth assays. After dissecting the arm and leg muscles from the mice (as described in the methods) we expanded our population briefly, before plating 10,000 cells on collagen-coated 10-cm plates. From there

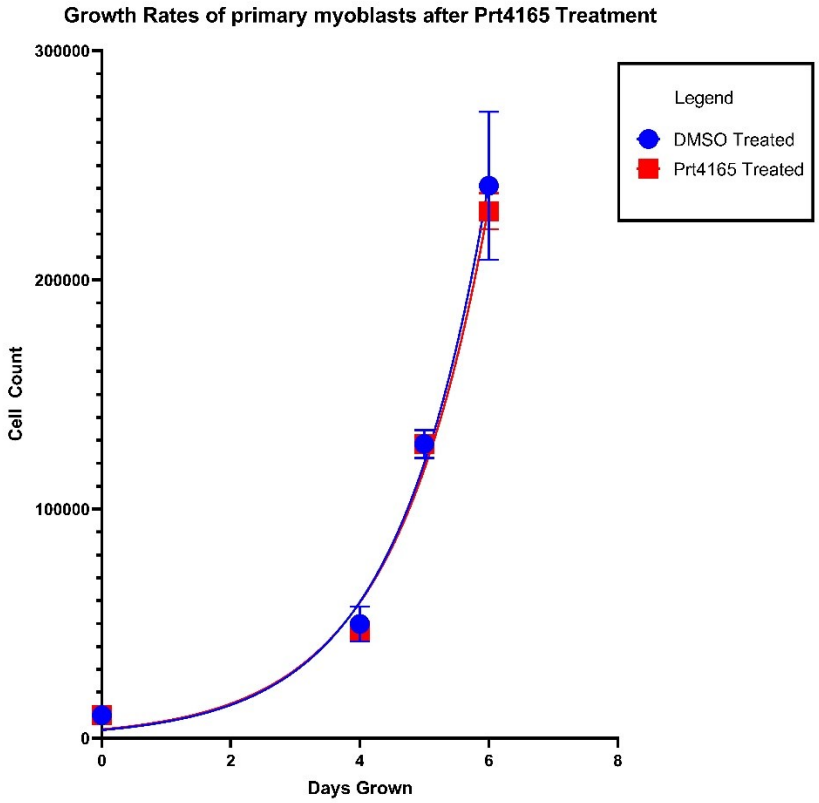

Figure 13: Primary myoblast growth rates are reduced when the Prc1 complex's enzymatic activity is inhibited.

A) Primary myoblasts were grown in primary growth media without any additional additions, before seeding 10,000 cells into a collagen-coated 10cm plate. The cells after seeding were treated with 10 µM Prt4165 (or an equivalent volume of DMSO for the control cells) as the cells grew for a further 4 days, with the media being refreshed daily. The cells were then trypsinized and counted at either 4,5, or 6 days. Cell counts were assessed using a hemocytometer and dead cells were excluded using trypan blue. Each data point in the average count of 3 independent plates for each sample (N=3). Statistical significance was calculated using Welch's t-test.

we changed media daily, supplementing this media with either 10 μM Prt4165, or an equivalent volume of DMSO. On days 4, 5, and 6 we counted the number of primary myoblasts we had in each sample (Fig. 13). From these results, it was clear that there was no difference in the growth rates of primary myoblasts which have their enzymatic activity inhibited.

When Ring1 or Rnf2 expression levels were reduced in C2C12 cells, the proliferation rates of the cells decreased, whereas the inhibition of the enzymatic activity of the Prc1 complex did not affect proliferation. This indicates that the enzymatic activity of the complex is dispensable for proliferation. One of the major roles of the enzymatic activity of the complex is to nucleate new Polycomb domains(201). When Ring1 and/or Rnf2 levels are reduced what likely occurs is that genes that required repression to maintain proliferation are de-repressed, leading to dysregulated proliferation. As the cells are not changing their fate, the nucleation of new Polycomb domains may be a dispensable function (at least in a constant *in vitro* environment) during proliferation. To confirm this hypothesis, one potential method would be to do bulk RNA-seq to determine the transcriptome within our Ring1 and Rnf2 depleted cell lines and the Prt4165 treated cell lines. This would allow us to determine which genes change their expression in response to the depletion of the Ring1 and Rnf2, and confirm that the enzymatic activity is not essential for regulating the key proliferation genes that the depletion of Ring1 and Rnf2 were essential for regulating.

4.3: The inhibition of the enzymatic activity of the Prc1 complex diminishes the fusion capacity of differentiating primary myocytes *in vitro*

The next assay we performed using Prt4165 was the differentiation of our primary myoblasts under Prt4165 treatment. After isolating our primary myoblasts from the mice, we once against briefly expanded our cell population, before plating our cells into collagen-coated 6-well plates. We then differentiated the cells for 2 days, changing the media each day and supplementing it with either 10 μM Prt4165 or an equivalent volume of DMSO. After two days, we fixed our cells and stained them with a pan-MyHC antibody to assess the cells' ability to differentiate. These results showed a marked decrease in the formation of myofibers, and an increase in the number of differentiated cells that had not been incorporated into myofibers (Fig. 14A). Based on these experiments we calculated the differentiation (Fig. 14B), and fusion (Fig. 14C) indexes. What we found was that this treatment had not impacted the cells' ability to begin differentiation, however, it had affected the ability of these cells to fuse into the myofibers.

In contrast to the case in proliferation, both the Prt4165 treatment, and reducing the levels of Ring1 and Rnf2 influenced the ability of the myocytes to productively form myotubes. As discussed in Chapter 3 we saw a decrease in the differentiative capacity when Rnf2 levels were reduced, but when the enzymatic activity was inhibited, there was no differentiation defect. This could potentially suggest that the nucleation of new Polycomb domains is not necessary for the entrance into differentiation. If this is the case, then what likely happens is that when Ring1 and Rnf2 are depleted, genes from alternate lineages are de-

