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Investigating the Function of Emerin during Skeletal Muscle
Differentiation in Human Stem Cells

by
Tracy Knight

DISSERTATION

Submitted in partial satisfaction of the requirements for degree of
DOCTOR OF PHILOSOPHY

in

Biochemistry and Molecular Biology

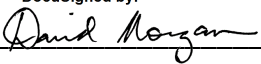
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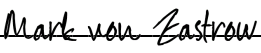
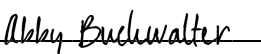
GRADUATE DIVISION

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Contributions

Chapter 2 is adapted from an unpublished manuscript with the following authors: Tracy A. Knight, Lucia Rossi, Jessica Mella, Joao Carrara, Helen Miranda, F. Saverio Tedesco, Abigail Buchwalter.

Chapter 3 is a summary of unpublished work with contributions from Jessica Mella.

Chapter 4 is a summary of unpublished work with contributions from Amy Chang and Adam Litterman.

“Listen to me, sister... Remember this: Nothing is written in the stars. Not these stars, nor any others. No one controls your destiny.”

-Gregory Maguire, *Wicked: The Life and Times of the Wicked Witch of the West*

Investigating the Function of Emerin during Skeletal Muscle Differentiation in Human Stem Cells

Tracy A. Knight

Abstract

The nuclear periphery is critical to establishing eukaryotic cell fate. The inner nuclear membrane (INM) protein emerin has been shown to play a role in cardiac and skeletal muscle development and maintenance. Mutations in the protein lead to X-linked Emery-Dreifuss Muscular Dystrophy, a degenerative disease characterized by both muscle wasting and cardiomyopathy. However, emerin-null mice do not develop muscular dystrophy phenotypes, making *in vivo* murine models impractical. To gain insight into emerin's functions in human cardiac and skeletal muscle, we need a tractable human model system. Here, we have adapted an induced skeletal muscle (iSM) model system to efficiently direct skeletal muscle differentiation from human induced pluripotent stem cells (hiPSCs).

Chapter 1 provides an overview of the nuclear periphery, skeletal muscle development, and current understanding of emerin function. Chapter 2 describes the use of iSMs to define the role of emerin in myogenesis. Chapter 3 summarizes experiments investigating the mechanism by which emerin regulates the Wnt effector β -catenin. Lastly, Chapter 4 highlights the challenges of using combinatorial CRISPR screening to investigate redundancy among LEM family proteins. This work establishes emerin's requirement during late myogenesis as well as the consequences of its loss in gene regulation and expression.

Table of Contents

Chapter 1: Introduction	1
The Nuclear Periphery	1
LEM Domain Proteins	2
Laminopathies & Emery-Dreifuss Muscular Dystrophy	3
Skeletal Muscle Development	5
Chapter 2: Characterization of emerin function during myogenesis in human iPSC-derived myotubes	8
Abstract	8
Introduction.....	9
Results.....	11
Discussion.....	20
Methods	23
Figures	31
Chapter 3: Determining the regulatory role of nuclear membrane protein emerin in functional pools of β-catenin	52
Abstract.....	52
Introduction.....	52
Results.....	53
Discussion.....	58
Methods	59
Figures	60

Chapter 4: Technical development of a combinatorial screen of LEM domain proteins	
using multiAsCas12a	68
Abstract.....	68
Introduction.....	68
Results.....	69
Discussion.....	71
Methods	71
Figures	73
Chapter 5: Conclusion and Future Directions	75
References	78

List of Figures

Figure 2.1. Emerin loss impairs iSM differentiation.....	31
Figure 2.2. Transgene-positive nuclei exhibit delayed cell cycle exit upon emerin loss.	33
Figure 2.3. p21 expression is diminished in iSMs upon emerin loss.	35
Figure 2.4. MyoD activation is unaffected by emerin loss.	37
Figure 2.5. Emerin loss leads to upregulation of proliferative genes and downregulation of myogenic differentiation genes.	38
Supplementary Figure 2.1. Validation of iSM immunodepletion protocol.	40
Supplementary Figure 2.2. Validation of <i>EMD</i> KO and <i>LMNA</i> KO hiPSCs.	42
Supplementary Figure 2.3. Additional <i>EMD</i> ^{KO} iSM differentiation defects and characterization of transgene, <i>EMD</i> transcript, and variable p21 levels.	43
Supplementary Figure 2.4. Additional <i>EMD</i> KO clones have a skeletal muscle differentiation defect.	45
Supplementary Figure 2.5. Myogenin expression is decreased in <i>EMD</i> KO iSMs.	46
Supplementary Figure 2.6. Analysis of transcriptional changes after <i>EMD</i> KO in Day 6 iSMs.	48
Supplementary Figure 2.7. Analysis of rescue-sensitive gene distribution among transcription factor targets, LADs, and H3K27me3-positive regions.	49
Figure 3.1. <i>EMD</i> null MEFs are more sensitive to Wnt induction.	60
Figure 3.2. U2OS response to Wnt activation is unaffected by <i>EMD</i> mutation.	62
Figure 3.3. <i>EMD</i> KO iPSCs are less sensitive to Wnt activation with LiCl.	63
Figure 3.4. Endogenous emerin co-immunoprecipitates with β -catenin.	64

Figure 3.5. Early atrial cardiomyocyte differentiation does not induce differences in Wnt response.....	66
Figure 3.6. Verification of additional Wnt inhibition phenotypes.	67
Figure 4.1. Analysis of multiAsCas12a knockdown efficiency.....	73
Figure 4.2. Determining the efficacy of all predicted <i>TMPO</i> sgRNA sequences	74

List of Tables

Supplementary Table 2.1. List of RT-qPCR and genotyping primers.	50
Supplementary Table 2.2. List of antibodies.	51

List of Abbreviations

EDMD: Emery-Dreifuss muscular dystrophy

H3K27me3: Tri-methylated histone 3 at lysine 27

H3K9me2: Di-methylated histone 3 at lysine 9

INM: Inner nuclear membrane

iPSC: inducible pluripotent stem cell

iSM: inducible skeletal muscle

LAD: Lamin-associated domain

LEM: LAP2-Emerin-MAN1 domain

MEF: Mouse embryonic fibroblast

PRC2: Polycomb repressive complex 2

Chapter 1: Introduction

The Nuclear Periphery

The nucleus is a critical and defining organelle of the eukaryotic cell. Beyond simply separating DNA from the cytosol, proper function of its sub-compartments is required to execute genetic programs. The nuclear periphery is the site of many transduced mechanical and chemical signals. It is comprised of the inner and outer nuclear membranes which are contiguous with endoplasmic reticular (ER) membrane, nuclear pores which allow import and export of material to and from the nucleus, and a meshwork of type V intermediate filaments known as the nuclear lamina.^{1,2} These intermediate filaments, called lamins, are encoded by the *LMNB1*, *LMNB2*, and *LMNA* genes in humans, the latter of which encodes both Lamin A and C splice isoforms.^{3–5} The nuclear lamina plays a critical role in many cellular processes including DNA replication and repair, transcriptional regulation, signal transduction, linkage of the nucleo- and cytoskeletons, and many others.^{6–9} One function of the lamina which is conserved across metazoa is the organization and compaction of chromatin.^{10–13}

There are approximately two meters of DNA within each cell's nucleus.¹⁴ To sufficiently condense it to fit inside an organelle just a few microns in diameter, DNA is wrapped around histone octamers which, altogether, each form a nucleosome containing 147 base pairs.¹⁵ Nucleosomes are compacted further to form the chromatin fiber. Chromatin is found in two distinct states: euchromatin, or “open,” transcriptionally active chromatin and heterochromatin, or “closed,” transcriptionally inactive chromatin.¹⁶ These states can be influenced via chromatin modification of specific histone residues. Certain modifications are associated with

heterochromatin, such as tri-methylation of lysine 27 on histone 3 (H3K27me3), while other modifications are associated with euchromatin, such as acetylation of the same site (H3K27ac).¹⁷ Chromatin modifiers are responsible for depositing these chromatin marks through which they regulate chromatin state. For example, polycomb repressive complex 2 (PRC2) deposits H3K27me3 marks, while the methyltransferase heterodimer G9a/GLP deposits H3K9me2.^{18–21} Chromatin state is also altered by ATP-dependent chromatin remodelers, which physically shift and slide histones along the DNA to adjust the accessibility of specific loci.^{22,23}

Heterochromatin is frequently associated with the nuclear periphery, while euchromatin tends to associate with the nuclear interior.^{8,24,25} The heterochromatic layer at the nuclear periphery is highly conserved among most eukaryotes.²⁶ Through use of the technique DamID (DNA adenine methyltransferase identification), regions of heterochromatin often found at the periphery have been identified, known as lamin associated domains (LADs).^{27,28} Association with LADs has been shown to be critical for development and in determining cell identity.²⁹

LEM Domain Proteins

The nuclear lamina is functionalized by proteins of the inner nuclear membrane (INM). The LAP2-Emerin-MAN1 (LEM) family of INM proteins in particular plays a critical role in mechanotransduction and nuclear integrity.^{30–32} These proteins share a conserved LEM domain which binds the chromatin crosslinker barrier-to-autointegration factor (BAF).^{33–36} Many LEM proteins have also been implicated in various signal transduction pathways necessary for mammalian development. For example, LAP2 β is associated with the Hedgehog (Hh) signaling pathway, while MAN1 has been shown to regulate TGF β signaling in humans.^{37,38}

The LEM protein emerin has been shown to play a role in cardiac and skeletal muscle development and maintenance.³⁹ Encoded by the *EMD* gene (previously known as *STA*), the 34-kDa, 254 amino acid protein contains a conserved N-terminal LEM domain and a single pass transmembrane domain which anchors it to the INM.⁴⁰ Emerin also binds directly to the nuclear lamina to form a ternary emerin-BAF-lamin A/C complex via its lamin-binding domain, which is predicted to be largely unstructured.^{33,41,42} It has also been proposed to bind myriad other factors within the nucleus, including GCL, β -catenin, YT521-B, Lmo7, Msx1, and HDAC3.^{43–49}

Emerin function requires its localization to the nuclear periphery.⁵⁰ Upon synthesis and insertion into the ER membrane, emerin localizes to the INM via lateral diffusion. Its localization and retention are dependent on the LEM domain as well as the presence of Lamin A within the nuclear lamina.⁵¹ It has also been shown to bind components of the Linker of Nucleoskeleton and Cytoskeleton (LINC) complex, which transduces mechanical cues from the cytoplasm and extracellular matrix to the nucleus.^{52–55} There has been increasing evidence over the past several decades that emerin influences the expression of specific genes. How it achieves this remains unclear, though it has been implicated in interactions with certain transcription factors and chromatin modifiers.^{43–49}

Laminopathies & Emery-Dreifuss Muscular Dystrophy

Disruption of many nuclear envelope proteins leads to a collection of diseases known as laminopathies. They are characterized by a number of different pathologies, including cardiomyopathy, muscular dystrophy, lipodystrophy, neuropathy, dysplasia, and/or premature aging.³⁷ A majority of these are caused by mutations in the *LMNA* gene, however etiologies have also been linked to mutations in genes encoding other nuclear peripheral proteins such as *SYNE1*,

ZMPSTE24, *LBR*, and *EMD*.⁵⁶ Despite emerin's ubiquitous expression throughout the body, cardiac and skeletal muscle are particularly susceptible to deterioration upon its mutation.⁵⁷

Mutations in emerin lead to X-linked Emery-Dreifuss Muscular Dystrophy (EDMD1), a degenerative disease characterized by early onset muscle contractures, progressive muscle wasting, and hypertrophic cardiomyopathy.^{57–60} It was first described in 1966 by Emery and Dreifuss who observed a congenital dystrophy within a large family in Virginia, likening it to Duchenne and Becker muscular dystrophies.⁵⁹ EDMD is the third most common hereditary muscular dystrophy, with a prevalence of 0.39 in 100,000 people worldwide reported in 2015.⁶¹ The average age of first observable symptoms is 5.6 years old with a life expectancy of 53.7 years; cause of death often stems from cardiac complications.⁶² In 1986, the cause of EDMD1 was attributed to mutations in the *EMD* gene sequence,⁶³ which was eventually mapped and cloned in 1994.⁵⁸ Years later, exon 2, which partially encodes the LEM domain, was identified as a hotspot for EDMD1-causing mutations.⁶⁴ Additional forms of the disease such as autosomal dominant or recessive EDMD (EDMD2/3/4) can be caused by mutations in other emerin- and lamina-associated genes such as *LMNA*, *SYNE1*, and others.^{57,65}

Most disease-causing mutations are destabilizing to the emerin protein and lead to its rapid degradation.^{66,67} Many *in vitro* and *in vivo* models of EDMD1 are consequently *EMD* null, however, it has proven difficult to model EDMD1 *in vivo*. The traditional mouse model fails to recapitulate phenotypes observed in the human disease due to functional compensation by the INM protein LAP1, which is more highly expressed in murine muscle than in human muscle.^{68,69} In the absence of a standard preclinical model, we still have a poor understanding of the

functional mechanism of EDMD1 and even less understanding of potential treatments beyond gene replacement therapies.⁷⁰

Skeletal Muscle Development

Skeletal muscle development requires a specific cascade of differentiation factor expression for proper embryonic development as well as for postnatal muscle repair.⁷¹ In the developing embryo, skeletal muscle is derived from the paraxial mesoderm.⁷² Notably, the pluripotent cell population from which this population is derived is characterized by high expression of genes such as *NANOG*, *SOX2*, and *POU5F1* (encoding Oct4).⁷² Primary and secondary myogenesis (multiple rounds of proliferation and lineage specification) occur during embryogenesis to generate functional muscle fibers. Postnatal myogenesis relies on a progenitor cell niche known as satellite cells.

In the developing embryo, *PAX3* is one of the earliest markers of myogenic precursor cells.⁷³ *PAX7*, on the other hand, marks the proliferative satellite cell niche postnatally. Once induced to differentiate after injury, hypertrophy, or other molecular cues, these cells will express *MYF5* and the “master regulator” of myogenesis, *MYOD1*. Additional signals are required to fully activate the myogenic program, however. In cycling cells, the retinoblastoma protein (Rb) is hyperphosphorylated, allowing E2F transcription factors to activate downstream cell cycle factors.⁷⁴ This activation upregulates HDAC1 and Cyclin/cyclin-dependent kinase (Cdk) activity, which inhibit MyoD activity through its de-acetylation and phosphorylation, respectively.⁷⁵ Upon initiation of the myogenic program, MyoD is activated through dephosphorylation by Dusp13/27 and simultaneous acetylation by p300/CBP.^{75,76} Upregulation of the Cdk inhibitor p21 and the subsequent hypo-phosphorylation of Rb are both critical regulatory steps in ensuring proper cell

cycle exit.⁷⁷ Chromatin state generally undergoes a variety of changes during differentiation as well. The chromatin remodeler BAF60C (unrelated to the chromatin crosslinker BAF) is specifically induced during myogenesis and primes the genome to express muscle lineage-specific genes.^{78,79}

Terminally differentiating myocytes begin to express later-stage factors like Myogenin which works in tandem with MyoD to activate remaining late myogenic factors. They will also begin to express a subset of cytoskeletal myosin heavy chains (MyHC). Myocytes then fuse, a process that requires the proteins Myomerger and Myomaker.^{80–82} Defects in this fusion and syncytium formation lead to Carey-Fineman-Ziter syndrome, a congenital myopathy characterized by muscle weakness, skeletal abnormalities, developmental delays.⁸³ As multinucleated myotubes, they will continue assembling parallel to each other to form larger myofibers that comprise the full muscle.

EDMD1-causing mutations impair skeletal muscle differentiation, but do not cause any known developmental defects during embryogenesis. This suggests that emerin is not necessary for primary/secondary myogenesis but is required for postnatal myogenic maintenance. Previous work in primary mouse myoblasts suggested that emerin played a role in the transition from cycling myoblasts to terminally differentiated myotubes via the Rb1/E2F and MyoD pathways, respectively.^{69,84} Interestingly, the LEM protein LAP2 α has been shown to regulate cell cycle progression by interfering with the Rb-E2F pathway.^{85,86} More recent studies have investigated the role of emerin in HDAC3 function in mouse myogenic progenitors.^{48,87–89} However, because emerin-null mice do not develop EDMD1 symptoms, mouse-derived cells may not be a suitable

model for investigating disease mechanism. Previously established mechanisms of emerin function in myogenesis have yet to be confirmed in human cells.

In this thesis, we will explore the use of an iPSC-derived model to study emerin function in a specifically human context *in vitro*. Using this model, we will investigate the mechanism by which emerin influences skeletal muscle differentiation and maintenance.

Chapter 2: Characterization of emerin function during myogenesis in human iPSC-derived myotubes

Abstract

Emerin plays a critical role in postnatal skeletal muscle differentiation, but its underlying function is still not fully understood. Studying this mechanism has proven difficult because emerin mutant mouse models develop little to no human-like phenotypes. Here, we establish an adapted protocol to develop human iPSC-derived inducible skeletal muscle (iSMs) as a tractable, *in vitro* model to study emerin loss in a relevant human context. Surprisingly, overexpression of the master myogenesis regulator MyoD is not sufficient to rescue a loss of emerin during myogenesis as previously hypothesized. Cells differentiated via induction with a MyoD-BAF60C transgene are less likely to form mature myotubes in *EMD* KO cells compared to wildtype, which can be fully rescued by re-expression of wildtype emerin into KO cells. *EMD* KO cells display an impaired ability to exit the cell cycle ahead of terminal differentiation and erroneously downregulate critical myogenic factors like p21 and myogenin. MyoD activation remains unchanged upon *EMD* KO, however, as determined by its acetylation and association with co-transcription factors E12/E47 (E2A). Targets of Polycomb Repressive Complex 2 (PRC2), which deposits heterochromatic H3K27me3 marks, are enriched among emerin-sensitive genes identified by RNA-seq. Further, while these genes are predicted to only be moderately enriched in lamin-associated domains, they are predicted to be highly enriched for H3K27me3. Altogether, we observe a requirement for emerin in regulating PRC2-target gene expression downstream of MyoD activation in human iSMs.