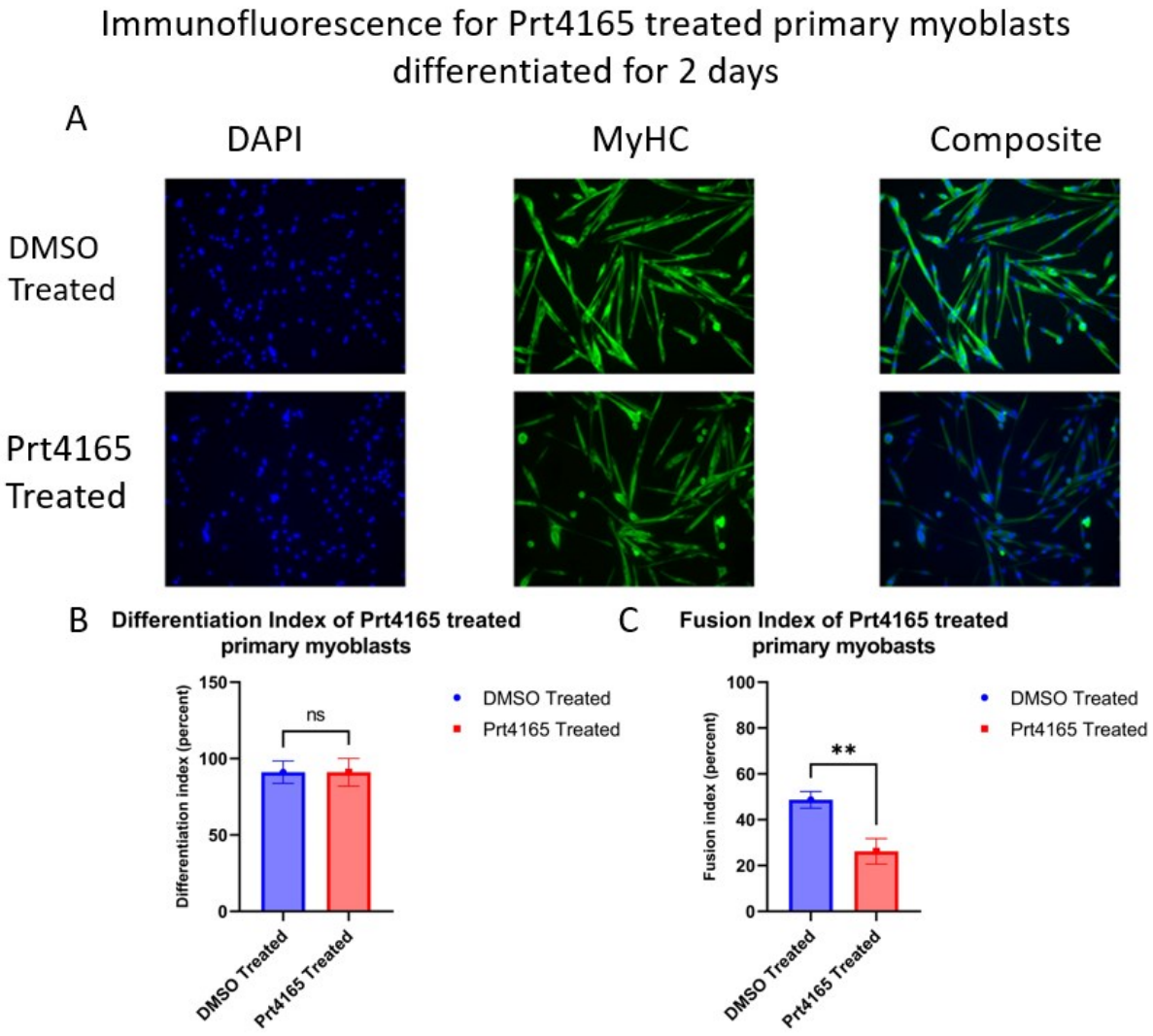

Figure 14: Enzymatic inhibition of the Prc1 complex in primary myoblasts decreases the fusion capacity of the myocytes.

A) Primary myoblasts were differentiated for 2 days (N=5) with either 10 µM Prt4165 or an equivalent volume of DMSO. Cells were fixed in 1% formalin for 30 minutes, then permeabilized in 2% Triton X-100. Nuclei were detected using 1X DAPI (D9542) and pan-MyHC proteins were detected using a 1:2 dilution of an MF20 hybridoma created in our lab. The images were taken on a Zeiss Axiovision Observer Z1 microscope. **B)** Differentiation index was calculated as the number of Myhc+ nuclei over the total number of nuclei. **C)** The fusion index was calculated as the number of nuclei in myofibers with more than 3 nuclei over the total number of nuclei. The calculated values were derived from the average of 5 images from each sample. The statistical significance was calculated using a student's T-test in GraphPad PRISM.

repressed, and proliferation-associated genes which were already marked with H3K27me3 fail to be properly repressed. Genes marked with H3K27me3 would be sufficient to nucleate the cPrc1 complexes, and if the binding of the cPrc1 complexes is displaced by the removal of the

Cbx proteins from the Prc1 complexes (by the ablation of Ring1/Rnf2 expression since Cbx binding is dependent on them(211)), they would not be able to perform these functions. In tentative support of this, in the Ring1 and Rnf2 depleted cells HoxA2 is one such gene which we confirmed was dysregulated (Fig. 6), although we did not assess the direct consequence of this gene's re-expression. HoxA2 is likely not causing the phenotype we observed in our Ring1 and/or Rnf2 depleted cells (and cannot in some cases as reducing the expression of only one of Ring1 or Rnf2 also caused a phenotypic disruption), however, if it has been dysregulated other genes would certainly be as well, and this could be determined using RNA-seq.

Alternatively, if the enzymatic activity is necessary for the transition into differentiation, it could be explained by the time point in which we performed the assay (48 hours after switching the cells to differentiation media). In this hypothesis, as the enzymatic activity is not completely inhibited, the cells will slowly begin differentiating. In this case, the phenotype we saw could be explained by a slowed transition into differentiation. To test this latter theory the experiment would need to be repeated, and RNA's isolated at early timepoints in differentiation to determine if there is a delay in the onset of the expression of differentiation genes.

Where we saw a major change in the myogenic capability of the enzymatically inhibited cells was during the fusion stage of differentiation (Fig. 15C). In the Ring1 shRNA assays, we also saw a pronounced defect far above the differentiation defect when Ring1 was depleted, however with Rnf2 the decrease in these cells fusion indexes were in-line with their differentiation defects. This could suggest that the Ring1 containing Prc1 complexes drives the acquisition of H2AK119ub1 at genes required for productive fusion. While these experiments give some indications on the specific functions of Ring1, Rnf2, and the enzymatic

activity of the Prc1 complex, they do not answer the specific mechanisms with which the enzymatic activity regulates fusion. An important question that needs to be resolved is which genes are dysregulated by the inhibition of H2AK119ub1 deposition, and are they driving the phenotype we have observed, or a byproduct of dysregulated fusion?

4.4: The inhibition of the enzymatic activity of the Prc1 complex does not impede fusion by affecting the transcription of myogenic transcription factors during differentiation

Given that we had seen a defect in fusion, we wanted to understand how the enzymatic activity of the complex was eliciting this effect. To this end, we evaluated the expression at the mRNA level of Pax7, MyoD, Myogenin, and Myf6, all of which have been shown to regulate different aspects of the differentiation process (or in the case of Pax7 its expression will entirely block the entrance into differentiation). We isolated RNA from Prt4165-treated, and DMSO-treated primary myoblasts which had been differentiated for two days then converted these RNAs to cDNAs. We then quantified the expression of Pax7 (Fig. 15A), MyoD (Fig. 15B), myogenin (Fig 15C), and Myf6 (Fig 15D) on these cDNAs. From these results only the transcription levels of MyoD were affected by the Prt4165 treatment, however, its overexpression would not be expected to impede fusion.

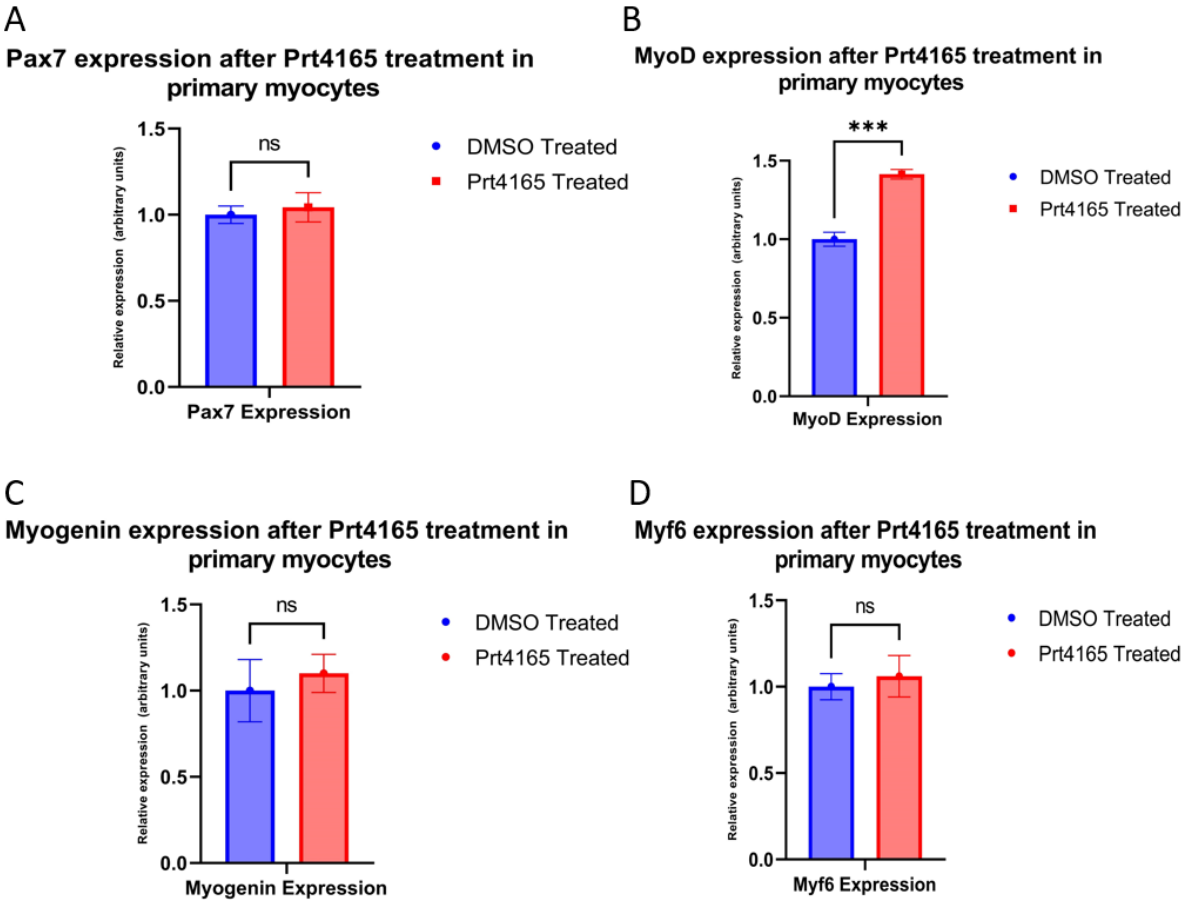

Figure 15: Only the expression of MyoD of the myogenic transcription factors is affected by Prt4165 treatment under differentiation conditions.

A-D) The mRNAs for the qPCR expression assays were extracted from primary myocytes differentiated for two days using stat-60 then probed with TaqMan probes A) Mm01354484_m1 for Pax7, B) Mm00440387_m1 for MyoD, C) Mm00446194_m1 for myogenin, D) and Mm00435126_m1 for Myf6. Each experiment was performed in triplicate, normalized to GAPDH (Mm99999915_g1). The statistical significance was calculated using a student's T-test in GraphPad PRISM.

One of the significant roles of MyoD during differentiation is to transactivate the expression of myogenin, among other differentiation-related genes(38). In our results, we did not see a change in the expression levels of myogenin (Fig. 15C). What is likely the case is that MyoD is not being appropriately downregulated in our sample from a knock-on effect of the poor fusion, although an RNA-seq experiment would be able to more robustly assess whether MyoD targets are being aberrantly expressed in our samples. During the later stages of differentiation, MyoD is downregulated(213). One of the factors which contribute to the

downregulation of MyoD is p202 (also known as Ifi202 in the literature) which is primarily expressed in the myotubes(214). As our Prt4165 treated samples are defective in forming myotubes, this could be indirectly causing the increase in the MyoD expression during the Prt4165 treatment. Therefore, it is likely that the MyoD overexpression was a byproduct of the poor fusion, and the phenotype we have observed cannot be explained by a misregulation of the MRFs or Pax7.

4.5: The inhibition of the enzymatic activity of the Prc1 complex decreases the expression of MyHC proteins

Looking back at the immunofluorescence experiments from section 4.5, the intensity of the immunofluorescence for the MyHC proteins appeared to be diminished by the Prt4165 treatment. Immunofluorescent staining cannot be used to quantitate the observation, so we went back to the cDNAs we had isolated and we quantified the expression for a panel of MyHC proteins (MyHC2, MyHC3, MyHC4, MyHC7, and MyHC8). From these results, there was a trend of decreased expression of the MyHC proteins broadly. More specifically, however, we saw a pronounced decrease in the expression of MyHC2, and MyHC4 (Fig. 16 A and C). The decrease in the expression of MyHC3 and MyHC8 was statistically significant, but the overall change was relatively mild (Fig. 16 B and E). While MyHC7 followed the same trend as the other MyHC proteins we evaluated, (Fig. 16 D), it was not statistically significant. Overall, these experiments showed that the enzymatic activity of the complex has a role in the positive regulation of the MyHC proteins expression.

Interestingly the MyHC proteins which were the most affected by inhibiting the deposition of H2AK119ub1 are the predominant MyHC proteins in two types of “fast-twitch” muscle fibers.

These MyHC proteins are the major MyHC type of Type2a (MyHC2) and Type2b (MyHC4) muscle fibers(215), which correspond to the same fiber types in humans(216). This would need to be followed up with more rigor, but this could indicate that the enzymatic activity of the Prc1 complex only affects the fusion of type 2 "fast-twitch" fiber types and that the enzymatic activity of the Prc1 complex is not essential in "slow-twitch" muscle fibers.

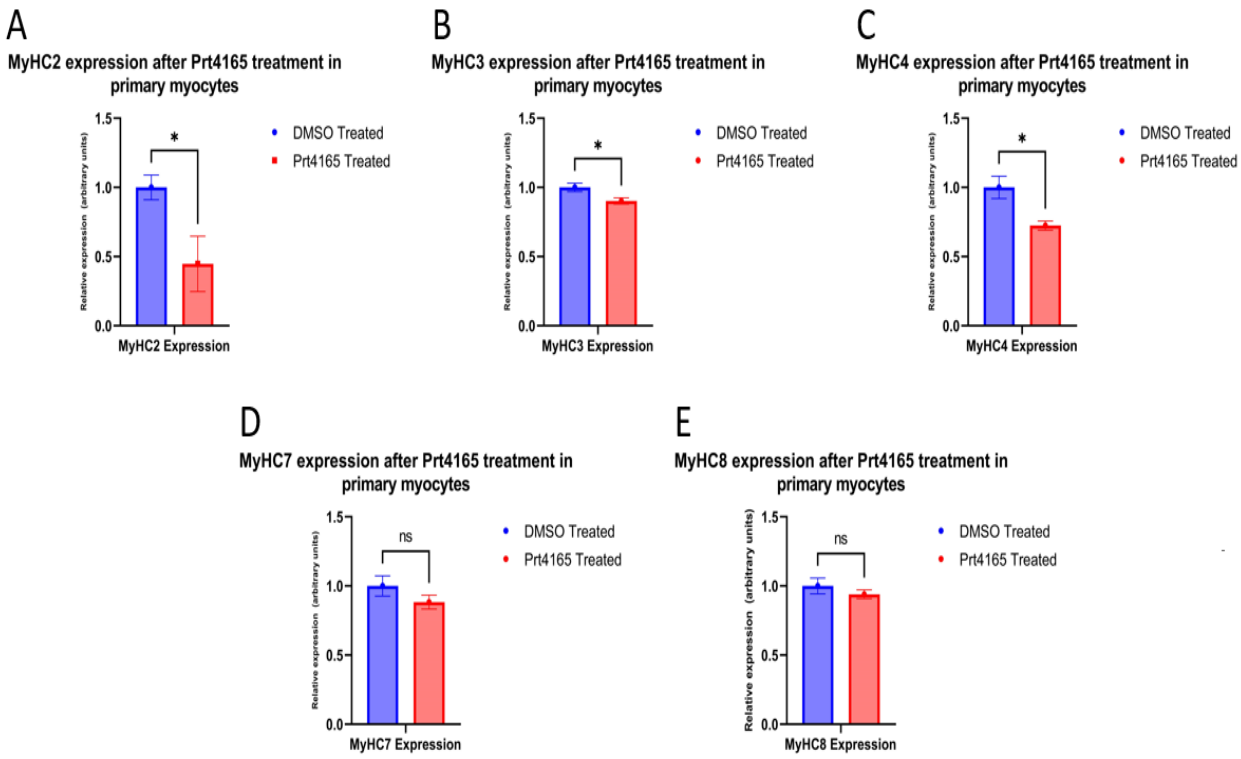

Figure 16: Inhibition of the enzymatic activity of the Prc1 complexes decreases the expression of MyHC proteins.