Introduction

Emery-Dreifuss muscular dystrophy (EDMD) is the third most common hereditary muscular dystrophy, and is characterized by muscle wasting, limb contractures, and cardiomyopathy.⁶⁰

EDMD is caused by mutations to several proteins that make up a nuclear structure known as the nuclear lamina. The nuclear lamina protects the nucleus from external forces, connects the nucleus to the cytoskeleton, and scaffolds chromatin organization.⁹⁰ The lamina is composed of the lamins, polymer-forming intermediate filament proteins that form a meshwork underneath the inner nuclear membrane (INM). The lamin meshwork is functionalized and bound by various soluble and membrane proteins. While the nuclear lamina exists in some form in all mammalian cell types, cardiac and skeletal muscle are uniquely vulnerable to mutations to lamina components. Mutations to the *LMNA* gene or to genes that encode lamin-binding proteins (*EMD*, *SYNE1*) cause EDMD and/or other related syndromes.⁹¹

Emerin is a broadly expressed inner nuclear membrane (INM) protein and an important component of the nuclear lamina. Loss-of-function mutations to the *EMD* gene, which is located on the X chromosome, cause an X-linked recessive form of EDMD.^{60,92} Emerin is a member of a small family of INM proteins that share a conserved LAP2 β -emerin-MAN1 (LEM) domain fold that binds to the DNA-crosslinking protein BAF (barrier-to-autointegration factor; also known as BANF1).³⁰ Emerin also binds directly to lamin A/C via an unstructured domain to form a ternary emerin-BAF-lamin A/C complex.⁹³ In addition to participating in this high-affinity complex, emerin has been linked to many other cytoskeletal, signaling, and chromatin components.^{44,45,48,94}

It has proven difficult to place emerin's functions into the disease-relevant context of skeletal muscle. Attempts to define the consequences of emerin loss of function in murine models have

delivered mixed results. The *Emd*^{-/-} mouse does not develop muscular dystrophy and instead exhibits only subtle cardiac and motor coordination phenotypes,^{69,95} yet *Emd*^{-/-} muscle progenitors exhibit differentiation defects when cultured *ex vivo*.^{69,96,97} These inconsistencies may be due to functional compensation by lamina-associated polypeptide 1 (LAP1), which is more highly expressed than emerin in murine, but not human, muscle.⁶⁸ To understand the unique vulnerability of human skeletal muscle cells to emerin loss of function, we require a tractable human model system.

Embryonic development of skeletal muscle begins with mesodermal specification, followed by the development of myogenic progenitors.⁷² The transcription factors MyoD and Myf5 drive commitment of progenitors to myoblasts and induce the myogenin transcription factor, which in turn drives terminal differentiation of myoblasts into multinucleated myotubes.⁷² Post-natal muscle growth and repair both involve the activation of a tissue-resident pool of quiescent muscle progenitors, often referred to as satellite cells. Upon activation, satellite cells express MyoD and briefly re-enter the cell cycle before terminally differentiating in response to myogenin expression.⁷² MyoD and Myf5 have overlapping functions in myoblast fate commitment,⁹⁸ while myogenin is uniquely essential for terminal muscle differentiation.^{99,100} This cell cycle to terminal differentiation transition is critically mediated by Polycomb Repressive Complex 2 (PRC2). PRC2 methylates histone 3 at lysine 27 (H3K27), which promotes heterochromatin compaction and decreases target gene expression. It is necessary to prevent premature differentiation in cycling cells as well as inhibit cell cycle re-entry in differentiated myotubes.^{101–103} BAF60C, a subunit of the SWI/SNF chromatin remodeling complex, is highly expressed in skeletal muscle and additionally plays a critical role in

facilitating MyoD-dependent gene transcription. BAF60C is also notably not present in pluripotent cells.^{78,104,105}

Emerin has been implicated in the regulation of MyoD and Myf5 expression,⁸⁷ suggestive of a role at the myoblast commitment stage. On the other hand, cultured *Emd*^{-/-} mouse myoblasts exhibit impaired terminal differentiation propensity,^{69,96,97} suggesting a role in the terminal differentiation cascade downstream of earlier MyoD and Myf5 function. Here, we directly test emerin's role in terminal muscle differentiation by adapting a transgene-driven directed differentiation protocol in human induced pluripotent stem cells (hiPSCs).^{105,106} In this induced skeletal muscle (iSM) approach, ectopic expression of MyoD and BAF60C in hiPSCs bypasses mesodermal differentiation and induces direct commitment to the myogenic lineage and formation of terminally differentiated, multinucleated myotubes. We generate iSMs from *EMD*^{-/-} and *LMNA*^{-/-} hiPSCs and demonstrate that loss of either *EMD* or *LMNA* blunts the ability of MyoD to drive myotube differentiation.

Results

Establishing a human induced skeletal muscle (iSM) culture model

Directed differentiation of hiPSCs is a powerful approach for modeling the functions of disease-linked genes in relevant cell types.¹⁰⁷ In this study, we establish a tractable *in vitro* model of emerin loss of function in human muscle. As cultured *Emd*^{-/-} mouse myoblasts exhibit impaired terminal differentiation propensity,^{69,96,97} we hypothesized that loss of emerin impairs the progression of human myoblasts through the terminal differentiation cascade downstream of MyoD and Myf5. To test this prediction, we adapted a transgene-driven induced skeletal muscle

(iSM) protocol in human induced pluripotent stem cells (hiPSCs).^{105,106} In this approach, ectopic expression of MyoD and the BAF60C subunit of the SWI/SNF chromatin remodeler in hiPSCs bypasses mesodermal differentiation and induces direct commitment to the myogenic lineage and subsequent formation of terminally differentiated, multinucleated myotubes (Fig. 1A). We constructed a single tetracycline-inducible cassette containing transgenes encoding the mouse MyoD and human BAF60C2 open reading frames separated by a P2A sequence to ensure proportional expression of each iSM-driving transgene. This cassette was then randomly integrated into the genome of WTC-11 hiPSCs by PiggyBac-mediated transposition.

To induce myotube formation, cells were grown in HuES medium with doxycycline for 2 days, then replated and grown in differentiation medium with doxycycline for up to 5 days (see Methods) (Fig. 1A). Earlier versions of iSM protocols involved a 2-day pulse of doxycycline in hESCs followed by embryoid body aggregation.¹⁰⁵ In hiPSCs, consistent with more recent reports by the protocol developers¹⁰⁶ and others,¹⁰⁸ we find that efficient iSM induction requires constitutive induction of MyoD and BAF60C expression throughout the time course.

Doxycycline treatment induces the appearance of myosin heavy chain (MyHC)-expressing cells within 3 days that fuse and mature into multinucleated, elongated myotubes over the next several days (Fig. 1B). After 6 days of iSM induction, approximately 90% of transgene-positive nuclei reside within MyHC-positive myotubes (Fig. 1D), demonstrating efficient induction of skeletal muscle differentiation.

Proliferating clusters of transgene-negative cells were sometimes observed (for example, see DAPI-stained, transgene-negative clusters at day 4 and day 5, Fig. 1B). As these cells lack the MyoD/BAF60C transgene, they presumably remain in a stem-like state and continue to

proliferate. These cells can be identified as transgene-negative by MyoD/BAF60C immunostaining and microscopy and excluded from further analyses. We determined that the pluripotency-specific cell surface marker CD57¹⁰⁹ is highly expressed by transgene-negative stem cell clusters, but not by transgene-positive mononucleated cells or mature myotubes (Suppl. Fig. 1) and confirmed that CD57 immunodepletion specifically removed stem cells from differentiated populations, yielding enriched populations of differentiated iSMs (Suppl. Fig. 1) This approach enables bulk analyses without contamination by transgene-negative cells.

Establishing EMD-null and EMD rescue hiPSCs

EDMD1 exhibits recessive inheritance,⁶⁰ and numerous truncating, frameshift, or missense mutations throughout the *EMD* gene are anticipated to destabilize the *EMD* transcript and/or protein.^{64,92} These features are characteristic of a loss-of-function disease mechanism. We therefore reasoned that *EMD* knockout (KO) would be an effective means to model EDMD1 muscular phenotypes *in vitro*. We also desired a means to efficiently conduct rescue experiments by re-introducing *EMD* or other genes of interest into *EMD* KOs. We accomplished both goals by CRISPR/Cas9 editing as described previously¹¹⁰ and summarized here. First, we introduced a dual integrase cassette exchange (DICE) landing pad¹¹¹ into one allele of the *AAVS1* safe harbor locus^{110,112} to generate WTC-11 DICE landing pad hiPSCs (referred to as parental). Next, we knocked out the *EMD* locus in the DICE hiPSCs, selecting two validated *EMD* KO DICE clones for further analysis (Suppl. Fig. 2). We also selected a CRISPR control clone for comparison (Suppl. Fig. 2). Finally, we confirmed that insertion of the emerin ORF into the *EMD* KO DICE landing pad resulted in constitutive rescue of emerin to a level slightly exceeding endogenous expression (Suppl. Fig. 2).

EMD-null and LMNA-null hiPSCs are less responsive to skeletal muscle induction

To evaluate the responsiveness of *EMD* KO cells to MyoD/BAF60C-induced differentiation, we integrated the iSM cassette into each clone of *EMD* KO hiPSCs and induced iSM differentiation with doxycycline. In both *EMD* KO clones, we noted a significant decrease in the proportion of transgene-positive cells that produced MyHC-expressing myotubes compared both to WT parental hiPSCs and to a CRISPR control hiPSC clone (Fig. 1C,D; Suppl Fig. 3); we further validated this phenotype in two independent *EMD* KO clones (Suppl. Fig. 4). This myogenic defect is not a consequence of reduced transgene expression, as MyoD and BAF60C are expressed at comparable levels in both genotypes (Suppl. Fig 3). Further, exogenous (mouse) MyoD efficiently induced endogenous (human) MyoD transcript expression in both WT and *EMD* KO cells (Fig. 4B), indicating that MyoD can drive its own feed-forward activation¹¹³ in both contexts. Importantly, iSM differentiation of *EMD* KO clones is fully rescued by re-expression of WT emerin via DICE integration at the *AAVS1* locus (Fig. 1C,D; Suppl. Fig 3).

As mutations to both *EMD* and *LMNA* cause EDMD, we next compared the consequences of *LMNA* ablation on iSM differentiation. To achieve this, we generated *LMNA* KO DICE landing pad hiPSCs as described above (Suppl. Fig. 2), then integrated the iSM cassette and induced differentiation. Transgene-expressing *LMNA* KOs are similarly impaired in their ability to produce MyHC-expressing myotubes, and this phenotype is again rescued by re-expression of WT Lamin A (Fig. 1E,F). Altogether, these data indicate that *EMD* and *LMNA* each enhance the efficiency of terminal skeletal muscle differentiation induced by MyoD and BAF60C.

EMD-null iSMs exhibit delayed cell cycle exit

As cell cycle exit normally precedes myotube fusion and MyHC expression, we evaluated proliferation in *EMD* KO iSMs by pulse-labeling cultures with 5'ethynyl-2'-deoxyuridine (EdU) at each day of the iSM differentiation time course (see Methods) (Fig. 2A). This analysis revealed a two-stage response in WT and *EMD* rescue iSMs. After replating in differentiation medium, MyoD and BAF60C-expressing cells undergo a drop in their proliferation rate (Fig. 2B-C, compare day 1 to day 2.5; from ~100% to ~40% MyoD/BAF60C cells EdU-positive). Over the following days, EdU labeling of transgene-expressing cells continues to decline (~20% MyoD/BAF60C cells EdU-positive at day 6). In the *EMD* KOs, the transgene-expressing cells respond similarly to WTs in the first phase (Fig. 2B-C, compare day 1 to day 2.5). However, transgene-expressing *EMD* KOs do not participate in the second phase of cell cycle exit and instead maintain a steady proportion of EdU-positive cells over the remainder of the iSM time course (Fig 2B-C; ~40% MyoD/BAF60C cells remain EdU positive through day 6). This abnormal proliferation was observed only in MyHC-negative cells, while MyHC-positive myotubes were nonproliferative (Fig. 2B, inset).

These results are consistent with prior reports in the *Emd*^{-/-} mouse model, where regenerating *Emd*^{-/-} muscle and cultured *Emd*^{-/-} myoblasts each showed transcriptional signatures of impaired cell cycle exit, including elevated expression of E2F target genes.⁶⁹ E2F-family transcription factors are normally inactivated by interaction with hypo-phosphorylated Rb during cell cycle exit. *Emd*^{-/-} mouse myoblasts exhibit elevated levels of hyper-phosphorylated Rb,⁶⁹ implying that Rb-mediated cell cycle exit may be impaired by emerin loss. To test whether Rb is also dysregulated in *EMD* KO human iSMs, we analyzed enriched day 6 iSMs. These analyses

revealed a moderate accumulation of hyperphosphorylated Rb in *EMD* KO iSMs that was reverted in *EMD* rescue iSMs (Fig. 2D-E).

MyoD promotes cell cycle exit by driving expression of the Cdk-inhibitory protein p21 (encoded by the *CDKN1A* locus).^{114,115} We tracked p21 induction over each day of differentiation in WT, *EMD* KO, and *EMD* rescue iSMs (Fig. 3A). Replating in differentiation medium quickly induced p21 expression in MyoD/BAF60C transgene-expressing cells, but to a lesser extent in *EMD* KOs than in WT or *EMD* rescue iSMs (Fig. 3A-B, compare day 1 to day 2). Levels of p21 expression remained persistently lower in *EMD* KOs through the differentiation time course (Fig. 3A-B, days 4 through 6). Similarly, transgene⁺ cells expressed less p21 in *EMD* KOs than WT over the course of differentiation (Fig. 3C). This effect was also apparent in bulk measurements of p21 expression in enriched iSM populations at both the RNA (Fig. 3D) and, more variably, at the protein level (Fig. 3E; Suppl. Fig. 3). This blunted p21 expression mirrors the persistence of EdU-labeled proliferating cells in *EMD* KO iSMs (Fig. 2). We also find that the expression of p57 (encoded by the *CDKN1C* locus), a related CIP that functions along with p21 in muscle differentiation, is moderately decreased.¹¹⁶ These findings imply that *EMD* KO iSMs are unable to establish and/or maintain cell cycle exit in response to pro-differentiation signals.

MyoD activation is unimpaired in EMD KO iSMs

The decrease of p21 expression in the absence of emerin (Fig. 3) could reflect impaired transactivation by MyoD, as the p21-encoding locus *CDKN1A* is a direct transcriptional target of this TF.^{114,115,117} MyoD's activity is controlled by a post-translational modification switch: Cdk phosphorylation in proliferating cells restrains MyoD activity, while acetylation promotes MyoD activity.^{118,119} MyoD acetylation can accumulate only in the absence of inhibitory

phosphorylation.¹¹⁹ To explore emerlin's potential influence on MyoD regulation, we immunoprecipitated MyoD from day 6 iSMs. Surprisingly, MyoD was robustly acetylated in WT, *EMD* KO, and *EMD* rescue iSMs (Fig. 4A). MyoD also forms a high-affinity, transcriptionally active heterodimer with the E12/E47 (also known as E2A) E-box proteins¹²⁰; we detect this robust interaction equivalently in WT, *EMD* KO, and *EMD* rescue iSMs (Fig. 4A). These data, along with the observation that the (mouse) MyoD transgene can effectively transactivate the endogenous (human) MyoD locus in each genotype (Fig. 4B), imply that emerlin loss does not impair MyoD activation.

Myogenin expression is decreased in EMD KO iSMs

Myogenin is a late, direct transcriptional target of MyoD^{99,100} that is essential for terminal muscle differentiation.⁷² We noted that *MYOG* transcript levels were decreased in *EMD* KO iSMs and rescued by re-expression of *EMD* (Suppl. Fig. 5D). We therefore evaluated myogenin expression over the course of differentiation in WT, *EMD* KO, and *EMD* rescue iSMs (Suppl. Fig. 5A). Transgene+ nuclei did not fully co-express myogenin until the final days of differentiation, consistent with the role of this transcription factor in terminal differentiation. Transgene+ nuclei expressed myogenin in similar proportions in WT, *EMD* KO, and *EMD* rescue iSMs (Suppl. Fig. 5B). However, there was a small but significant decrease in the proportion of myogenin+ nuclei contributing to MyHC+ myotubes in *EMD* KOs (Suppl. Fig. 5C), comparable to the proportional decrease of transgene+, MyHC+ nuclei that we observed in *EMD* KOs (Fig. 1). These results indicate that *EMD* loss moderately impairs the expression and/or activity of myogenin.

Transcriptional signatures of impaired cell cycle exit and differentiation in EMD KO iSMs

To probe the effects of *EMD* loss on the iSM transcriptome, we performed RNA-sequencing (RNAseq) on three biological replicates of enriched day 6 WT, *EMD* KO, and *EMD* rescue iSMs. There was a strong, positive correlation ($R^2 = 0.737$) between genes differentially expressed in *EMD* KO iSMs and genes responsive to *EMD* rescue, indicating that re-introduction of *EMD* generally brings expression of these genes back towards a WT state (Fig. 5A). In total, we identified 626 DEGs that were differentially expressed by at least 1.5-fold in *EMD* KOs and rescued by re-introduction of *EMD*, which we refer to hereafter as rescue-responsive genes. Gene Set Enrichment Analysis (GSEA) revealed enrichment of cell cycle regulators among genes upregulated in *EMD* KOs (Fig. 5B), such as *AURKA*, *CCNA2*, and *CDK1*, consistent with our observations of impaired cell cycle exit (Fig. 2). We also observe upregulation of homeobox genes critical to embryonic development like *DLX4* and *SIX3* (Fig. 5A). Amongst genes downregulated in *EMD* KOs, GSEA identified factors related to skeletal muscle function and terminal differentiation (Fig. 5B), including muscle-specific myosins (*MYH3*, *MYH7*, *MYH7B*, *MYH8*), transcription factors (*MYOG*), and CIPs (*CDKN1A*, *CDKN1C*) (Supl. Fig. 6).