A-E) The mRNAs for the qPCR expression assays were extracted using stat-60 then probed with TaqMan probes A) Mm01332564_m1 for Myh2, B) Mm01332463_m1 for Myh3, C) Mm01332541_m1 for Myh4, D) Mm00600555_m1 for Myh7, and E) Mm01329494_m1 for Myh8. Each experiment was performed in triplicate, normalized to GAPDH (Mm99999915_g1). The statistical significance was calculated using a student's T-test in GraphPad PRISM.

4.6: Summary of the results for Chapter 4

In this chapter, we have assessed the effect of the Prc1 complex's enzymatic activity on the regulation of myogenesis in primary myoblasts *in vitro.* What we have discovered is that the enzymatic activity of the Prc1 complex is not necessary for the proliferation, or the entrance into differentiation for primary myoblasts. Where the enzymatic activity of the Prc1 complex is required is during fusion, where H2AK119ub1 deposition promotes the fusion of the myocytes into the myotubes. We then further quantified the effects of the enzymatic activity of the Prc1 complex and found that the MyHC2 and MyHC4 are downregulated by the inhibition of the Prc1 complex's enzymatic activity.

While we cannot directly confirm the mechanism by which the enzymatic activity of the Prc1 complex promotes fusion, there are several possibilities for why the myocytes failed to fuse into the myotubes when it was inhibited. These include defects in expressing fusion factors such as myomaker and myomerger(217), defects in chemokine expression on the myotubes or receptors on the myocytes(218), or defects in cellular motility(219) (Fig. 17A). Given the data we had compiled thus far, the most likely explanation for the fusion defect is that the myocytes have defective motility. The MyHC proteins have been shown to drive motility functions in myogenic precursors(220–222), and we have confirmed that Prt4165 treatment reduced the expression of the MyHC proteins. The MyHC proteins have roles in remodeling the actin cytoskeleton with numerous functions within cell biology, including cellular motility(222, 223). *In vitro,* experiments with purified MyHC proteins from rat muscle fibers on coverslips have shown that these proteins can directly move actin filaments and that in low-density regions of MyHC coverage on these slips, the actin filaments move both slower and erratically(224). In our experiments with a reduction in MyHC2 and MyHC4 expression,

this could mimic the effects of the low-density regions from the coverslip experiments, and lead to the disjointed movement of our myocytes. If the cells are indeed moving both more slowly, and/or erratically, this could easily explain the fusion defect in the presence of normal levels of differentiated myocytes. This would lead *in vivo* to myocytes which were near the site of the injury being capable of migrating to the site of the injury and fusing into the damaged myotubes giving the myofibers some regenerative capacity, but more peripherally located myocytes would be unable to reach the site of the damage (Fig. 17B). This model would lead to smaller myofibers being formed in the muscles of mice as we saw in our diminished fusion into our fibers *in vitro.* While this model offers a good rationalization for the phenotype, and the expected effects *in vivo,* future efforts will need to be put in to understand the exact molecular mechanism as to how the inhibition of the Prc1 complexes enzymatic activity elicits this phenotype.

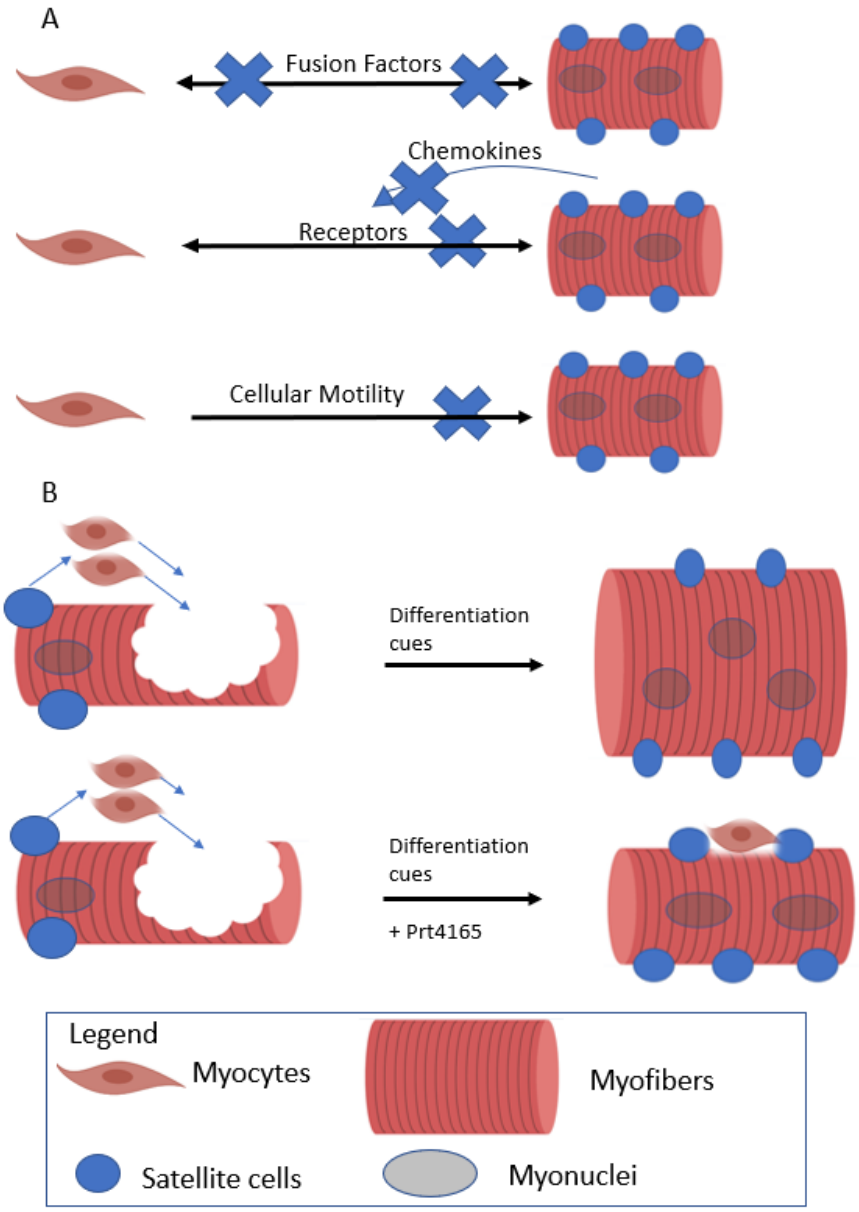

Figure 17: Proposed model on the enzymatic function of the Prc1 complex in the context of myogenic fusion.

A) As the differentiation process in myogenesis ensues, the myocytes begin to prepare themselves for fusion into the myotubes. Failure to do so results in low-caliber myofibers being formed, impeding the health of the organism. Three of the most likely defects in fusion can be defects in expressing fusion factors, chemokine/receptor combinations, or cellular motility. If the myocytes do not express myomaker, they cannot properly bind to the myotubes, but if the myotubes do not express myomerger they cannot accept the Myonuclei. If the myotubes do not express chemokines, or the myocytes do not express the receptors to sense these chemokines, the myocytes would need to find the fusion locations by random sampling of their environment, obviously, an inefficient way to generate fusion events. Lastly, if the myocytes are improperly motile, fusion events can be rare as the myocytes are unable to reach the myotubes to fuse into them. **B)** In our proposed model, when the enzymatic activity of the Prc1 complex is inhibited, the myocytes motility is inhibited. Myocytes formed closer to the site of the damage (or *in vitro,* the myocytes already formed) can fuse into the myofibers, however, myocytes which are further away would not be able to fuse into the myofiber while the signaling environment is permissible to this activity.