Dysregulated genes are not enriched in lamina-associated domains (LADs)

The nuclear lamina scaffolds heterochromatic lamina-associated domains (LADs) which cover approximately 40% of the genome. Due to emerin's association with the lamina, we hypothesized that rescued genes may be enriched within LADs. To assess this, we compared our list of rescue-responsive genes to Lamin A and Lamin B1 genome-binding datasets deposited in the ChIP-Atlas database.¹²¹ Surprisingly, this analysis did not indicate an enrichment of rescued

genes within LADs associated with either Lamin A or Lamin B1. While many LADs are constitutive, their genomic coverage does vary across cell types. Therefore, to confirm this result in muscle cells, we compared our list of rescue-responsive genes with published DamID LAD maps from murine C2C12 myoblasts and myotubes¹²² and from human myotubes and myoconverted fibroblasts.¹²³ These comparisons indicated that upregulated rescue-responsive genes were modestly enriched within LADs (Suppl. Fig. 7). However, as many dysregulated genes are not found within LADs, we conclude that gene expression changes induced in *EMD* KO iSMs cannot be explained solely by LAD disruption.

E2F, bHLH, and PRC2 targets are enriched among dysregulated genes in EMD KO iSMs

To conduct an unbiased search for candidate transcription factors or co-regulators that underlie differential gene expression in *EMD* KO iSMs, we compared our list of rescued genes to the full breadth of genome-binding datasets in the ChIP-Atlas database: 1,597 targets in total, including various transcription factors, chromatin modifiers, and chromatin architectural proteins. Top factor categories targeting these genes included the bHLH family of myogenic transcription factors, the E2F-DREAM cell cycle regulatory pathway, and polycomb repressive complex 2 (PRC2) (Fig. 5C-E). bHLH TF targets included genes involved in terminal muscle differentiation, which were mainly downregulated; in contrast, E2F targets included a cluster of upregulated, pro-proliferative genes. PRC2 targets included both up- and down-regulated genes. These findings suggest a general defect in PRC2-dependent gene regulation in *EMD* KO iSMs.

Upregulated genes are enriched for H3K27me3

The PRC2 complex is responsible for the di- and tri- methylation of H3K27.¹⁰³ To further explore the relationship between *EMD* loss and dysregulation of PRC2 target genes, we cross-referenced our rescue-responsive genes to publicly available ChIP-seq datasets mapping H3K27me3 occupancy in human myotubes.^{124–126} This analysis revealed that genes upregulated in *EMD* KO are strongly enriched for H3K27me3 in normal myotubes (Fig. 5F). Genes downregulated in *EMD* KO also show moderate enrichment for H3K27me3 (Fig 5F; Suppl. Fig. 6). Both H3K27me3 and lamin associated domains (LADs) demarcate heterochromatic domains,^{127,128} but their correlation with each other is highly cell-type and context dependent.^{129–131} While we noted that upregulated *EMD*-sensitive genes are modestly enriched in myotube LADs, cross-referencing LAD and H3K27me3 datasets revealed that it is actually the subset of LADs that are H3K27me3-modified in myotubes that show a strong enrichment for upregulated *EMD*-sensitive genes, and a moderate enrichment for downregulated genes (Fig. 5F). We also confirmed that LADs identified from these datasets were significantly more enriched for H3K27me3 in human myotubes non-LADs (Suppl. Fig. 7). Altogether, these results demonstrate that H3K27me3 modification, rather than LAD association, is a general shared feature among genes dysregulated by *EMD* loss.

Discussion

Elucidating the functional role of emerin in human skeletal muscle differentiation and maintenance is critical for understanding the mechanism and potential treatment routes for EDMD1. However, EDMD1 disease modeling has been limited by the lack of a phenotype in *Emd* KO mice. There have been some improvements in EDMD1 animal models recently; a rat

knockout model was reported to develop defects in both cardiac and skeletal muscle, consistent with symptoms observed in human patients.¹³² Despite these advances, the field still lacks an efficient model to study this disease in a human context. Here, we establish iSMs as a tractable human model of EDMD1. This model provides an accessible resource to study emerin function in human cells.

The severity of the *EMD* KO phenotype in iSMs is particularly notable in comparison to other cellular models of muscular dystrophy. For instance, a previous study used a similar transgene expression system to induce Duchenne muscular dystrophy (DMD) patient-derived iPSCs to form myocytes and reported minimal differences in differentiation propensity or morphology between controls and DMD mutant myocytes.¹⁰⁸ However, DMD myocytes still exhibited pronounced abnormalities in calcium influx and secretion of creatine kinase which was rescued by treatment with an exon-skipping antisense oligonucleotide. These DMD models utilized patient-derived iPSCs harboring disease-causing nonsense or missense mutations. Similarly, EDMD1 mutations are destabilizing and characteristically lead to nearly undetectable protein levels in patients, making *EMD* KO a reasonable strategy to model the disease *in vitro*. The clear differentiation defect of *EMD* KO iSMs emphasizes the necessity of emerin for *in vitro* skeletal muscle development.

Previous studies using cellular models have reported a requirement for emerin function at early stages of myogenesis. Emerin has been reported to be required for positioning gene loci encoding the early myogenic differentiation factors Pax7, Myf5, and MyoD at the nuclear periphery.⁸⁷ Emerin has also been implicated in activation of MyoD and its downstream targets in *EMD* KO mouse muscle, despite the lack of an overt muscular dystrophy phenotype.⁶⁹ Here,

in contrast, we report a requirement for emerlin downstream of MyoD expression, as MyoD overexpression is unable to rescue the overt phenotype caused by emerlin loss in human iSMs. In response to MyoD overexpression, *EMD* KO iSMs show persistent DNA replication in MyoD-expressing cells, retain hyperphosphorylated Rb (Fig. 2), and less effectively induce p21 (Fig. 3).

We also demonstrate the strong association between emerlin-sensitive genes and PRC2. Several studies have shown that the timing of PRC2 activity is critical in myogenesis both for silencing myogenesis genes to prevent premature differentiation as well as silencing cell cycle genes in late myogenesis to promote terminal differentiation. In satellite cells, activity of the PRC2 component EZH2 maintains the proliferating satellite cell population by limiting expression of myogenesis genes.¹³³ Interestingly, the *CDKN1A* locus, which encodes p21, is a direct target of EZH2 in cycling C2C12 myoblasts.¹³⁴ Notably, p21 induction is blunted in *EMD* KO iSMs, suggesting that emerlin may promote the timely removal of EZH2-mediated repression at this locus during myogenesis (Fig. 3). In late myogenesis, the PRC2 complex is remodeled by EZH2 degradation and replacement with EZH1, a structurally similar but less active H3K27 methyltransferase.^{135–137} In this current study, we find that a loss of emerlin leads to improper upregulation of many H3K27me3-marked genes. Surprisingly, we also find limited association between emerlin-sensitive genes and myotube LADs. Due to these findings, we speculate that emerlin influences PRC2/EZH2 targeting and/or activity during myogenesis, both within and outside of LADs.

We recognize certain limitations to this current study. While iSMs are a valuable system for studying skeletal muscle differentiation in a human context, they bypass native control of lineage specification by ectopic overexpression of MyoD and BAF60C. Additionally, while we

undertook comparative studies leveraging existing genomic datasets, it will be valuable to map LADs and H3K27me3 genomic enrichment specifically in iSMs. We are unable to comment on the direct requirement of PRC2 in iSMs due to this limitation – we simply observe that its targets are enriched among genes dysregulated in *EMD* KOs but cannot say whether emerin loss alters PRC2 localization or activity. In conclusion, our work will enable future studies into the essential functions of emerin in human myogenesis.

Methods

Cell Culture

WTC-11 hiPSCs (healthy 30-year-old, XY) were obtained from and verified by the Berkeley Stem Cell Center. hiPSCs were cultured at 37C in 5% CO₂ under normoxic conditions in either mTeSR+ medium (Stem Cell Technologies 100-0274/100-0275) or 1:1 mTeSR+:HiDef-B8 (Defined Biosciences LSS-204) in vessels coated with 1:15 Geltrex (Gibco A14132-02) without penicillin/streptomycin. Cells were passaged as clumps using ReLeSR (Stem Cell Technologies 100-0483) for routine culture and as single cells using Accutase (Innovative Cell Technologies AT-104) for counting and electroporating. Cells were plated in 10uM ROCK inhibitor Y-27632 (Selleck Chemicals 101763-964), then changed to fresh hiPSC media after 24 hours, with media changed ever 48 hours thereafter.

Generation of DICE hiPSCs

Genome editing was performed by electroporating 1×10^6 cells with 12.5 pmol Cas9-NLS protein and 12.5 pmol sgRNA using a 100-μL Neon electroporation system (1300 V, 30 ms, 1 pulse). For DICE landing pad integration, 2 μg of the HDR donor plasmid containing the landing

pad cassette was included in the electroporation mixture. Following transfection, cells were selected with 0.25 µg/mL puromycin for 3 days. Single-cell clones were isolated by limiting dilution in 96-well plates, expanded, and genotyped by PCR using primers spanning the AAVS1 homology arms to identify heterozygous landing pad integration.

Emerin knockout cell lines were generated from a single landing pad clone using two sgRNAs targeting the 5' and 3' ends of the coding sequence. Deletion of the targeted locus was confirmed by PCR, and loss of emerin expression was verified by immunoblotting and immunofluorescence microscopy. For site-specific integration of emerin constructs, 1×10^6 landing pad cells were electroporated with 2 µg of an attB-containing donor plasmid and 500 ng of a BxbI-P2A-PhiC31 integrase plasmid using the same electroporation conditions. After recovery, BFP-negative, donor-positive cells were isolated by FACS using a SONY SH800 cell sorter. Cell lines were routinely tested for mycoplasma every 3–4 months and were consistently negative.

Generation of iSM DICE hiPSCs

Tetracycline-inducible iSM transgene vector was amplified by PCR from two separate vectors gifted by the lab of Dr. Lorenzo Puri. Final vector included rtTA-Advanced-T2A-BlastR controlled by a UbC promoter and mMyod1-P2A-hBAF60C-FLAG controlled by a tight TRE promoter, both flanked by SV40 poly(A) sequences. These features were all enclosed between 5' and 3' piggyBac integration sites. Full vector sequence is available upon request.

Expected sequence was confirmed by whole plasmid sequencing. The iSM transgene vector was introduced into DICE hiPSCs using a 100 µL Neon electroporator set to 1150V, 20ms, and 2

pulses. Cells were placed under Blasticidin selection (Research Products International B12150-0.1) to select for mMYOD1/hBAF60C-positive cells (transgene+) for at least 7 days at 10ug/mL.

Differentiation

For differentiation into iSMs, hiPSCs were seeded in hiPSC media and allowed to recover under Blasticidin selection before switching to HuES media containing 500ng/mL doxycycline (Alfa Aesar J67043). HuES media was made by mixing Dulbecco's modified Eagle medium:F12 (Gibco 11330-032), 20% Knockout Serum Replacement (Thermo Scientific 10828-028), 1% non-essential amino acids (Caisson Labs NAL03-100ML), and 0.2% Beta-mercaptoethanol (Gibco 21985-023). After 24hr, cells were replated in HuES + doxycycline + ROCK inhibitor. After another 24hr, media was switched to DM media + doxycycline. DM media was made by mixing Dulbecco's modified Eagle medium:F12, 1X insulin-selenium-transferrin-ethanolamine (ITS-X; Gibco 51500-056) and 1X N-2 (Gibco, 17502-001). Cells were maintained in DM media + doxycycline until collection or imaging, replacing with fresh media every 48 hr.

iSM cleanup was performed on freshly collected Day 6 iSMs. Fresh cell pellets were suspended in anti-CD57 (BioLegend 393330) diluted in 0.5% BSA in PBS for 90min, rotating end-over-end at 4C. Sheep anti-mouse M280 Dynabeads (Thermo Scientific 11201D) were calibrated in 0.5% BSA briefly before the cell-antibody mixture was added and set to rotate end-over-end at 4C for another 90min. Beads were removed using a magnetic stand and cell pellets were washed in 0.5% BSA before being flash frozen.

Immunofluorescence and EdU incorporation staining

8-chamber μ -slide (ibidi 80806) were coated with 1:15 Geltrex for 10min at 37C. Cells were seeded at the time of replating during differentiation, then fixed in 4% PFA (Electron Microscopy Sciences 15710) in PBS for 5 minutes at room temperature at various time points. Cells were permeabilized in IF buffer (0.1% Triton-X, 0.02% SDS, 10mg/mL BSA in 1X PBS) for 20min at room temperature. Primary and secondary antibodies diluted in IF buffer were incubated for 2hr and 1hr at room temperature, respectively, with IF buffer washes in between. DNA was stained with Hoechst during secondary antibody incubation. See antibody table for list of antibodies used and working concentrations.

After 16hr EdU incubation, cells were fixed as described above. The Click-iT EdU Cell Proliferation Kit for Imaging (ThermoFisher C10338) was used according to supplier's instructions to label EdU with Alexa-555 fluorophore. The above immunofluorescence staining protocol immediately followed EdU labeling.

Co-immunoprecipitation & Immunoblotting

Immunoprecipitation was performed by lysing cell pellets in RIPA buffer (50mM Tris-HCl pH 8.0, 150mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, EDTA-free Protease Inhibitor Cocktail (Roche, 11836170001)). Cell lysates were incubated with the mouse monoclonal anti-MyoD antibody (BD 554130) at 1:120, rotating end-over-end overnight at 4C. Lysates were then added to 50uL Sheep anti-mouse M280 Dynabeads (calibrated in RIPA buffer) and incubated rotating end-over-end overnight at 4C. Beads were separated from lysate on a magnetic column, washed three times in IP wash buffer (0.1% BSA, 2mM EDTA in PBS) and

eluted in 2X sample buffer (5X = 10%SDS, 25 mM Na₃PO₄, 50% glycerol, 0.5 M DTT, .05% Bromophenol blue) by boiling at 95C for 10min.

For detection of lamins and membrane-bound proteins, cells were pelleted and lysed in 8 M urea, 75 mM NaCl, 50 mM Tris pH 8.0 and cOmplete, Mini, EDTA-free Protease Inhibitor Cocktail. For detection of phosphorylated and/or soluble proteins, cell pellets were lysed on ice for 10min in buffer containing 1% NP-40 (Sigma I3021), 150mM NaCl, 50mM Tris-HCl pH7.5, 1mM EDTA, cOmplete, Mini, EDTA-free Protease Inhibitor Cocktail, and 2X PhosSTOP Phosphatase Inhibitor Cocktail (Roche, 4906845001). Lysates were run on 4-20% Mini-PROTEAN TGX precast gels (BioRad 4561096) and transferred to a MeOH-activated PVDF membrane for 16hr at 30mA, 4C or for 1hr at 100V, 4C. Blots were probed with anti-EMD, anti-Rb1, anti-alpha tubulin, anti-p21, anti-H4, mouse monoclonal anti-MyoD, rabbit polyclonal anti-MyoD, anti-TCF3/E2A, anti-pan-acetylated lysine, anti-Lamin A/C, anti-BAF60C, anti-MyHC, anti-Myf5, anti-Lamin A/C, and anti-vinculin primary antibodies diluted in 5% milk TBST (see Table 2.2 for additional antibody information). Secondary antibodies were diluted at 1:5000 in 5% milk TBST. For ppRb1 detection, blots were blocked in 5% milk TBST, incubated in with anti-ppRb1 in 5% BSA, followed by anti-Rabbit HRP in 5% BSA. Blots were developed using high-sensitivity ECL substrate (Azure Biosystems AC2103) and imaged using a BioRad ChemiDoc XRS+.

RNAseq Analysis

Three iSM replicates of each genotype were differentiated in separate wells of 6-well plates, then collected on Day 6 for cleanup as described above. Snap-frozen, enriched iSM pellets were sent to Signios Bio for RNA extraction, library preparation, and sequencing. Paired-end sequencing was performed (read length of 150 bp, ~40 million reads per library). Raw sequencing reads

were mapped to the GENCODE V49 primary assembly human genome build (GRCh38) using STAR (version 2.7.10a): `--runMode alignReads --readFilesCommand zcat --clip3pAdapterSeq CTGTCTCTTATA CTGTCTCTTATA --clip3pAdapterMMp 0.1 0.1 --outFileNamePrefix ${base}_ --outFilterMultimapNmax 1 --outReadsUnmapped None --outSAMtype BAM SortedByCoordinate --twopassMode Basic`. Bam files were indexed using SAMtools (version 1.10) and used to generate RPKM normalized coverage files using the deepTools2 function `bamCoverage` (version 3.4.5). A read counts table was generated for all conditions using the `subread featureCounts` package (version 1.6.4) and GENCODE V49 primary annotation. Read counts table was used as an input for the Bioconductor DESeq2 package (version 1.40.2) to generate normalized counts and perform differential gene expression analysis.

MyoD ortholog analysis was performed independently from all other RNAseq analysis. The mouse *Myod1* transgene CDS was appended as a synthetic, single exon chromosome/transcript to both the genome FASTA and the transcriptome FASTA. A transcriptome-only Salmon pufferfish index was built from the combined transcriptome FASTA (Salmon version 1.10.1) to which transcript reads were mapped: `salmon quant --index salmon_index/ --libType ISR --mates1 ${SAMPLE}_R1.fastq.gz --mates2 ${SAMPLE}_R2.fastq.gz --output salmon_quant/${SAMPLE}/ --threads 1 --validateMappings`. A quant's file was generated for each replicate and used as the input for DESeq2. The `tximport` package was used to perform transcript-to-gene mapping before generating normalized counts (R version 4.4.x).