Chapter 5: Discussion

5.1: The findings in relation to our hypothesis

In this project, we hypothesized that the Prc1 complex is essential for regulating the progression of myogenesis through its ability to ubiquitinylate histones. What we have shown is that the monoubiquitinylation of H2AK119 is not essential for regulating the progression of myogenesis, except in the later stages when the myocytes are undergoing fusion. The Prc1 complex was shown to regulate the myogenic progression through the proliferative, differentiative, and fusogenic stages, however, the enzymatic activity was shown to be dispensable during proliferation and differentiation. Delving deeper into the function of the enzymatic activity, we found that the MyHC proteins were downregulated when the enzymatic activity was inhibited in primary myoblasts, but this was not due to a change in the expression of myogenic regulatory factors which are known to be effectors of differentiation. There are several possibilities for why the myocytes failed to fuse into the myotubes, including defects in expressing fusion factors such as myomaker and myomerger(217), defects in chemokine expression on the myotubes or receptors on the myocytes(218), or defects in cellular motility(219) (Fig. 17A). In our proposed model of the enzymatic function of the Prc1 complex during primary myoblasts differentiation, we propose that when the enzymatic activity of the Prc1 complex was inhibited, the decrease in the expression of the myosin heavy chain kinase proteins causes a defect in the myocytes' motility, leading to the decrease in the myocytes' fusion ability. This leads to a decrease in the nuclei in myotubes, and a concomitant increase in the nuclei which have undergone differentiation but failed to fuse (Fig 17B). Thus, we have found that our hypothesis is partially true. The enzymatic activity is dispensable for

proliferation and early differentiation *in vitro;* however, it is essential for the fusion stage of myogenesis.

5.2: Comparison of the role of the enzymatic activity in our study to previously established work

The major finding of our study was that while the Prc1 complex has roles in regulating the proliferation and differentiation of skeletal muscle cells, the enzymatic activity of the Prc1 complex is dispensable for proliferation, and the entrance into differentiation, and is only necessary for the positive regulation of fusion.

The role of the Prc1 complex's enzymatic activity has been studied in embryogenesis(143), early implantation(144), ESC differentiation(200), and in more differentiated cells, neural progenitor stem cells during development(225). When Rnf2 was completely ablated from the mouse genome, the mouse embryos failed to pass the gastrulation phase (E6.5-7~) and were not viable(226). In contrast, in Ring1B$^{I53A/I53A}$ mice, while there were defects in achieving their developmental milestones and the embryos developed in sub-Mendelian ratios, the Ring1B$^{I53A/I53A}$ were capable of developing past the gastrulation phase(143). These studies clearly show that the enzymatic activity is dispensable for some of the roles of the Prc1 complex.

The studies on the role of the enzymatic activity of the Prc1 complex were further refined in a study in neural progenitor stem cells. In this paper, they showed that in Ring1A$^{-/-}$;*Rnf2*$^{i53a,D56K/i53a,/D56K}$ mice (mutation for Rnf2 was a conditional floxed allele), the enzymatic activity is essential to transiently repress poised genes for neurogenic lineages(225). As these neural progenitor cells differentiate into neural lineages or lose their neural potential by

differentiating into astrogliogenic neural progenitor cells, they either lose the H2AK119ub1 mediated repression in the neural lineages or transition to an H2AK119ub1 independent repression (mediated by the cPrc1 and Prc2 complexes). While the maintenance on the stably repressed neural genes was maintained even when the catalytic activity of the Prc1 complex was abrogated, the catalytic activity was removed after the repression had already been established, and this study did not determine whether the enzymatic activity was dispensable if the initial repressive environment was not set up before differentiation.

A separate study looked at the effect of PCGF1 and independently Ring1/Rnf2 double depletion in the differentiation of ESCs into embryoid bodies(139). What was found from their studies was that the PCGF1 containing Prc1 complexes were essential for establishing the repressive environment by depositing *de novo* H2AK119ub1 at key genes, before they gained their stable repression by the recruitment of the cPrc1 and Prc2 complexes. Indeed, when they removed PCGF1 while simultaneously inducing the differentiation of ESCs to embryoid bodies, the induction of repression at these genes was abrogated(139). This indicates that in at least some circumstances, the transient repression of genes by H2AK119ub1 is explicitly necessary to establish the more permanent cPrc1 and Prc2 mediates repression during differentiation.

The experiments that our model most closely resembles are these latter experiments, as we inhibited the deposition of de novo H2AK119ub1 as we began the differentiation of our cells similar to how they depleted PCGF1 levels as the ESCs differentiate into embryoid bodies(139). At the phenotypic level, our data guides our theory towards a motility defect gained by the inhibition of *de novo* H2AK119ub1, and subsequent repression of the MyHC fast-twitch proteins.

While the precise mechanism of this remains purely speculative, there are plausible theories which can be made. The fast-twitch MyHC genes are clustered closely together in the genome(227). Recently, a putative super-enhancer for these genes has been described, which coordinates transcription factors to strongly transactivate a single fast MyHC protein in single nuclei(228). The MyHC genes before differentiation remain repressed, although the precise factors behind this repression are not known. In our studies, what we found was then when the enzymatic activity of the Prc1 complex is ablated, MyHC2 and MyHC4 were downregulated. Both genes are physically close together in the genome, and subject to regulation by this super-enhancer. Potentially the inhibition of the Prc1 complexes enzymatic activity could result in the downregulation of MyHC2 and MyHC4 by failing to repress an unidentified (at least in this specific context) repressive transcription factor in response to differentiation cues (Fig. 18). This would result in the indirect repression of MyHC2 and MyHC4 by this mechanism. However, this does not fully explain why MyHC8 would not also be repressed by the inhibition of the Prc1 complexes enzymatic activity, as it responds to the same super-enhancer. The MyHC8 gene is the furthest of the 3 MyHC genes in this cluster whose expression we tested. A part of how super-enhancers mediate transcriptional activation is through chromosomal looping(229), and potentially this could allow it to escape the repression mediated by this mechanism. In this model, when the ncPrc1 complexes' enzymatic activity is not inhibited, this would allow these complexes to establish a *de novo* repressive environment around the repressive transcription factor, and the subsequent repression of the loci through a cascade of Polycomb binding.

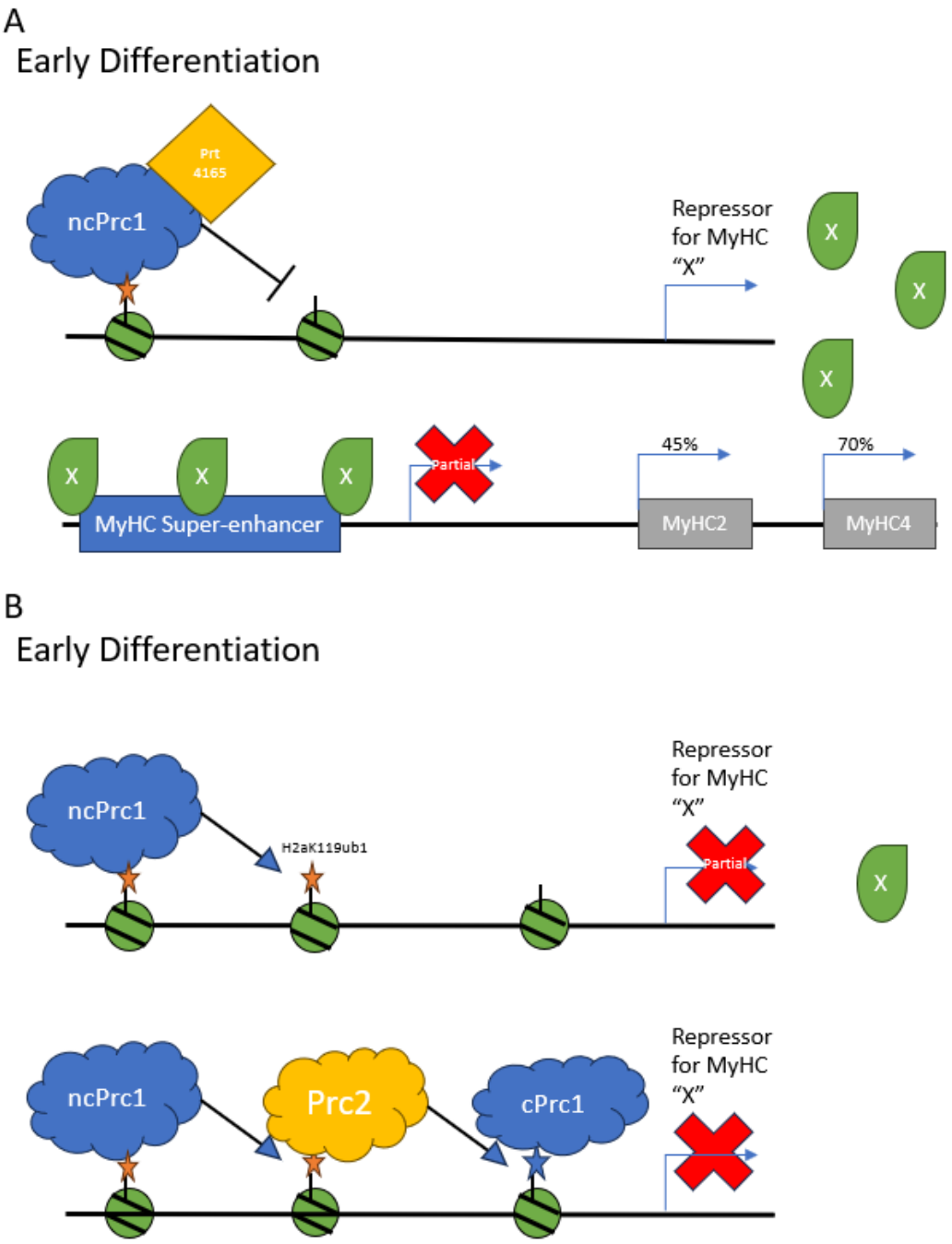

Figure 18: Proposed mechanism on how the enzymatic activity of the Prc1 complex regulates the expression of MyHC2 and MyHC4.

A) During early differentiation, the ncPrc1 complexes are targeted to the loci of unknown transcription factor "X". When Prt4165 is inhibiting the enzymatic activity of the Prc1 complex, it is unable to ubiquitinylate nearly histones, and this leads to the failure of gene "X's" repression. This creates a defect in removing the repressive environment around the super-enhancer for the fast-twitch MyHC genes, partially reducing the expression levels of MyHC2 and MyHC4. **B)** When the enzymatic activity of the ncPrc1 complexes is not inhibited, during the onset of differentiation the ncPrc1 complexes will be targeted to gene "X" and catalyze the ubiquitinylation of H2AK119 and the transient repression of the same gene. This establishes the environment for the recruitment of the Prc2 complex and its subsequent catalysis of H3K27me3 on nearby histones, and the recruitment of the cPrc1 complexes leading to the chromatin condensation and stable repression of the target gene.

While we did not directly assess the *de novo* deposition of H2AK119ub1, based on the mechanics of how the MyHC genes are expressed the model in Figure 18 is a reasonable hypothesis. The MyHC genes begin expression during the differentiation of the myoblasts(230) and require repression before differentiation begins. When the Prc1 complex's enzymatic activity was inhibited, the MyHC genes were downregulated, which almost certainly precludes the possibility of the Prc1 complex being directly targeted to these genes.

While this model is highly speculative, it is a reasonable model to test. Using CUT&Tag or ChIP-seq it could be determined where the Prc1 complex and H2AK119ub1 are targeted, and then RNA-seq could be used to determine what genes were upregulated by the inhibition of the Prc1 complex's enzymatic activity. Then, potential transcription factors could be identified in these datasets, and these transcription factors could be depleted from our cells to directly determine if these genes can phenocopy the enzymatic inhibition. Taking it one step further a CUT&Tag or ChIP qPCR could be performed with the putative repressive transcription factor to determine if it does bind to, or near the MyHC super-enhancer. These experiments would provide a direct link between the acquisition of H2AK119ub1 at these loci being essential for establishing repression as in the paper from the Kozeki lab(139) but in the regulation of adult stem cells differentiation. The other paper from the Gotoh lab showed that potentially similarly regulated genes acquired H2AK119ub1-independent mechanisms of repression after the acquisition of the initial H2AK119ub1(225). To test this expression assays could be done on the MyHC genes after myotubes were treated with Prt4165. This would assess whether the Prc1 complex's enzymatic activity was still necessary to maintain repression and link together the results from these two papers.

5.3: Contrasting the role of the E3 ubiquitin ligases of the Prc1 complex, to previous studies on the Prc1 complex in muscle tissues

5.3.1: Contributions of Polycomb proteins to skeletal muscle myogenesis

Previous work in muscle cells has looked into the effect on myogenesis of removing Bmi1 from the genome(153). This study found that there was a significant (an approximately 65% decrease in EdU^+ cells when cultured in growth media *in vitro*) decrease in the myoblasts proliferation rate, which lead to defective regeneration in the mouse muscle tissues. In their study, they did not find that reducing Bmi1 levels directly led to defective differentiation, but indirectly affected differentiation due to poor expansion of the myogenic progenitors. It is important to note that reducing the levels of Bmi1 in their cells would not abrogate the formation of the cPrc1 complexes entirely, as they found that the depletion of Bmi1 did not affect the expression of Mel18(153).

In our work on the Prc1 complex, we reduced the levels of Ring1 and Rnf2, which would exclude the binding of Cbx proteins to what remains of the Prc1 complex, and would fairly effectively disrupt cPrc1 complex binding(181, 231). The results we found were in line with the Robson paper on the effect of Bmi1 disruption on the proliferation of the myoblasts(153). Our studies with enzymatic inhibitors showed no effect on proliferation, which leads to the conclusion that the proliferative effects are being predominantly driven by the cPrc1 complexes. Interestingly, Mel18 containing Prc1 complexes have been shown to suppress proliferation in multiple different cellular contexts(232–234), although when Bmi1

and Mel18 levels are reduced, the proliferation rate of these cells are also reduced(235). The results from other labs suggest that the cPrc1 complexes have a significant independent function, at least within the proliferative phase, but when the targeting of these complexes is disrupted, the net phenotype is dominated by the contributions of Bmi1.

The Prc1 complexes co-regulate many of their target genes alongside the Prc2 complex through the recruitment of Prc2 complexes to H2AK119ub1, and the subsequent recruitment of cPrc1 complexes to H3K27me3(99, 134, 139, 201). During proliferation *in vivo,* EZH2 (the enzymatic subunit of the Prc2 complex) knockout mice fail to proliferate appropriately, but there was no defect in their differentiative abilities when EZH2 was conditionally knocked out as differentiation was induced(236). Our data has led us to suspect that the regulation of proliferation for the Prc1 complex was through the cPrc1 complexes, which would bind to H3K27me3 at loci for the regulation of proliferation, particularly genes from alternate lineages. This interpretation of our data is in line with the results from the Pell lab(236), and these data together suggest that the Prc1 and Prc2 complexes co-regulate their target genes during proliferation. This does raise the question as to why the inhibition of H2AK119ub1 deposition does not affect proliferation, as we would have expected to see the displacement of the Prc2 complex's ability to bind and contribute to the spread of Polycomb domains(201). It has been shown that established Polycomb domains containing the cPrc1 complexes can be resistant to the removal of the Prc1 complexes enzymatic activity, and can maintain the repression of Prc1 targeted genes with the cPrc1 H2AK119ub1 independent pathway(225). Thus, if the cPrc1 complexes are the predominant form of the Prc1 complex for the regulation of proliferation, the similar results between our work on the Prc1 complex, and the Pell lab's work on the Prc2 complex(236) fit together well.

Where our works shows that role of the Prc1 complexes differentiate from the Prc2 complex in myogenesis is during fusion, where the conditional ablation of EZH2 did not affect terminal differentiation or myofiber formation(236), unlike the case when the H2AK119ub1 or Ring1 and Rnf2 levels are depleted. It is clear that the Prc1 complex has significant roles outside of the Prc2 complex during differentiation. Given that the enzymatic activity of the Prc1 complex functions independently of the Prc2 complex to seed new Polycomb domains, this suggests that the acquisition of new targets is essential for the regulation of fusion. Similar results have also been found from the Dynlacht lab, where they found that the ablation of the Prc2 complex does not impede differentiation and even slightly enhances it(152). This study also examined the effect of Rnf2 ablation within C2C12 cells and found that it is essential for the entrance into differentiation. Where we expanded this work was to extend the data into fusion, and to incorporate Ring1 depletion on its own and with Rnf2 depletion. In our work, we have replicated the Dynlacht findings for Rnf2's role in differentiation(152), but also found that Ring1 has a relatively small contribution to differentiation during myogenesis. As we showed in Chapter 3, Ring1 would appear to be critical for fusion during myogenesis.