GSEA was conducted with the `gseGO` function from the `clusterProfiler` R package (version 4.14.3) using the “Biological Processes” subontology. Gene set size range was set to a minimum

of 5 and a maximum of 5,000, with a Benjamini-Hochberg method-adjusted p-value cutoff of 0.01 and simple permutation n of 10,000.

TFEA analysis

Rescue-sensitive genes were compared against targets of all human antigens listed in the ChIP-Atlas database with sufficient data (version 3.0).¹²¹ Background was calculated using the union of 19,032 genes listed as targets of at least one of all 1,597 antigens tested. TF families (i.e. E2F, bHLH, etc.) were assigned to each gene based on enrichment of that gene as a TF family target (if a gene was a target of more than one family, it was assigned to the family with the highest proportion of TFs targeting that gene).

LAD and H3K27me3 Analysis

Mouse myoblast and myotube LAD data was acquired directly from Robson et al.¹²² Human orthologs were mapped using the biomaRt package to map MGI to HGNC homology (only 1:1 orthologs were used). Human myotube and myo-converted fibroblast LAD data was acquired directly from Benarroch et al.¹²³ and used as listed. Background was calculated using all human protein-coding genes from GENCODE v45.

H3K27me3 ChIP-seq data were acquired from ENCODE dataset ENCSR000ATI (human myotubes, Histone ChIP-seq). Pseudoreplicated peaks were used for gene assignment.

GENCODE v46 primary assembly basic annotation GTF for GRCh38 was used to build a protein-coding gene list background. Genes were classified as “H3K27me3-marked” within a dataset if at least one peak within the BED file of that dataset overlapped with that gene’s TSS +/- 5kb promoter window.

Image Analysis & Computation

Images were acquired on a Nikon Ti microscope equipped with a CSU-X1 spinning disk confocal using a 10X 0.45 numerical aperture air objective. All z-stacks were acquired at $0.3\mu\text{m}$ steps. Raw 16bit files were saved as ND2 files using Nikon Elements (version 6.10.2). All image processing and quantification was done with Fiji (ImageJ2 release 2.17.0). Images are shown as single slices within a z-stack. MyoD was used to create masks of nuclei to measure mean intensities. Percent positive within transgene+ nuclei quantification was determined by presence of mean signal above a negative background threshold. Percent of nuclei within MyHC+ myotubes was quantified manually. Graphing and statistical analysis was done using GraphPad Prism (version 11.0.1).

Figures

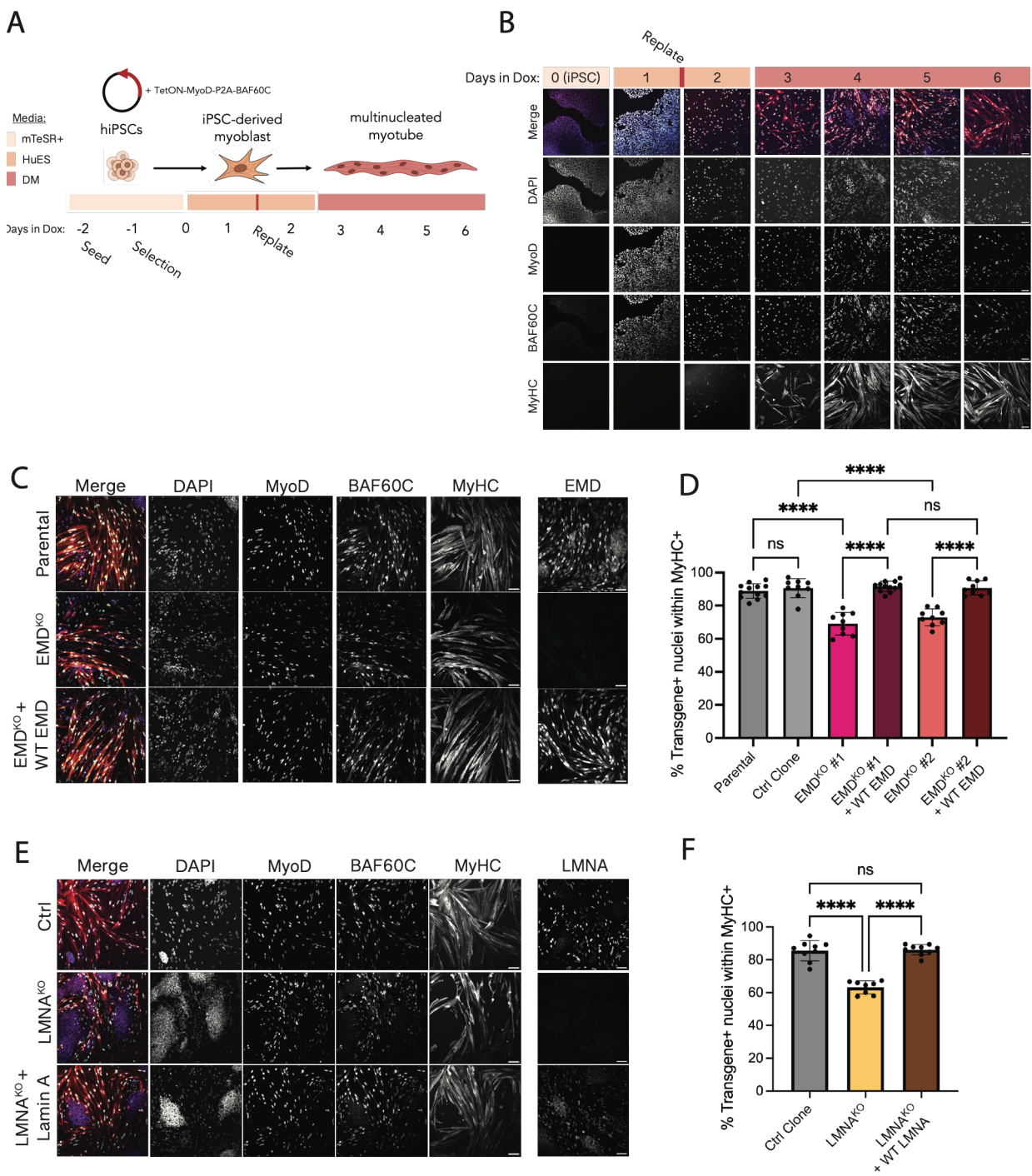


Figure 2.1. Emerin loss impairs iSM differentiation.

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A) Diagram of the iSM differentiation protocol. B) Time course of WT (parental) DICE iSM differentiation, following the protocol described in (A). C) Representative images of Day 6 iSMs upon emerin KO and rescue with untagged emerin. D) Quantification of transgene-positive nuclei within MyHC-positive myotubes in (C) (n = 3 biological replicates per genotype). E) Representative images of Day 6 iSMs upon LMNA KO and rescue with untagged Lamin A. F) Quantification of transgene-positive nuclei within MyHC-positive myotubes in (E) (n = 3 biological replicates per genotype). All scale bars = 100um. Proportions significantly different by one-way ANOVA: ****p < 0.0001.

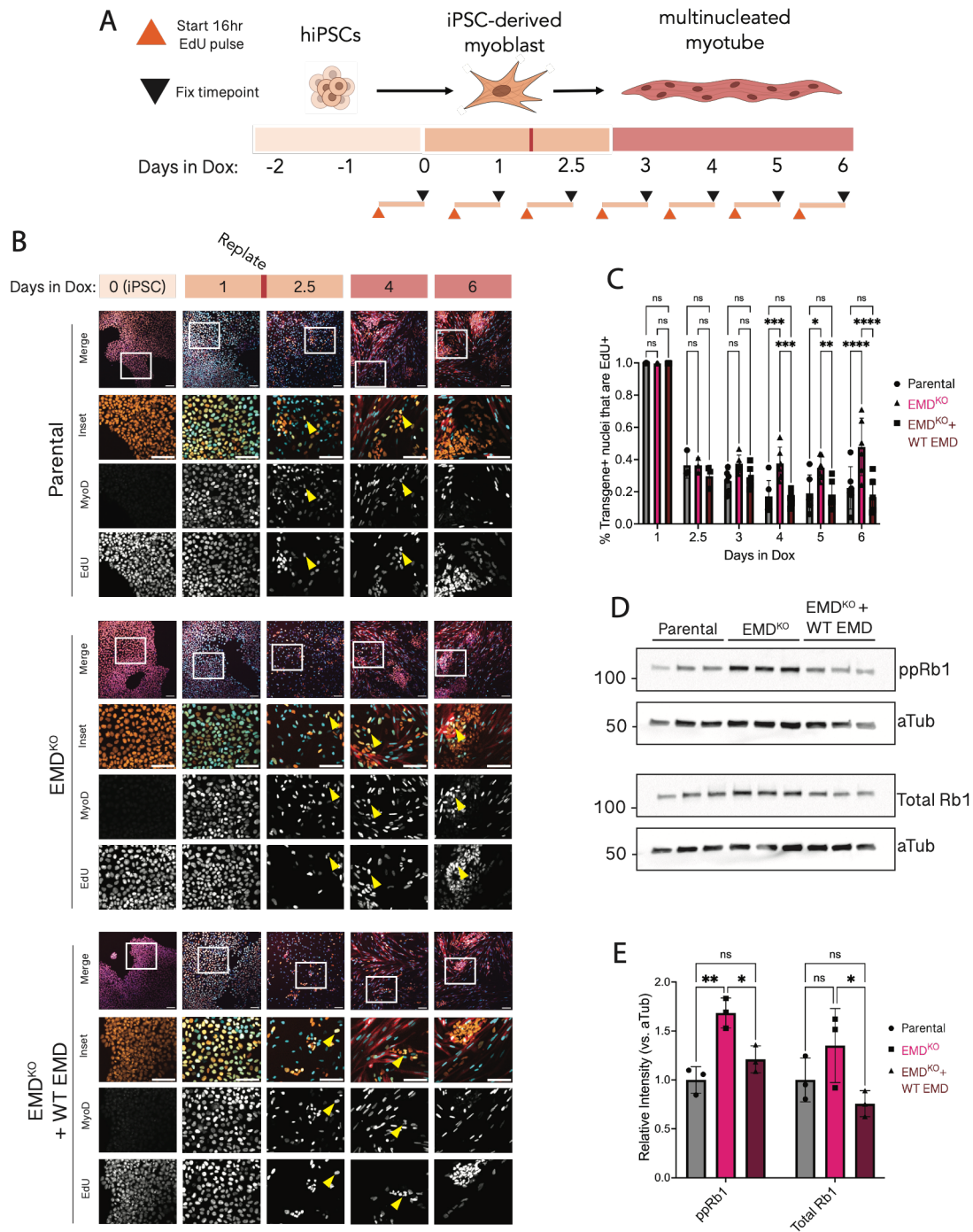


Figure 2.2. Transgene-positive nuclei exhibit delayed cell cycle exit upon emerin loss.

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A) Diagram of the EdU labeling protocol. B) Time course of parental wildtype, EMD KO, and EMD KO rescued with untagged WT EMD co-stained for MyoD, MyHC, and incorporated EdU after 16hr pulse. Yellow arrowheads indicate transgene+/EdU+ population, quantified in (C). C) Quantification of transgene-positive nuclei that are also EdU-positive (n = 2 biological replicates per genotype). D) Western blot of hyper-phosphorylated Rb1 (ppRb1) and total Rb1 in Day 6 iSMs. Three biological replicates per genotype. E) Densitometric quantification of (D) as compared to acetylated Tubulin loading control. All scale bars = 100um. Proportions significantly different by two-way ANOVA: *p < 0.05, **p < 0.01, *** p < 0.001.

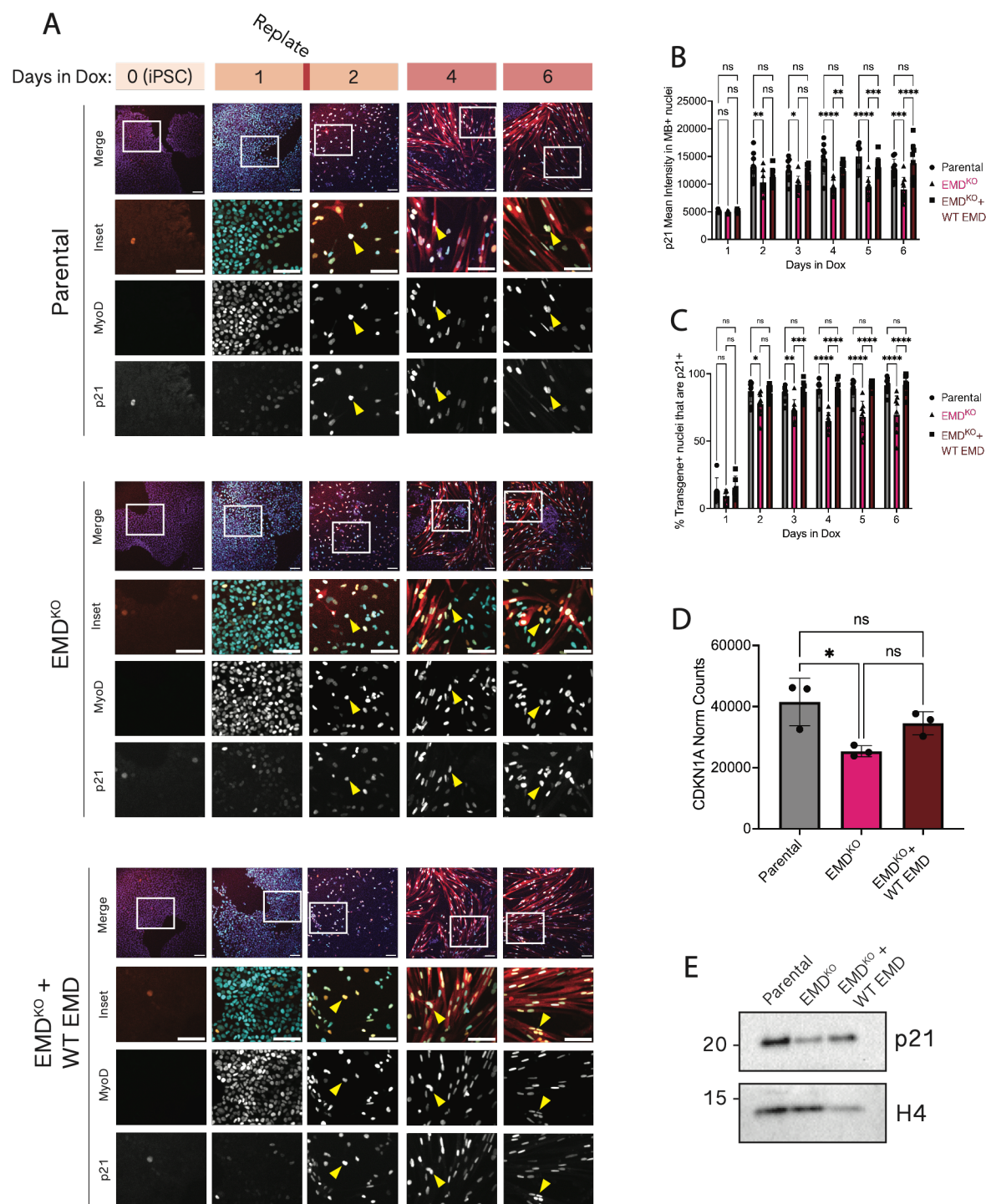


Figure 2.3. p21 expression is diminished in iSMs upon emerin loss.

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A) Time course of iSM differentiation, co-stained for MyoD, MyHC, and p21. Yellow arrowheads indicate transgene+/p21+ population, quantified in (B). B) Quantification of transgene-positive nuclei that are also p21-positive (n = 3 biological replicates per genotype). C) Quantification of average p21 signal in transgene-positive nuclei (n = 3 biological replicates per genotype). D) Normalized transcript counts of *CDKN1A* in Day 6 iSMs. E) Western blot for p21 in Day 6 iSMs. All scale bars = 100um. Proportions significantly different by two-way ANOVA in (B) and (C), one-way ANOVA in (E): *p < 0.05, **p < 0.01, *** p < 0.001

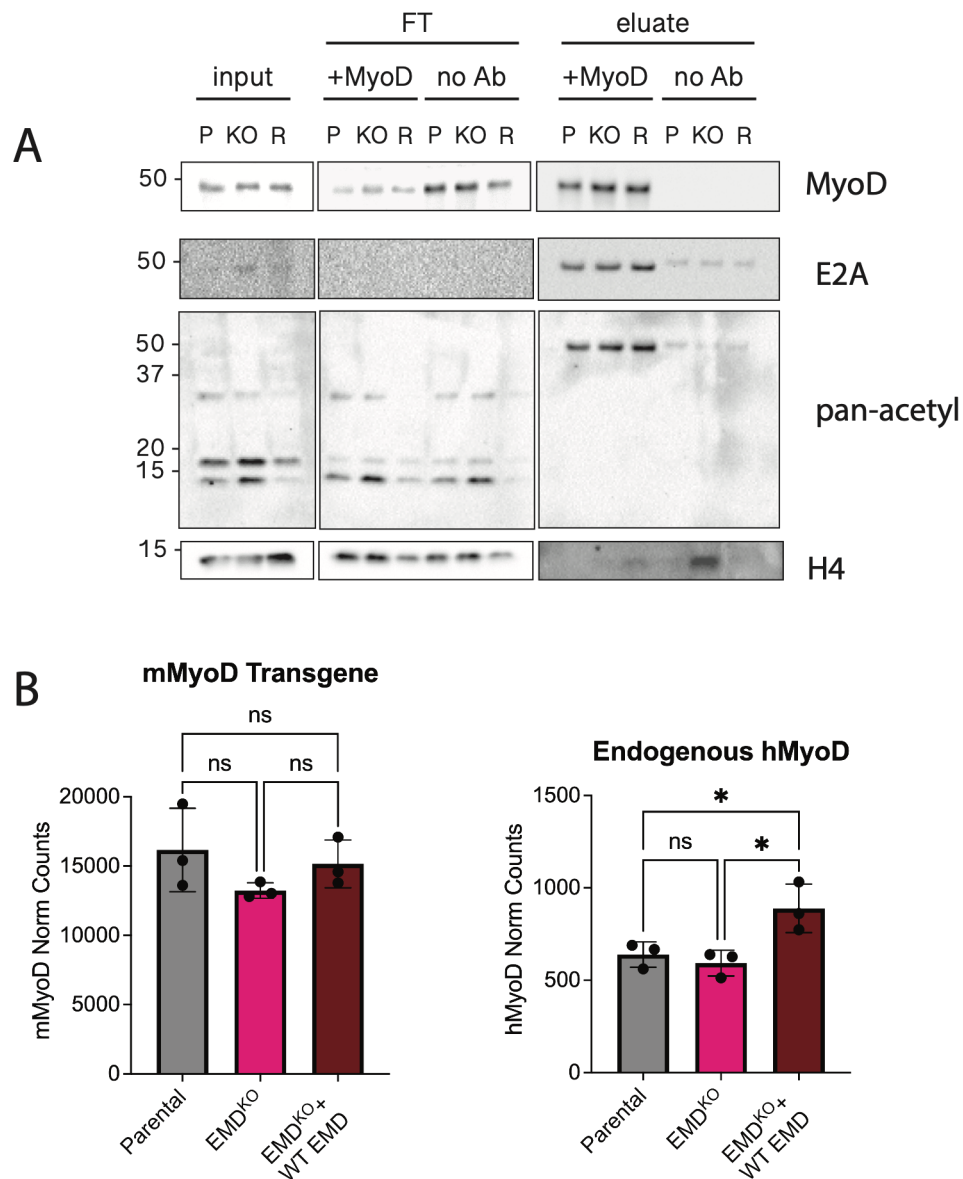


Figure 2.4. MyoD activation is unaffected by emerin loss.

A) Representative immunoblots for immunoprecipitation of MyoD and co-immunoprecipitation of E2A (repeated with 3 biological replicates). FT = flow-through, P = parental, KO = EMD^{KO} #1, R = EMD^{KO} #1 + WT EMD (Rescue). B) Normalized transcript counts of the mouse *Myod1* transgene and endogenous human *MYOD1*. Counts significantly different by one-way ANOVA: * $p < 0.05$.

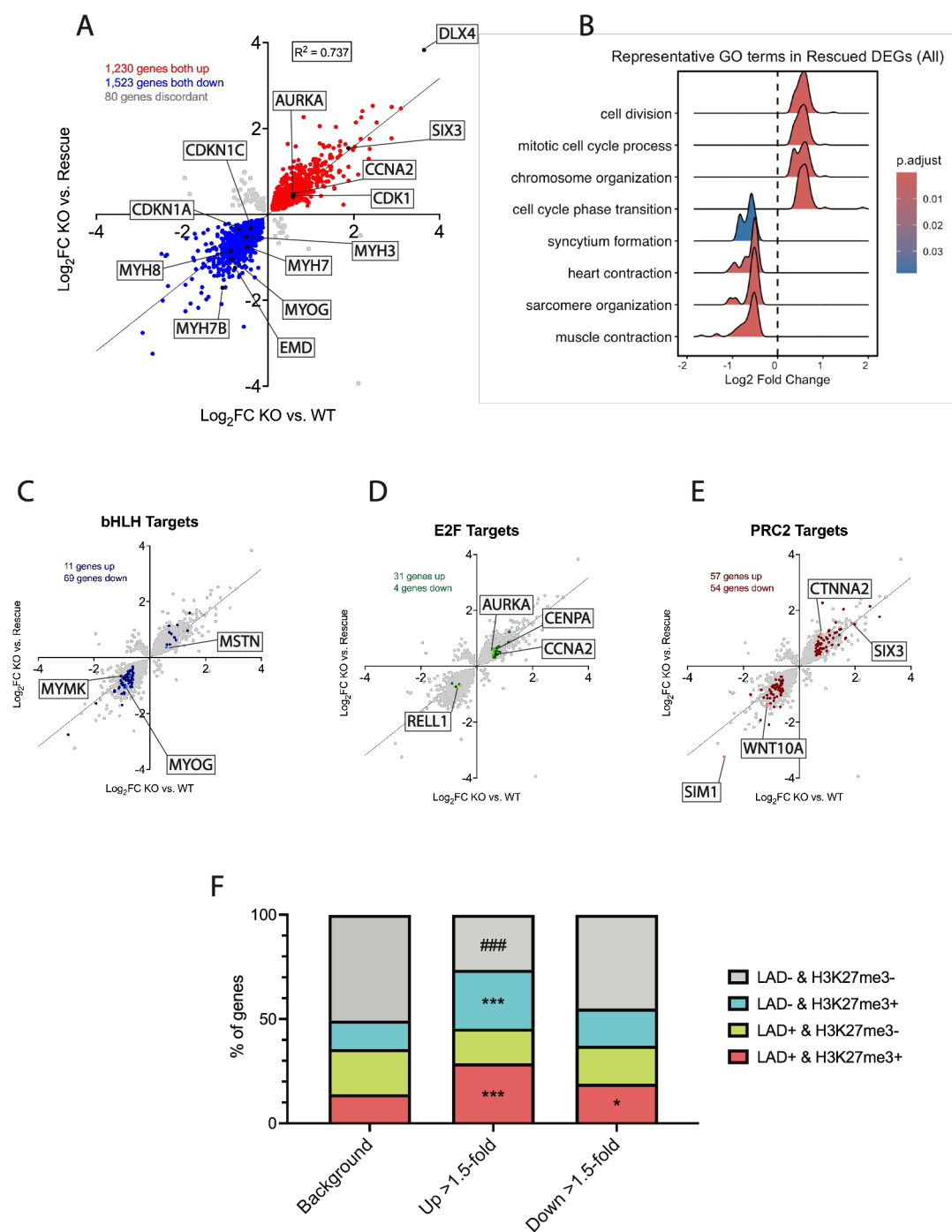
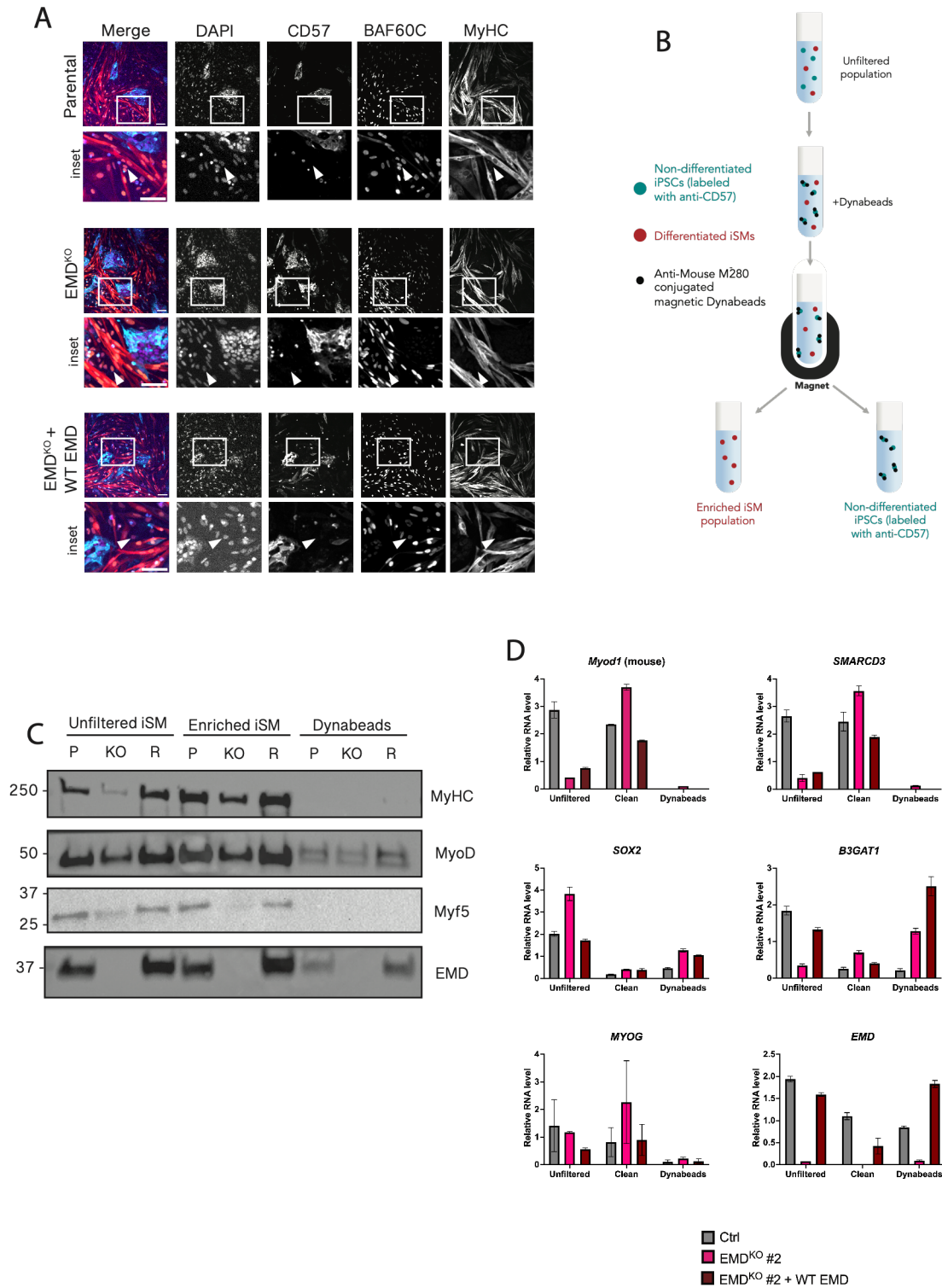


Figure 2.5. Emerin loss leads to upregulation of proliferative genes and downregulation of myogenic differentiation genes.

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A) Identification of emerlin-sensitive genes. Log₂ fold-change (FC) values in EMD^{KO} vs. Parental (KO vs. WT) plotted against log₂FC values in EMD^{KO} vs. EMD^{KO} + WT EMD (KO vs. Rescue). Best-fit shown as solid black line ($y = 0.7939x - 0.005017$; $R^2 = 0.737$). Genes of interest labeled in black with gene names. B) Representative GO terms enriched among all emerlin-sensitive genes shown in (A) (identified by gene set enrichment analysis). C-E) Identification of enriched transcription factor (TF) targets among emerlin-sensitive genes. Genes identified as targets of the bHLH TF family (C), the E2F-DREAM TF family (D), and the PRC2 complex (E) by the ChIP-Atlas database are highlighted against the same scatterplot shown in (A). Representative target genes are labeled. Also see Supplementary Fig. 7. F) Distribution of rescue-responsive genes among LAD and/or H3K27me3 regions reported in human myotubes. Background corresponds to 20,036 protein-coding genes found in both published datasets. Proportions significantly different from background by two-sided Fisher's exact test: * $p < 0.05$, *** $p < 0.001$ (increased) and #### $p < 0.001$ (decreased).

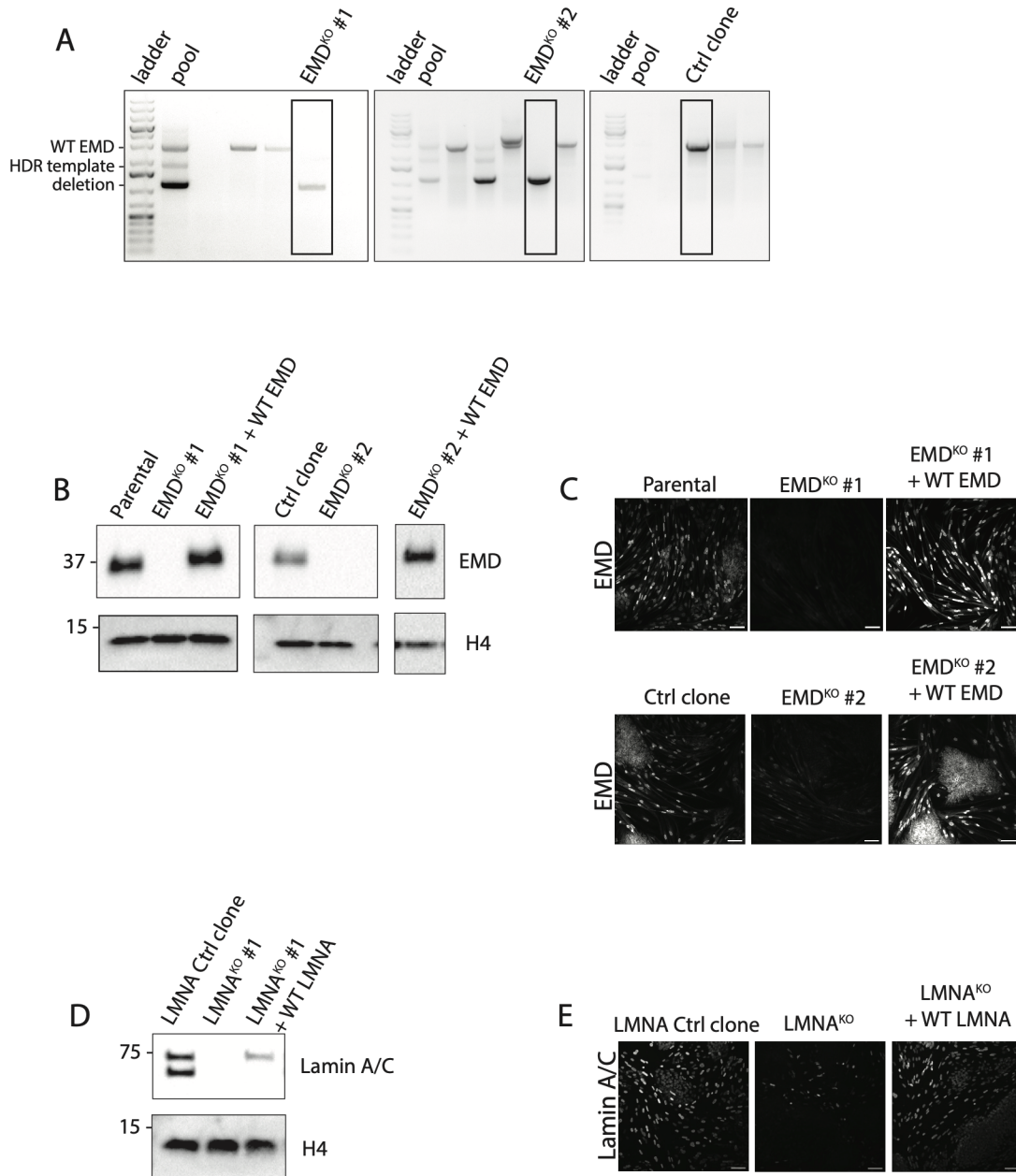


Supplementary Figure 2.1. Validation of iSM immunodepletion protocol.

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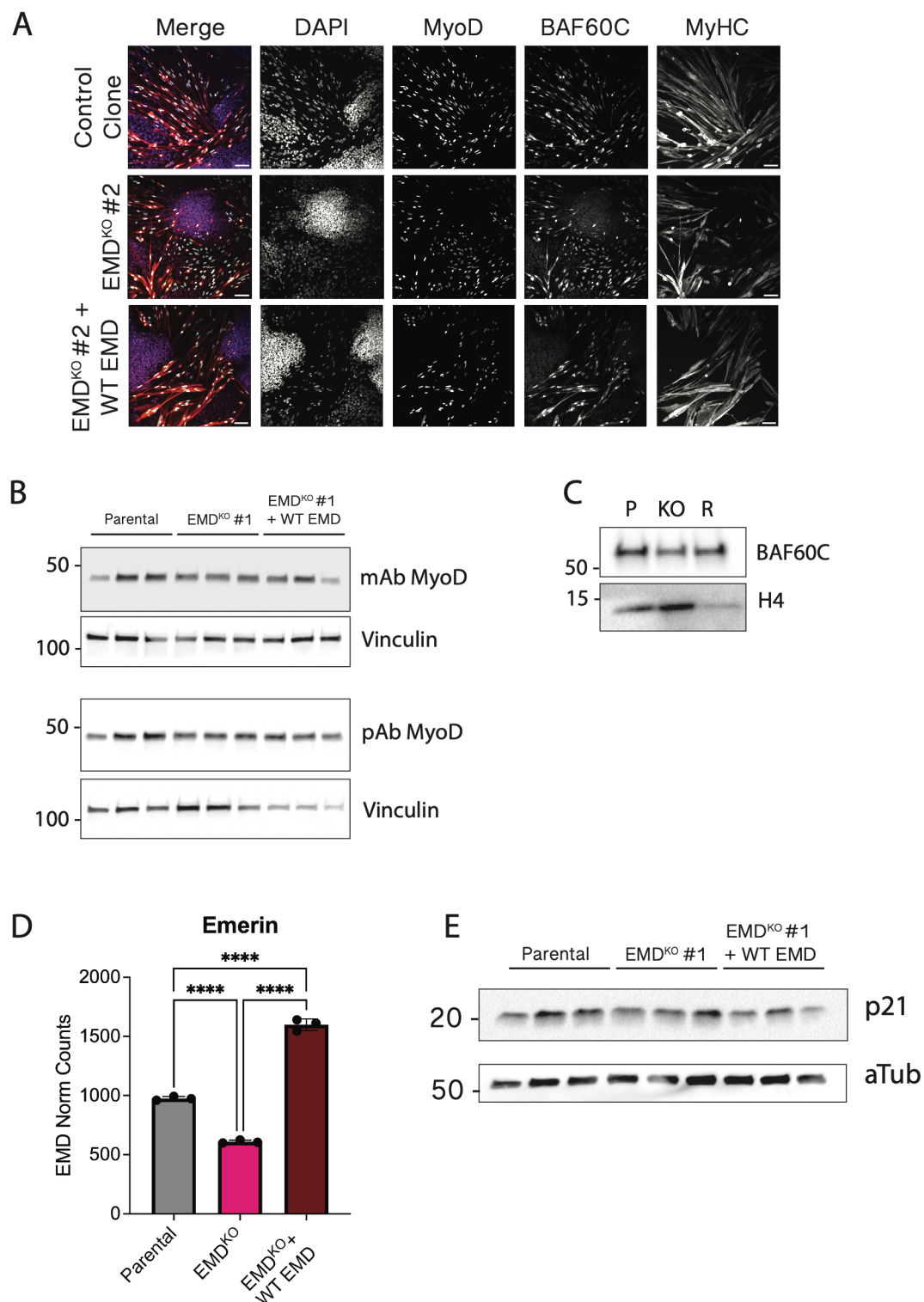
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A) Immunofluorescence staining of Day 4 iSMs, co-stained for BAF60C, MyHC, and CD57. White arrows indicate examples of BAF60C+/CD57- nuclei. B) Diagram detailing iSM cleanup protocol. C) Western blot of myogenic factors and emerin in unfiltered and enriched iSM populations, as well as CD57+ cells captured by Dynabeads. P = parental, KO = EMD^{KO} #1, R = EMD^{KO} #1 + WT EMD (Rescue). D) RT-qPCR of select genes in CRISPR control clone, EMD^{KO} #2, and EMD^{KO} #2 + WT EMD Day 6 iSMs, comparing unfiltered, enriched, and Dynabead-bound populations (n = 3 technical replicates per condition). RNA level calculated relative to GAPDH control. Gene transcripts quantified: *Myod1* (mouse MyoD transgene), *SMARCD3* (encoding BAF60C), *SOX2*, *B3GAT1* (encoding CD57 synthase), *MYOG* (encoding myogenin), and *EMD*. All scale bars = 100um.