5.3.2: The role of the enzymatic activity of the Prc1 complex in myogenesis

Previous work in skeletal muscle cells has indirectly suggested that H2AK119ub1 can regulate myogenesis by studying the effect of overexpressing and reducing the levels of RYBP. A major role for RYBP in cells has been shown to be its stimulation of the enzymatic activity of the ncPrc1 complexes(136, 137). Previous work in skeletal muscle cells has also shown that depleting the levels of RYBP increases the differentiation index of the cells(194), and presumably would also decrease H2AK119ub1 levels, although this was not analyzed in the paper.

In our work, we have seen that the inhibition of the enzymatic activity of the Prc1 complex does not affect the differentiation index of the cells, a direct contradiction to these results. It is unclear why this would be the case, however, RYBP has been shown to bind to the genome with function consequences outside the Prc1 complex(196, 197), which could explain the discrepancy. The authors of the paper also did not assess H2AK119ub1 levels, and Yaf2 can compensate for RYBP for the ncPrc1 complexes binding and promotion of its enzymatic activity(136), and this was not analyzed within their system.

In light of our assays which directly tested the effect of H2AK119ub1 on the regulation of fusion in a myogenic context, Yaf2 was likely compensating for RYBP's roles in targeting and regulating the ncPrc1 complexes within their system. This would make their observation that the depletion of RYBP promotes myogenic fusion (albeit with a strong potential of this occurring at the cost of satellite cell self-renewal) more likely attributed to the roles of RYBP outside of the Prc1 complex, which has been documented by other researchers(196, 197).

5.3.3: The effect of Ring1a and Rnf2 depletion on HoxA2 expression

Hox genes are well-studied targets of the Polycomb complexes(208–210, 237). In our study, depleting the levels of only one of Rnf2 or Ring1 did not result in the re-expression of HoxA2, however, reducing the levels of both proteins did. The results from these experiments suggest that Ring1 and Rnf2 containing Prc1 complexes can compensate for one another in the regulation of HoxA2. While it could be tempting to generalize this finding to all Hox genes, previous studies have found that removing only one of Ring1 or Rnf2 can affect the regulation of Hox gene expression(210, 237). In the Rnf2 paper, the authors looked at the expression of 10 hox genes and found that Rnf2 depletion resulted in the re-expression of 9 of these genes(237). The Ring1 paper had a panel of 33 Hox genes including HoxA2, and found only

3 genes were re-expressed after Ring1 depletion, and HoxA2 was not affected by Ring1 depletion(210). Together these results show that it cannot be assumed that the contribution of the E3 ubiquitin ligases of the Prc1 complex to the regulation of Hox genes is homogenous, however at specific Hox genes like HoxA2 in muscle cells, the E3 ubiquitin ligases are interchangeable.

5.4: Limitations of this study

There are several limitations to this study to be noted before moving on to future directions on this project. The first limitation which has been mentioned at several points is the lack of a suitable antibody for Ring1. Several published papers have gotten around this limitation by using a flag (or otherwise) tagged Ring1 protein, and then probing the tag for Western blots to determine the depletion of the protein. This may be an excellent step forward for this project, as accurately assessing the Ring1 levels would greatly increase the significance of section 3, and further refine the analysis of the results.

A second limitation is that the depletion levels of Ring1 and Rnf2 were incomplete using this system, only getting to around 50% at best. Some effects of these proteins could be masked due to this, but it does offer insights into the dose dependency of Ring1 and Rnf2 concentrations. Related to this, depleting Ring1 and Rnf2 with these shRNAs was not tried in primary myoblasts. As these cells have a limited time in which they are viable, we deemed it unlikely that we could successfully ensure that the cells had properly incorporated these shRNAs, while also maintaining a significant enough population of cells to perform our experiments.

5.5: Future Directions

A major unanswered question posed by this study is the specific transcriptomic and mechanistic effects of the Prc1 complex within myogenesis. From the data we have generated, the role of the enzymatic activity of the Prc1 complex in primary myocytes is to promote the upregulation of the MyHC proteins associated with fast twitch myofibers and the fusion of the myocytes into the myofibers. The lack of the Prc1 complex's enzymatic activity leads to defective motility and poor fusion; however, the motility defect remains to be confirmed. A scratch test was first attempted; however, this also removed the collagen from the plate that the myogenic cells need to bind, and very few cells were capable of infiltrating the void created during the assay. What could instead be done to test this hypothesis is to use live cell microscopy. Using a machine such as the Cellomics arrayscan vti would allow us to track the motility rates of all cells within a 96-well plate under Prt4165 or a DMSO vessel control treatment to confirm this hypothesis.

While confirming the motility defect hypothesis would help understand the function of the enzymatic activity of the Prc1 complex within myogenesis, there remain questions as to how the Prc1 complex is regulating motility (if this is true of course). The Prc1 complex has been shown to both up, and down-regulate its direct target genes, although most of its targets are repressed(142, 168, 198). To determine how the Prc1 complex's enzymatic activity affects transcriptomic activity, we could use CUT&Tag to determine the direct target genes, and then use RNA-seq to determine what the full transcriptomic effect of the enzymatic activity is. With CUT&Tag we could perform the assay on Rnf2, Ring1 (if an antibody can be found that works for it), Bmi1, Rybp, H2AK119ub1, H3K27me3, and H3K4me3 during proliferation, and differentiation conditions. This panel of antibodies, and these time points will allow us to

determine how the bivalent domains are resolved as the myoblasts begin to differentiate, where the cPrc and ncPrc1 complexes are binding, and what genes specifically are being regulated by the enzymatic activity of the Prc1 complexes. We can also perform bulk RNA-seq at these time points, which will allow us to couple the targeting data, to the transcriptomic effects. These two experiments will allow us to determine if the Prc1 complex's enzymatic activity directly or indirectly regulates the expression of MyHC2 and MyHC4. If it is indirect, then it may offer insights into which genes H2AK119ub1 are essential for regulating properly during differentiation. In the indirect case, we would deplete the levels of key genes identified in the RNA-seq and CUT&Tag experiments to confirm that these genes are driving the phenotype that we had observed. While these experiments were pitched to explain how the Prc1 complex affects motility, they are independent of this hypothesis. If the motility hypothesis is wrong, these experiments regardless should be able to explain how the inhibition of the enzymatic activity of the Prc1 complex affected the ability of the myocytes to fuse into the myotubes and would greatly strengthen the impact of this project.

Another interesting addition which could be made to the study of the enzymatic activity is the use of Rnf2I53A mutant mice. These mice have been shown to ablate the enzymatic activity of Rnf2, without affecting its incorporation into the Prc1 complexes(143, 144). This would allow us to study the role of the enzymatic activity *in vivo* which is not currently possible in a directed manner using Prt4165 that would not also affect the regulation of nearby non-muscle cells complicating the interpretation of these potential experiments. While Ring1 would still be active, Rnf2 is expressed at a higher level in the skeletal muscle of mice during the early timepoints during regeneration, and would thus be the major driver of H2AK119 deposition. A knock-in of this mutant in a B6.Cg-Pax7tm1(cre/ERT2)Gaka/J background

would segregate the Rnf2I53A mutant into the satellite cells and pituitary gland, although the latter likely would not affect the muscle regeneration system and should not pose a problem to the study. These mice would also allow us to test the role of the enzymatic activity in activation, which is not possible starting *in vitro* as the isolated myoblasts have already undergone activation by the time we could treat them with Prt4165. As the Polycomb proteins are important for the regulation of quiescence in Bmi1 knockouts(153), this would allow us to determine if the enzymatic activity has roles in these time points as well. As quiescence and proliferation (activation) are distinct states, it is reasonable to hypothesize that the seeding of new Polycomb domains may play a role in the activation of satellite cells.