Supplementary Figure 2.2. Validation of *EMD* KO and *LMNA* KO hiPSCs.

A) Genotyping PCR for *EMD* KO #1, *EMD* KO #2, and CRISPR control. Upper band corresponds to wildtype emerin locus, lower band corresponds to deletion within the *EMD* locus. B) Western blot of CRISPR control clone and both *EMD* KO clones with corresponding rescue. C) Immunofluorescence staining of CRISPR control clone and both *EMD* KO clones with corresponding rescue. D) Western blot of control, *LMNA* KO, and *LMNA* KO + Lamin A rescue, blotted for Lamin A/C. E) Immunofluorescence staining of control, *LMNA* KO, and *LMNA* KO + Lamin A rescue. All scale bars = 100um.

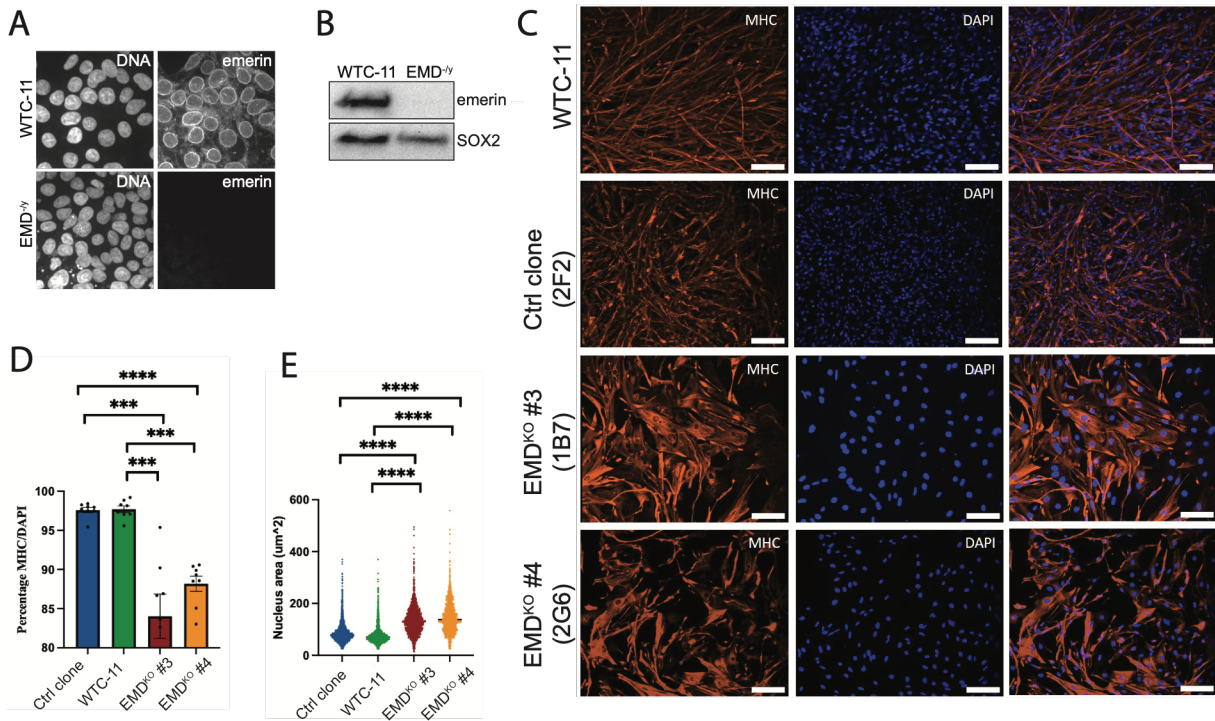


Supplementary Figure 2.3. Additional EMD^{KO} iSM differentiation defects and characterization of transgene, *EMD* transcript, and variable p21 levels.

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A) Representative images of Control, EMD KO #2, and EMD KO #2 + WT EMD Day 6 iSMs, quantified in Figure 1D. B) Three distinct biological replicates immunoblotted using mouse monoclonal (BD cat#) and rabbit polyclonal (Ptg cat#) anti-MyoD antibodies. C) Immunoblot of BAF60C levels. P = parental, KO = EMD^{KO} #1, R = EMD^{KO} #1 + WT EMD (Rescue). D) Normalized transcript counts of *EMD* in Parental, EMD^{KO} #1, and EMD^{KO} #1 Rescue. E) Three distinct biological replicates immunoblotted to demonstrate p21 variability by Western blot (compare with Figure 3E). All samples in this figure are from Day 6 iSMs. All scale bars = 100um. Proportions significantly different by one-way ANOVA: ****p < 0.0001.

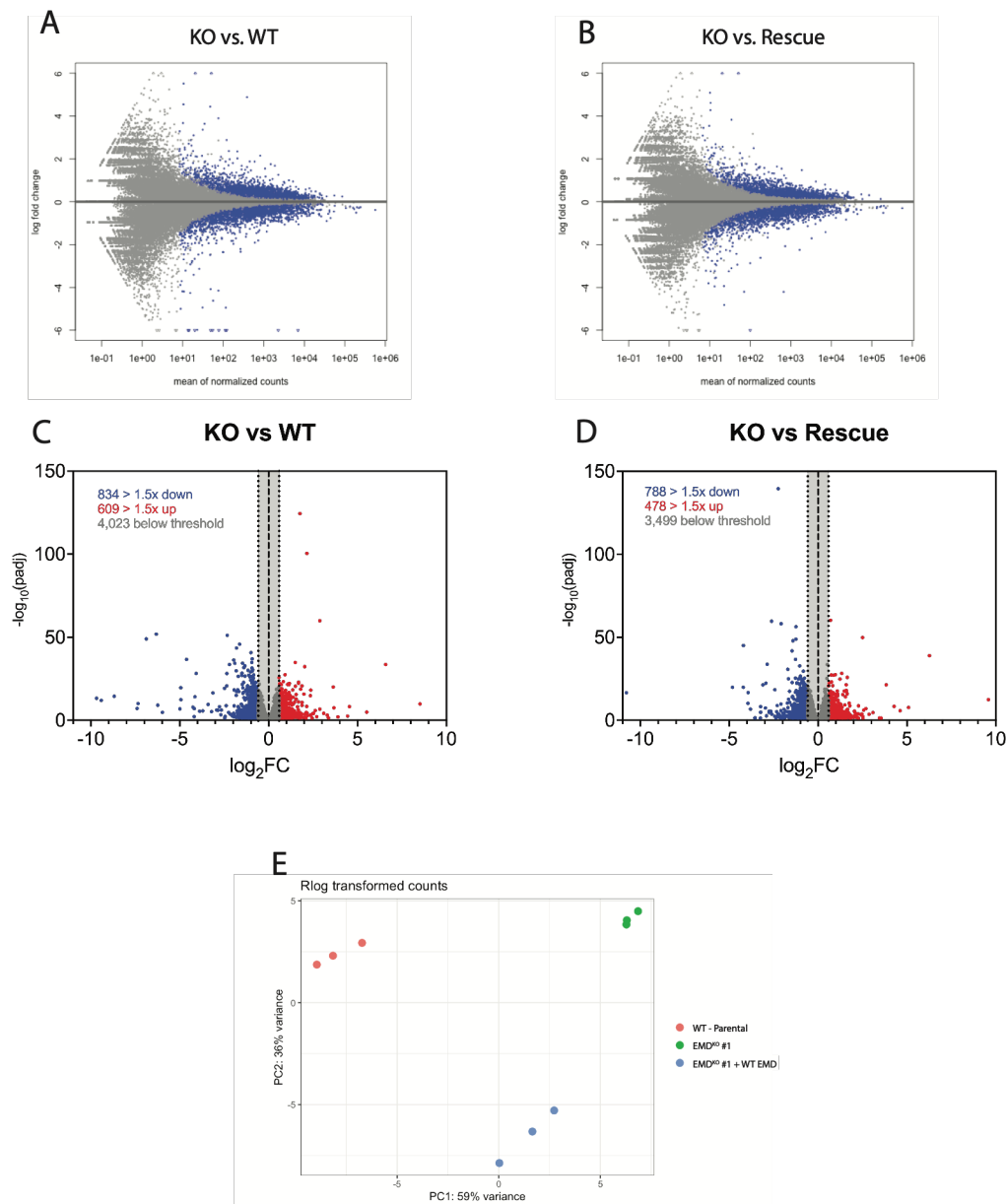


Supplementary Figure 2.4. Additional EMD KO clones have a skeletal muscle differentiation defect.

A-B) Representative EMD^{-/-} iPSC clone lacking detectable emerin expression by immunostaining (A) and Western blotting (B). Note preserved expression of pluripotency marker SOX2. C) Representative images of MHC-positive skeletal muscle myotubes in WTC-11 parental hiPSCs, CRISPR control (2F2), and two *EMD* KO clones (1B7 & 2G6). D) Skeletal muscle differentiation efficiency scored as % of cells positive for myosin heavy chain (MHC) for WTC-11, CRISPR control clone (2F2), and two *EMD* KO clones (1B7 & 2G6). E) Nucleus area calculated for each nucleus within each field of view across replicates in WTC-11, CRISPR control (2F2), and two *EMD* KO clones (1B7 & 2G6).

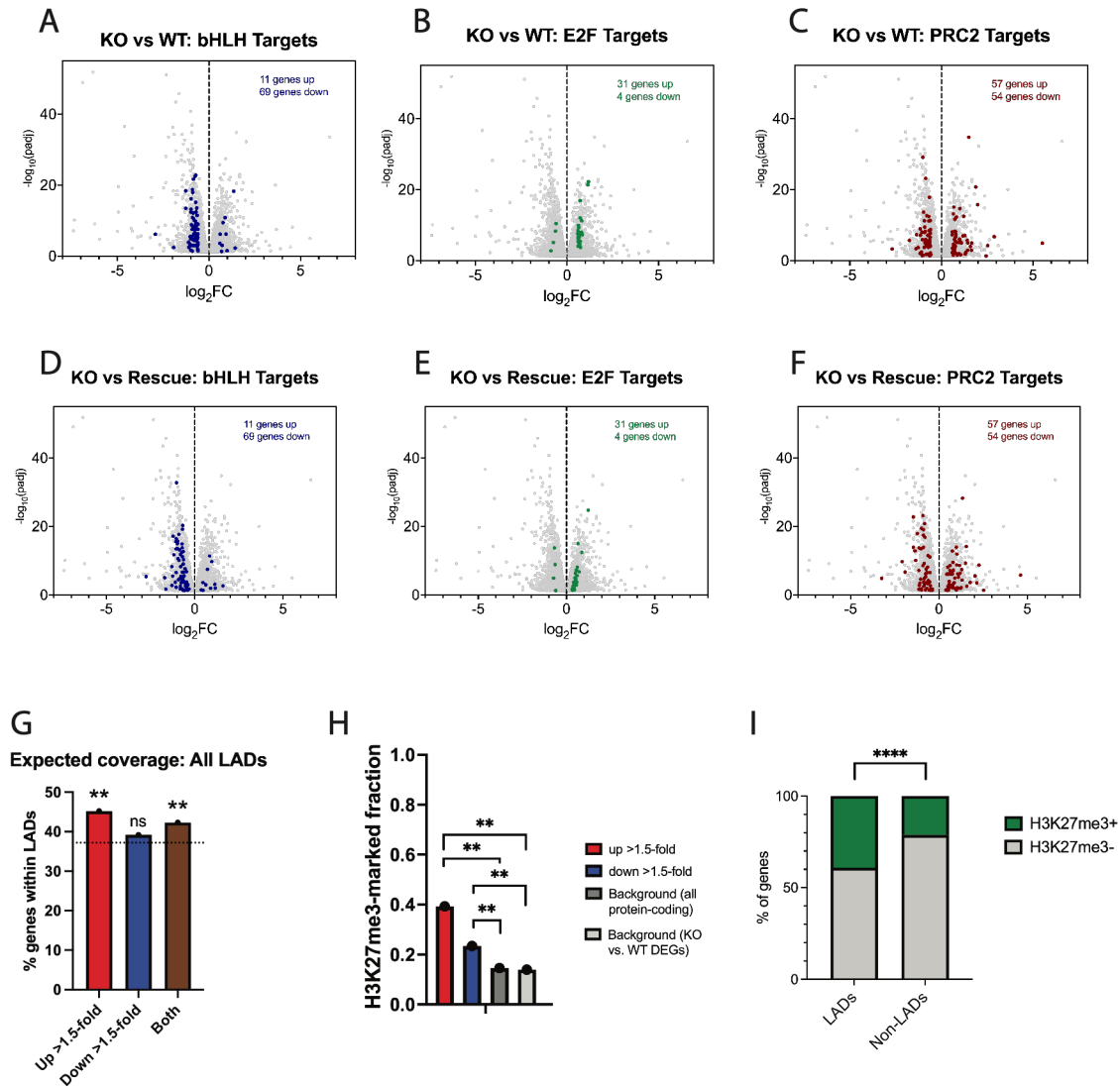
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A) Time course of iSM differentiation, co-stained for myogenin, MyoD, and MyHC. Yellow arrowheads indicate transgene⁺/Myog⁺ population, quantified in (B). Quantification of transgene-positive nuclei that are also myogenin-positive (n = 3 biological replicates per genotype). C) Quantification of myogenin-positive nuclei within MyHC-positive myotubes in (A) (n = 3 biological replicates per genotype). D) Normalized transcript counts of *MYOG*. All scale bars = 100um. Proportions significantly different by two-way ANOVA in (B) and (C), one-way ANOVA in (D): **p < 0.01, *** p < 0.001, ****p < 0.0001.



Supplementary Figure 2.6. Analysis of transcriptional changes after EMD KO in Day 6 iSMs.

A-B) MA plots comparing gene expression in KO vs. WT (A) and KO vs. Rescue (B). 5,466 differentially expressed genes in (A) and 4,765 in (B) with $p\text{-adj} < 0.05$ shown in blue. C-D) $\log_2\text{FC}$ of differentially expressed genes shown in (A) and (B), plotted against $-\log(p\text{-adj})$. 609 genes are upregulated > 1.5-fold, 834 downregulated > 1.5-fold in (C). 478 genes are upregulated > 1.5-fold, 788 downregulated > 1.5-fold in (D). E) Principal component analysis (PCA) plot for three biological replicates across the three genotypes analyzed by RNA-seq.



Supplementary Figure 2.7. Analysis of rescue-sensitive gene distribution among transcription factor targets, LADs, and H3K27me3-positive regions.

A-F) Log2FC of differentially expressed genes between KO vs. WT (A-C) and KO vs. Rescue (D-F), plotted against $-\log_{10}(\text{p-adj})$. Gene targets of bHLH TFs (A & D), E2F TFs (B & E), and the PRC2 complex (C-F) are highlighted. G) LAD enrichment among up and downregulated rescue-sensitive genes, or the union of both. Dotted line represents background (average LAD coverage across all human protein-coding genes). H) Fraction of genes per set predicted to be H3K27me3-marked in human myotubes. Up and downregulated rescue-sensitive genes were compared to both an all protein-coding gene background and the full DEG list of KO vs. WT background. I) Percentage of all genes within LADs and non-LADs that are predicted to be H3K27me3-marked in human myotubes. All percentages significantly different when compared to background(s) by two-sided Fisher's exact test: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

Supplementary Table 2.1. List of RT-qPCR and genotyping primers.