Lastly, there is a tenuous link between the results from the MyHC expression level experiments when the Prc1 complex's enzymatic activity was inhibited and potential disease states. A class of rare myopathies known as myosinopathies have been shown to be caused by mutations and or a lack of expression of MyHC proteins, including MyHC2(238–240). MyHC IIa myopathy (an E706K mutation leading to protein truncation and depletion of total MyHC2 levels) presents with distinct rimmed vacuoles in the myofibers when taking cross-sections from patient muscle samples(240). Other patients have presented with MyHC2-related myopathies, where there was a lack of MyHC2 expression or very low levels of expression due to mutations in the MyHC genes(238). As we have shown that the enzymatic activity of the complex regulates the expression of MyHC2, it would be interesting to see in the Rnf2I53A mice whether the phenotypes from these myopathies would be recreated.

As mentioned in the limitations, the questions surrounding Ring1 protein levels were unable to be answered due to technical limitations with the antibodies. A proper assessment of the protein levels of Ring1 would greatly clarify the results from the Ring1 and Rnf2 depletion

experiments (Chapter 3). There are two methods which could remedy this situation. The first would be to generate a new antibody for Ring1. In the long term, this would be a great addition to the field, as our lab is likely not the only lab dealing with this issue. The second method could be to insert a flag tag into the Ring1 locus and perform Western blots for the tag instead. This is a readily available method, but there is also the possibility of the tag affecting the endogenous function of the protein. As the Prc1 can form complicated multiprotein complexes, this is a valid concern, and we would need to confirm that the normal functions of the complex were not affected by the inclusion of this tag to Ring1.

5.6: Concluding remarks

In this study, we have explored the functions of the Prc1 complexes for both the ncPrc1-driven enzymatic activity, and the complete role of the E3 ubiquitin ligases of the Prc1 complexes myogenesis. We have found that the E3 ubiquitin ligases of the Prc1 complexes are essential for regulating proliferation and differentiation *in vitro.* In our studies of the enzymatic activity of the complex, we found that the deposition of H2AK119ub1 regulates the ability of the cells to properly fuse into myotubes. Circumstantial evidence suggests that this effect is due to a reduction in the cell's motility rates, although whether this activity is a direct or indirect effect remains to be determined. While mechanistic work still needs to be performed to understand the phenotype, we have charted a clear path forward to resolving this question. Once complete, this project will delineate the functions of the Prc1 complex in regulating myogenesis, helping to fill in the gaps in our understanding of epigenetics, and specifically the deposition of H2AK119ub1 in myogenesis.

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Curriculum Vitae

Education

2019/9 – 2023/09:
M Sc. candidate, Cellular and Molecular Medicine, University of Ottawa
Degree Status: In Progress
Supervisor: Dr. Jeffrey Dilworth
2014/9 - 2019/6:
Bachelor's Honours, B. Sc. (Honours), Biochemistry, University of Saskatchewan
Degree Status: Completed
Supervisor for honours project: Dr. Adelaine Leung

Research Activity

2019/9 – 2023/08:
M. Sc. candidate, Cellular and Molecular Medicine, Ottawa, University of Ottawa

Research: In this project I am analyzing how the Prc1 complex contributes the regulation of skeletal muscle regeneration. Using shRNA's and enzymatic inhibitors to the Prc1 complex we determined how the Prc1 complex affects skeletal muscle regeneration, and began isolating some of the unique contributions of the Prc1 complex components. We determined that the Prc1 complex promotes both proliferation and differentiation in skeletal muscle cells during regeneration through its non-enzymatic functions. The enzymatic activity of the Prc1 complex only regulates the fusion of the myocytes into myotubes (this occurs at the late differentiation timepoint). The leading hypothesis for the defective fusion is that in the absence of the Prc1 complex's enzymatic activity, the motility of the cells is severely impeded preventing the myocytes from reaching the myotubes.

2019/5 - 2019/8:
Summer Student, Cellular and Molecular Medicine, Ottawa, University of Ottawa
Research: This research was the beginning of my M. Sc. work. I learned the basic techniques (cell culture, IP's, etc..) that I have used to further my research during my M. Sc. candidacy.
2018/8 - 2019/4:
Honors Research Project, Biochemistry, Saskatoon, University of Saskatchewan

Research: In this project I cloned a small fragment of DISC1 into a bacterial expression vector and optimized a purification protocol to attempt to derive a DISC1 construct suitable which was abled to be crystalized.

Affiliations

2019/9 – 2023/08:
M. Sc. candidate, Cellular and Molecular Medicine, University of Ottawa, Ottawa, Canada
2019/9 – 2023/08:
Ottawa Hospital Research Institute, Ottawa, Canada

Research Funding History

2021/5 - 2022/4:
Queen Elizabeth II Graduate Scholarship in Science and Technology (QEII-GSST), Scholarship
2022/5 - 2023/4:
Queen Elizabeth II Graduate Scholarship in Science and Technology (QEII-GSST), Scholarship

Event Participation

2022/10 - 2022/10:
Attendee, CEEHRC Annual Meeting 2022, Esterel Quebec, Conference
Conference on the latest developments in epigenetics research (with a focus on Canadian research. At this conference I also presented a poster on H2AK119 monoubiquitinylation in skeletal muscle, updated with new results from the previous year.
2021/11 - 2021/11:
Attendee, CEEHRC Annual Meeting 2021, Conference
Conference on the latest developments in epigenetics research (with a focus on Canadian research. At this conference I chaired session 5 (Epigenetics of Cell Renewal and Cell Fate). At this conference I also presented a poster on H2AK119 monoubiquitinylation in skeletal muscle.
2020/2 - 2020/2:
Poster Evaluator, OHRI Post-Doc Research Day, Conference
This was an event hosted by the OHRI postdoctoral association to highlight the research done by the post-docs in the institute. I judged two posters (single blind) during the event and provided written feedback to the owners of the posters.
2019/10 - 2019/10:
Attendee, NMD Ottawa 2019, Conference
Conference on the latest developments in the neuromuscular dystrophy field.

Knowledge and Technology Translation

2020/9 – 2023/02:
Lay author, Podcast host (two sessions), CEEHRC Trainee Committee
Activity Description: As part of the CEEHRC trainee committee I have contributed 4 articles to https://thisisepigenetics.ca/. These articles are written with the intent of disseminating scientific information in a manner which would be understandable and engaging to non-experts. I also recorded two podcasts interviewing scientific researchers to engage science on a more personal level.

Committee Memberships

2020/9 – 2023/02:
CEEHRC Trainee Committee
This committee was formed to promote knowledge translation among the general public and in the long term for researchers, teachers, and clinicians.

2017/9 - 2019/4:

Biochemistry Student Association Member, University of Saskatchewan

I was a member of the Biochemistry Student Association. I participated by voting on proposals submitted by the committee members and by participating in events planned by the committee as a part of the biochemistry community.

Presentations

2019/4:
Title: Cloning and purifying a GSK3B and DISC1 complex.
Honors Research Project Presentation, Saskatoon, Canada
Description: For this presentation the honors students in the Biochemistry department at the University of Saskatchewan presented their undergraduate research project. The presentation was a short 10-15 presentation with questions.

2023/02:
Title: The role of the Prc1 complex in skeletal muscle myogenesis.
OHRI trainee presentations, Ottawa, Canada
Description: For this presentations I presented the key data I had collected for my master's program at the time, and presented it to the department at the Ottawa Hospital Research Institute. The presentation was 20 minutes long, with a 10 minute question period at the end.

Publications

Journal Articles

1. Massenet J, Gardner E, Chazaud B, Dilworth FJ. Epigenetic regulation of satellite cell fate during skeletal muscle regeneration. Skelet Muscle. 2021 Jan 11;11(1):4. doi: 10.1186/s13395-020-00259-w. PMID: 33431060; PMCID: PMC7798257.