Target	Forward sequence	Reverse sequence
mMyoD (Caputo et al. 2020)	AATGGCTACGACACCG CCTACTA	AGATGCGCTCCACTATGCT GGACA
hBAF60C (Caputo et al. 2020)	GCGCGCAAAGCCACGA AA	ATCCGGGCTCCAGACGGC ATC
hEMD	CTCGGCCGGTTTTGGTA G	GTCAGCTCGGTATCCGAA AG
hB3GAT1	GCAGCCTCCTTCGAGA ACTT	AGCCCTTCTTGCCCTCATT C
hSox2	GGGAAATGGGAGGGGT GCAAAAGAGG	TTGCGTGAGTGTGGATGG GATTGGTG
hMyogenin (Caputo et al. 2020)	AATGCAGCTCTCACAG CGCCTC	TCAGCCGTGAGCAGATGA TCC
hGAPDH	CGCTCTCTGCTCCTCCT GTT	CCATGGTGTCTGAGCGAT GT
hEMD (genotyping)	GGGGAACCCTAGGCTC TG	GGGAGCAAGCACTACTCT GG

Supplementary Table 2.2. List of antibodies.

Target	Isotype	Application	Dilution	Catalog No.
MyoD	Mouse IgG1	IF	1:150	BD 554130
		IP	1:120	
		WB	1:1000	
MyoD	Rabbit	IF	1:400	Proteintech 18943-1-AP
		WB	1:2000	
BAF60C	Rabbit	IF	1:100	Proteintech12838-1-AP
		WB	1:1000	
MyHC	Mouse IgG2b	IF	1:50	DSHB MF 20
		WB	1:100	
EMD	Rabbit	IF	1:300	Proteintech 10351-1-AP
		WB	1:2000	
p21	Rabbit	IF	1:400	Cell Signaling Technology 2947T
		WB	1:1000	
Myogenin	Mouse IgG1	IF	1:50	DSHB F5D
Vinculin	Mouse IgG1	WB	1:4000	Sigma-Aldrich V9264-100UL
LMNA	Mouse IgG2a	IF	1:500	Active Motif 39288
		WB	1:1000	
Histone H4	Rabbit	WB	1:1000	Active Motif 39070
Pan-acetyl-K	Rabbit	WB	1:1000	Cell Signaling Technology 9814S
E2A	Rabbit	WB	1:1000	Cell Signaling Technology 4865S

Chapter 3: Determining the regulatory role of nuclear membrane protein emerin in functional pools of β -catenin

Abstract

This chapter details experiments on the reported inhibitory effects of emerin on the Wnt effector protein β -catenin.

Introduction

As discussed in previous chapters, emerin has been implicated in chromatin tethering, mechanotransduction, and maintaining nuclear envelope structural integrity. Interestingly, it has also been identified as a negative regulator of the canonical Wnt pathway.^{43,138} Canonical Wnt signaling plays a critical role in development and stem cell differentiation. Defects in the regulation of its components, particularly of its main effector protein β -catenin, lead to defects in the cell cycle, cell fate decisions, and at an organismal level, metastatic cancers. Despite evidence that emerin antagonizes the Wnt pathway through β -catenin, the way it inhibits β -catenin remains unknown.

There are multiple points of β -catenin regulation within the canonical Wnt pathway. In the absence of a Wnt ligand, cytosolic β -catenin undergoes rapid turnover by the degradation complex which is comprised of several negative regulators. β -catenin is bound by the tumor suppressor gene product adenomatous polyposis coli (APC) to be phosphorylated by associated kinases such as GSK-3 β , ubiquitinated, and finally degraded by the 26S proteasome. When not bound to β -catenin, TCF/LEF family transcription factors in the nucleus bind repressive

elements to inhibit expression of Wnt targets. When the pathway is activated by Wnt ligand binding to its transmembrane receptor, the degradation complex is sequestered and inhibited at the plasma membrane. “Stable” or unphosphorylated β -catenin translocates to the nucleus to bind TCF/LEF where these co-transcription factors go on to activate multiple downstream gene targets.^{139–142} There are many additional regulators that can modulate this transduced signal, such as the inhibitor ICAT which binds β -catenin and prevents its interaction with TCF/LEFs.¹⁴³

In mammalian cells, emerin appears to not only downregulate expression of β -catenin transcriptional targets but also decrease the amount of “active” or non-phosphorylated β -catenin within the cell. Through a combination of co-immunoprecipitation and protein overlay assays, it was discovered that emerin seemingly binds β -catenin via an APC-like (APC-L) domain near its transmembrane domain. EDMD1 patient fibroblasts also exhibit excessive nuclear β -catenin as well as an ectopic growth phenotype characteristic of overactive Wnt signaling.⁴³ Several models of emerin inhibition have been proposed, including binding/sequestration, degradation, and/or nuclear export of β -catenin. In this chapter, we attempt to recapitulate published phenotypes of β -catenin activation in the absence of emerin in multiple mouse and human cell lines.

Results

EMD KO may sensitize MEFs to β -catenin induction

To initially probe whether emerin plays an inhibitory role in the canonical Wnt pathway, we acquired EMD^{+/y} and EMD^{-/y} mouse embryonic fibroblasts (MEFs). Both cell lines expressed β -catenin, while only EMD^{+/y} expressed emerin, confirmed by Western blot and immunostaining (Fig. 3.1A-B). Lithium chloride (LiCl) acts as a Wnt pathway activator by inhibiting the GSK-3 β

kinase.¹⁴⁴ While treatment with LiCl and varying concentrations did not alter nuclear β -catenin signal in WT MEFs, there was a significant increase in nuclear β -catenin in EMD^{-/-} MEFs (Fig. 3.1C-D). This suggested that EMD loss may sensitize cells to Wnt activation.

EMD mutant does not sensitize U2OS cells to β -catenin nuclear translocation

The LEM domain of emerin has been shown to be required for several of its functions. To determine if the LEM domain is required for emerin's inhibition of β -catenin activity, we generated U2OS osteosarcoma cell lines stably expressing either Flag-tagged wildtype or Δ LEM emerin constructs. Immunofluorescent staining of these cells revealed that, while LiCl could induce a mild increase in nuclear β -catenin in all conditions, this increase was not significant, nor was it significantly higher in Δ LEM cells than WT (Fig. 3.2). These data suggest that, either this cell type is less sensitive to emerin mutation than fibroblasts, or the LEM domain is dispensable for emerin's regulation of β -catenin.

hiPSC response to β -catenin induction does not change upon EMD KO

Previous studies have been conducted using fibroblasts or cancer cell lines to elucidate the relationship between emerin and Wnt. However, establishing a model for emerin-Wnt interaction in pluripotent cells could allow investigation of this mechanism during cardiomyocyte or skeletal muscle differentiation *in vitro*. To achieve this, we generated two EMD KO human iPSC clones via CRISPR-Cas9 (clones 1B7 and 2G6, corresponding to EMD^{KO} clones #3 and #4 in Chapter 2, respectively) from the parental wildtype WTC-11 cell line. When treated with 40mM LiCl, while WTC-11 cells exhibited significant β -catenin nuclear localization, neither EMD KO clone exhibited significant translocation, nor was there significant amounts of nuclear β -catenin

present in the untreated conditions as observed in other studies (Fig. 3.3A-B). These data are contrary to our previous findings in MEFs. However, while LiCl is a commonly used Wnt activator, most standardized iPSC differentiation protocols activate the Wnt pathway with the GSK-3 β inhibitor CHIR99021 (CHIR). To determine optimal conditions in these hiPSCs, we activated the Wnt pathway in WTC-11 and EMD KO cells using both CHIR99021 as well as recombinant Wnt3a protein (rWnt3a). Clone 2G6 appears to contain generally lower amounts of both active and total β -catenin. In both WT and the 1B7 EMD KO clone, CHIR99021 induced higher levels of active β -catenin compared to rWnt3a (Fig. 3.3C). From these results, we continued to use CHIR to activate the Wnt pathway in hiPSCs.

Emerin does not impact β -catenin degradation

One proposed mechanism for how emerin antagonizes the Wnt pathway is through facilitating the degradation of β -catenin. It is unknown whether this degradation would be via phosphorylation, ubiquitination, or some other post-translational modification enabled by emerin's interaction. To determine if emerin generally has an impact on β -catenin degradation, WT and EMD KO 1B7 iPSCs were incubated in 25uM of the ribosomal inhibitor cycloheximide for the indicated number of hours to prevent translation of additional β -catenin protein. Both total β -catenin and emerin can be seen to decrease from 0 to 16-hour treatment. There was no observable difference in degradation rate between WT and EMD KO cells, implying that emerin does not impact the turnover of β -catenin.

Characterization of emerin- β -catenin interaction

Emerin's APC-like domain is predicted to interact with β -catenin. Previous studies have demonstrated emerin- β -catenin interaction through co-immunoprecipitation experiments, though in humans, these interactions were shown in patient-derived fibroblasts and mouse skeletal stem cells.^{43,145} To determine emerin's ability to interact with β -catenin in hiPSCs specifically, we co-immunoprecipitated β -catenin with endogenous emerin. We observe the presence of β -catenin when blotting for the total protein (see yellow arrowhead, Fig. 3.4A), but not when blotting for the active form only. This suggests either that active (phospho-) β -catenin is not abundant enough to be co-immunoprecipitated with endogenous emerin under these conditions or that emerin is less likely to interact with active β -catenin.

We also generated a Δ APC-L emerin mutant to investigate the necessity of the APC-L domain in emerin- β -catenin interaction. However, attempts to co-immunoprecipitate β -catenin were confounded due to very low levels of the emerin mutant (Fig. 3.4B). Interestingly, the Δ APC-L mutant co-immunoprecipitated with a modest amount of β -catenin compared to WT emerin, despite its low abundance and lack of a putative β -catenin binding motif. This preliminary result could suggest that the putative APC-L domain is dispensable for emerin- β -catenin interaction in iPSCs. Though multiple replicates and follow-up experiments would be needed to validate this, the enrichment of Δ APC emerin in the eluate hints that even a small amount of mutant protein could still interact with β -catenin.

EMD KO does not sensitize iPSCs to Wnt signaling during early atrial cardiomyocyte differentiation

The canonical Wnt pathway is necessary for many developmental pathways. Differentiation of iPSCs into atrial cardiomyocytes, for example, requires 48 hours of Wnt induction. To determine if a more physiologically accurate treatment could invoke differences between WT and EMD KO iPSCs, cells were incubated in cardiomyocyte differentiation media (CDM) plus CHIR99021 for 48 hours. We observed a visible increase in β -catenin localized to the nucleus in both genotypes when induced in CDM plus CHIR, while mTeSR+ plus CHIR only yielded a modest increase in nuclear β -catenin. This increase, however, was not significantly higher in EMD KO cells as previously hypothesized (Fig. 3.5A-B). Increased active β -catenin was also observed by immunoblot when grown in CDM media + CHIR99021 for 24 or 48 hours, but the overall effect appeared to decrease in the EMD KO condition (Fig. 3.5C). From these data, we concluded that inducing an initial differentiation program was still insufficient to recapitulate previous findings in EMD KO iPSCs.

iPSCs are unable to recapitulate additional EMD KO phenotypes in Wnt signaling

EDMD1 patient fibroblasts have been found to be more proliferative *in vitro* when compared to healthy patient control fibroblasts.⁹⁴ This phenotype is thought to explain the increased cardiac fibrosis observed in EDMD1 patients. We quantified iPSC proliferation of WTC-11 controls and EMD KOs over 5 days in culture at two different seeding densities. However, no significant difference was observed between wildtype and EMD KO cells (Fig. 3.6A-B). Given the inability of iPSCs to recapitulate published phenotypes of EMD loss so far, we compared their EMD sensitivity with that of MEFs, which demonstrated a mild but significant phenotype (Fig. 3.1). To

determine any effects of EMD KO on the Wnt pathway beyond translocation of β -catenin, RT-qPCR was performed on both EMD^{+/-} and EMD^{-/-} MEFs and multiple EMD KO iPSCs with wildtype WTC-11s. We quantified expression of four common direct β -catenin target genes: *AXIN2* and *TCF7* in iPSCs and *Bmp4* and *Ccnd1* in MEFs. None of these genes were differentially expressed at the transcript level in EMD KOs in either cell type as seen in previous work¹⁴⁵ (Fig. 3.6C-D). Altogether, it was concluded that these cell lines and/or Wnt induction methods were not sufficient to recapitulate published phenotypes of emerin loss.

Discussion

Understanding the functional relationship between emerin and the Wnt pathway is critical in establishing the molecular effects of EDMD1. Previous work investigating emerin's inhibition of Wnt signaling has demonstrated robust phenotypes, particularly when using EDMD1 patient-derived cells.⁴³ It's possible that there is some context-specific characteristic of the disease in humans that is not easily or intentionally replicated *in vitro*. Recent studies have also demonstrated how 3D cell culture can effect experimental outcomes over traditional 2D culture, particularly when analyzing mechanosensitive factors of the nuclear periphery.¹⁴⁵ This chapter emphasizes the necessity of using the proper experimental system to understand emerin's mechanism in the context of Wnt signaling.

There have been numerous reports of emerin's interactions with multiple other transcription factors and nuclear peripheral proteins (see Chapter 1). AlphaFold2 predicts emerin to be largely unstructured, making identifying discrete binding domains beyond the LEM and transmembrane domains challenging. Many proposed binding domains overlap considerably with each other.³⁹

Emerin's regulation of β -catenin may therefore be a more complex mechanism than previously appreciated, one that may rely on other nuclear factors' interactions with emerin.

Methods

MEFs were gifted by the Lammerding lab at Cornell University and cultured in high glucose DMEM (GenClone 25-500) + 10% fetal bovine serum + 1X penicillin/streptomycin (Gibco 15070-063). U2OS cells were cultured in McCoy's 5A (Gibco 16600-082) + 10% fetal bovine serum + 1X penicillin/streptomycin. Cardiomyocyte differentiation media (CDM) was made by combining RPMI 1640 (Gibco 11875-093) + 1X B27 -insulin (Gibco A18956-02). All cell lines were incubated at 37C in 5% CO₂. All other methods were conducted as described in Chapter 2.

Figures

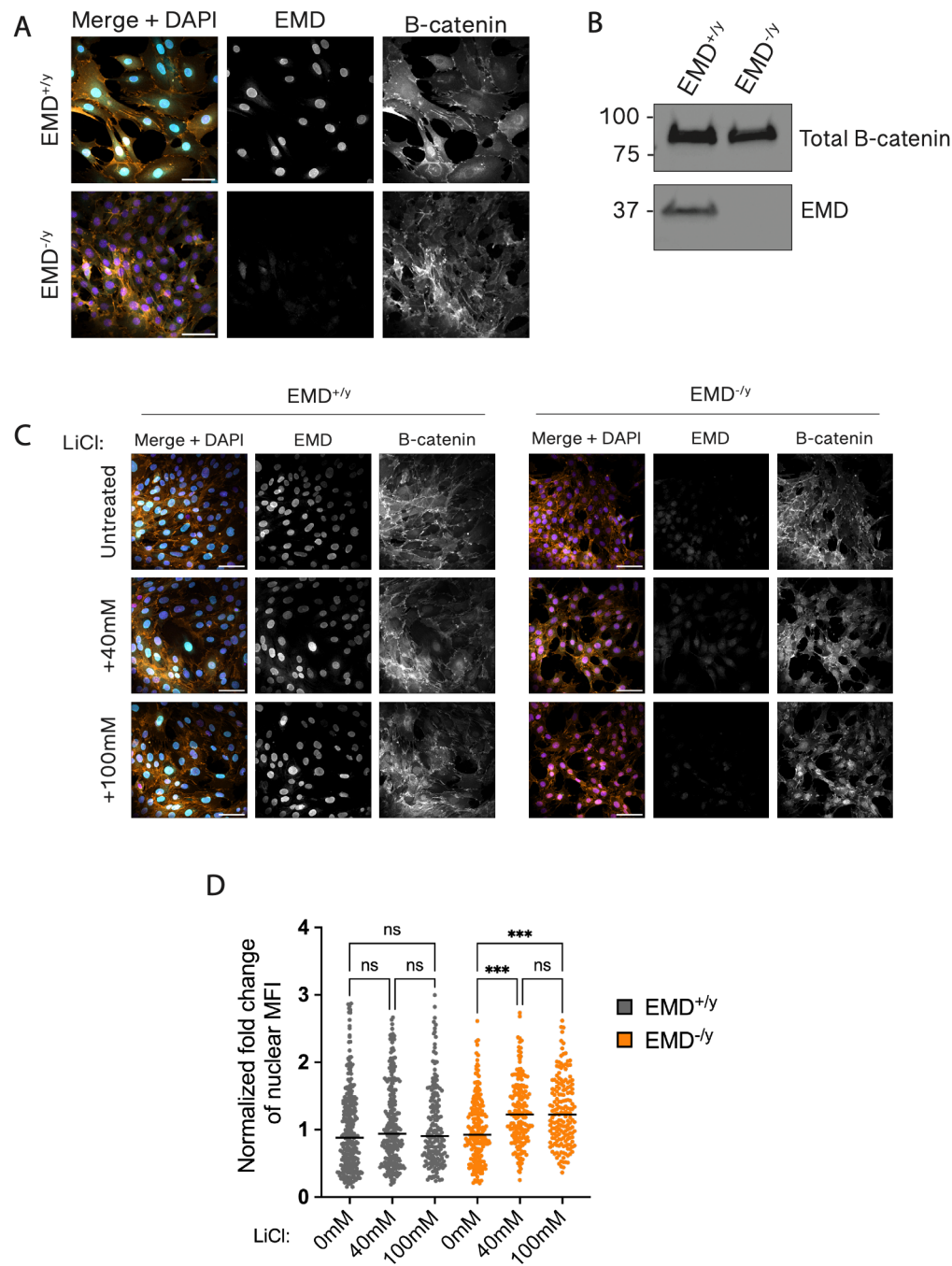


Figure 3.1. EMD null MEFs are more sensitive to Wnt induction.

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A-B) Baseline expression of β -catenin and emerin confirmed by immunofluorescence (A) and Western blot (B). C) Representative images of wildtype and EMD null MEFs treated with 0, 40, or 100mM LiCl. Note nuclear localization of β -catenin in EMD^{-y} cells upon LiCl treatment. D) Quantification of (C). Each point represents one nucleus, normalized to average mean fluorescent intensity (MFI) of EMD^{+y}, 0mM LiCl. ~200 nuclei measured across 3 images (n = 1 biological replicate per genotype). All scale bars = 100um. Normalized MFI significantly different by two-way ANOVA: ***p < 0.001.

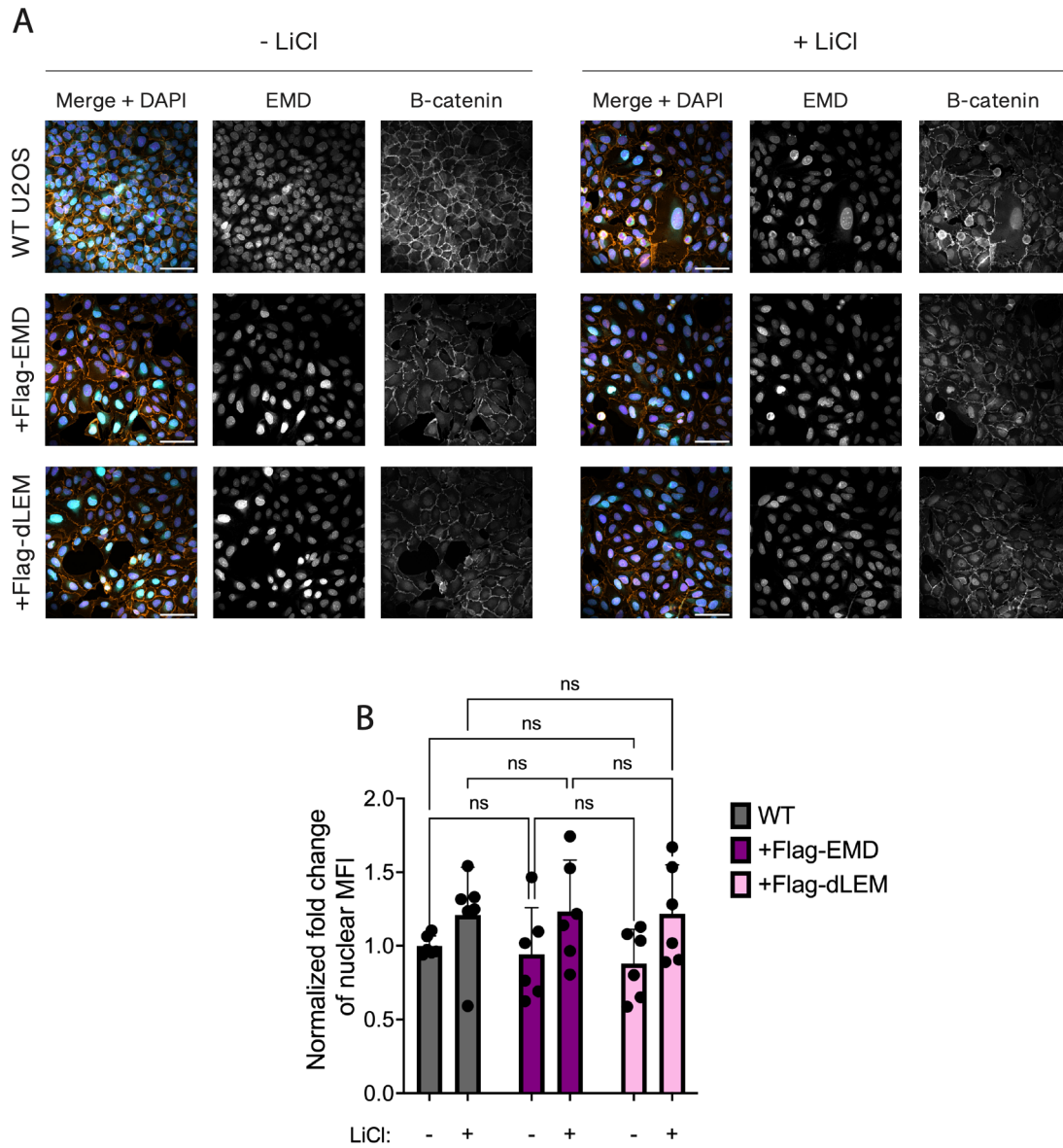


Figure 3.2. U2OS response to Wnt activation is unaffected by EMD mutation.

A) Representative images of U2OS cells with and without LiCl treatment. B) Quantification of (A). Each point represents average nuclear signal, normalized to average nuclear MFI of WT U2OS -LiCl, measured across 6 images (n = 2 biological replicates per genotype). All scale bars = 100um.

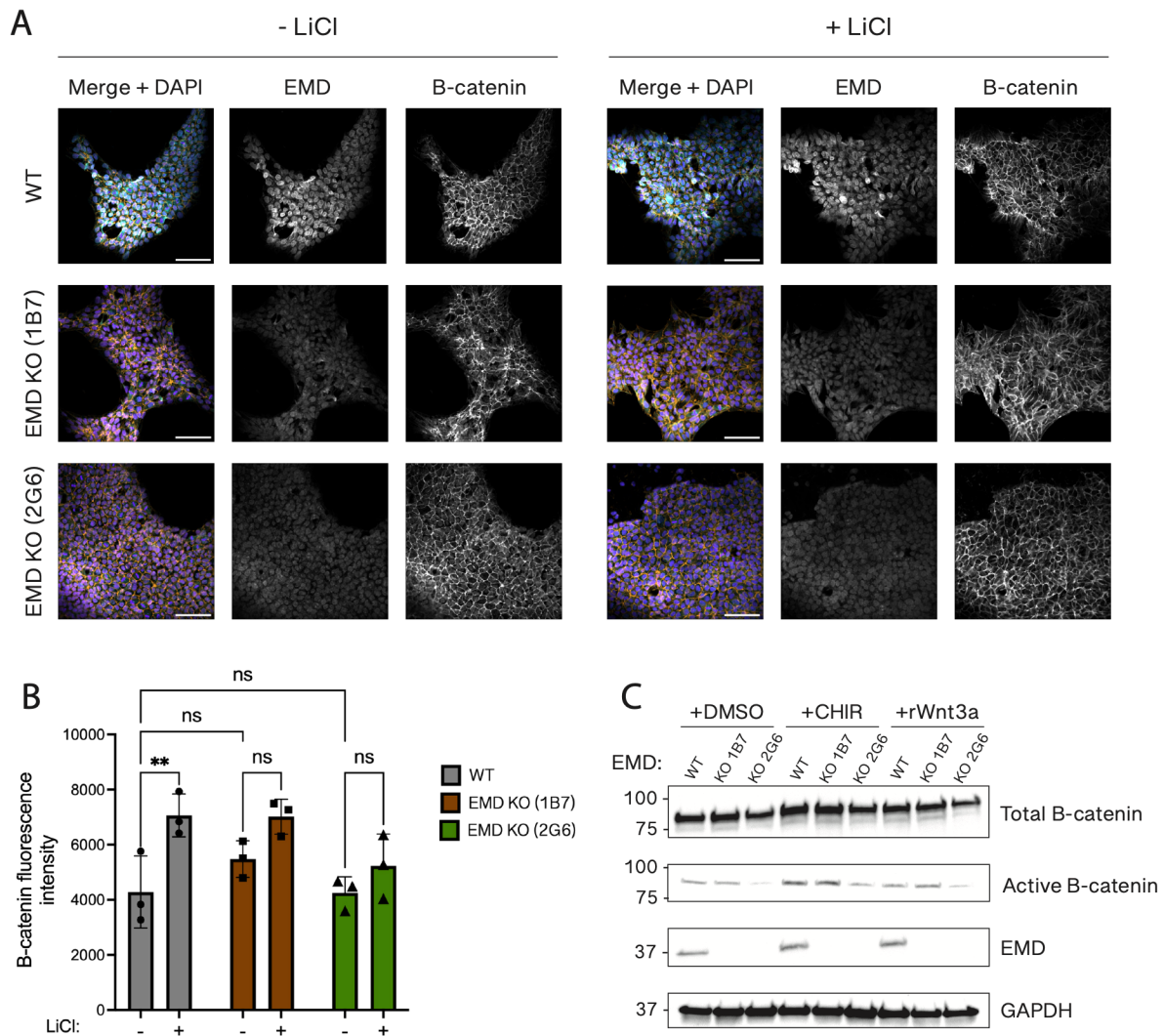


Figure 3.3. EMD KO iPSCs are less sensitive to Wnt activation with LiCl.

A) Representative images of human iPSCs (wildtype and new EMD KO clones) with and without LiCl treatment. B) Quantification of (A). Each point represents average nuclear signal across 3 images (n = 1 biological replicate per genotype). C) Immunoblot of whole cell lysates from wildtype and EMD KO clone iPSCs, treated with DMSO, CHIR, or rWnt3a. All scale bars = 100um.

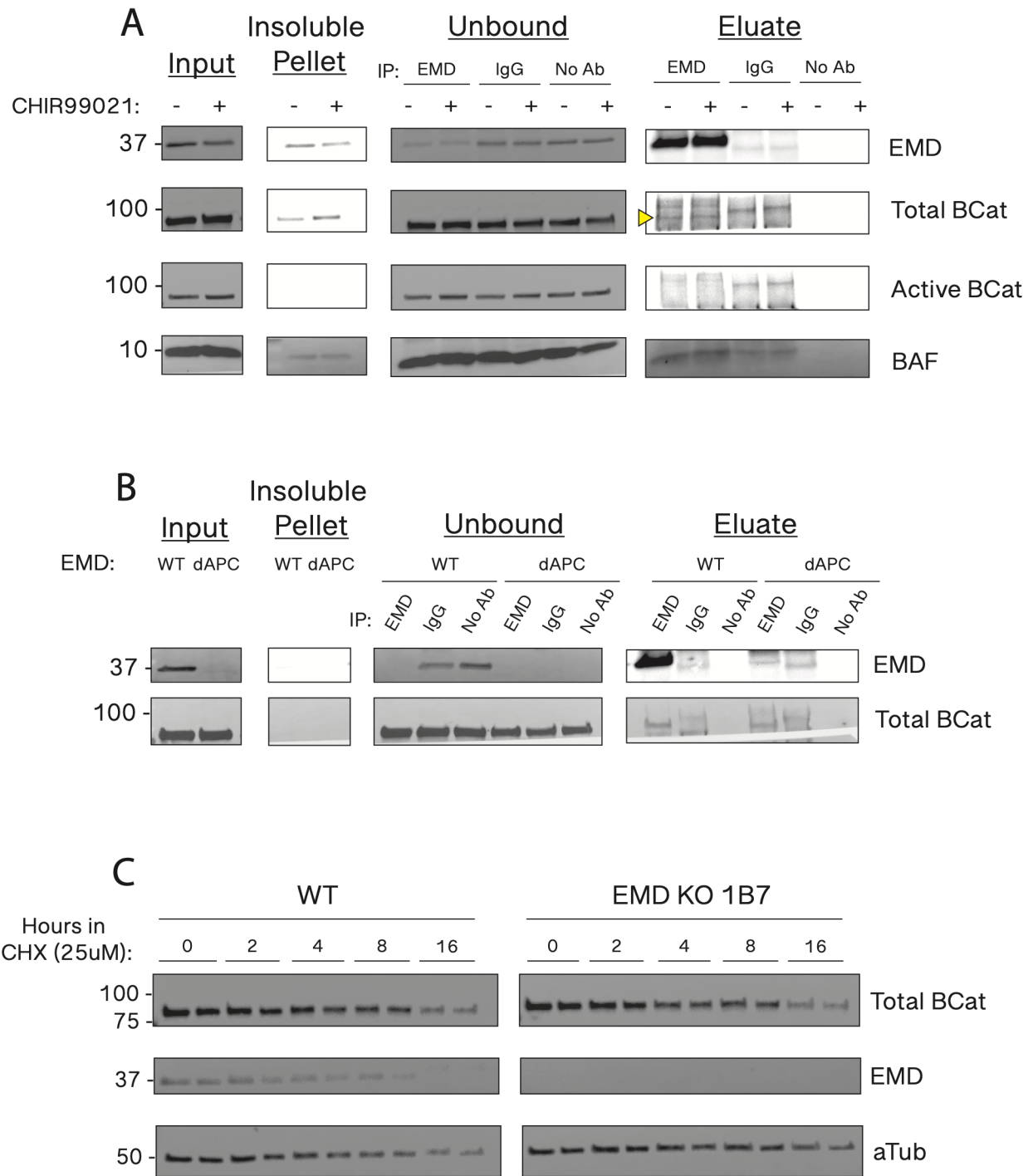


Figure 3.4. Endogenous emerlin co-immunoprecipitates with β -catenin.

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A) Immunoprecipitation of endogenous emerin from WTC-11 wildtype iPSCs. Note depletion of endogenous emerin by an anti-emerin antibody in the unbound fraction. Yellow arrowhead indicates specific β -catenin band at 92kDa. B) Immunoprecipitation of endogenous emerin from WTC-11 and Δ APC EMD mutant iPSCs. C) Immunoblots of wildtype and EMD KO clone 1B7 treated with cycloheximide (CHX) for the indicated times. 2 technical replicates per timepoint.

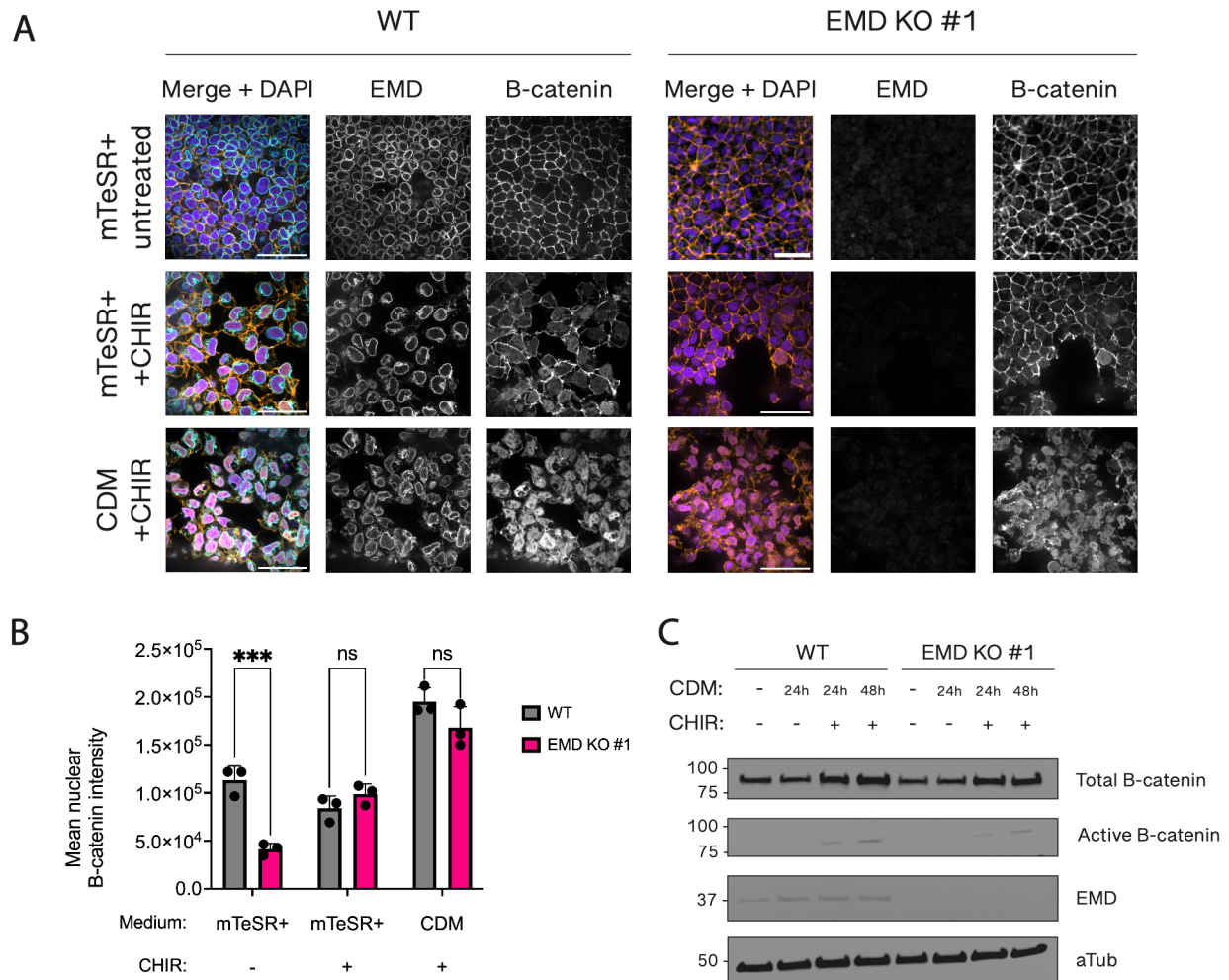


Figure 3.5. Early atrial cardiomyocyte differentiation does not induce differences in Wnt response.

A) Representative images of wildtype and EMD KO clone #1 grown in mTeSR+ or CDM, +/- 7uM CHIR99021, for 48 hours. B) Quantification of (A). Each point represents average nuclear signal across 3 images (n = 1 biological replicate per genotype). C) Immunoblot of whole cell lysates from wildtype and EMD KO clone #1. Cells grown mTeSR+ or CDM for 24 or 48 hours, +/- CHIR99021. All scale bars = 50um. Mean nuclear intensity significantly different by two-way ANOVA: ***p < 0.001.

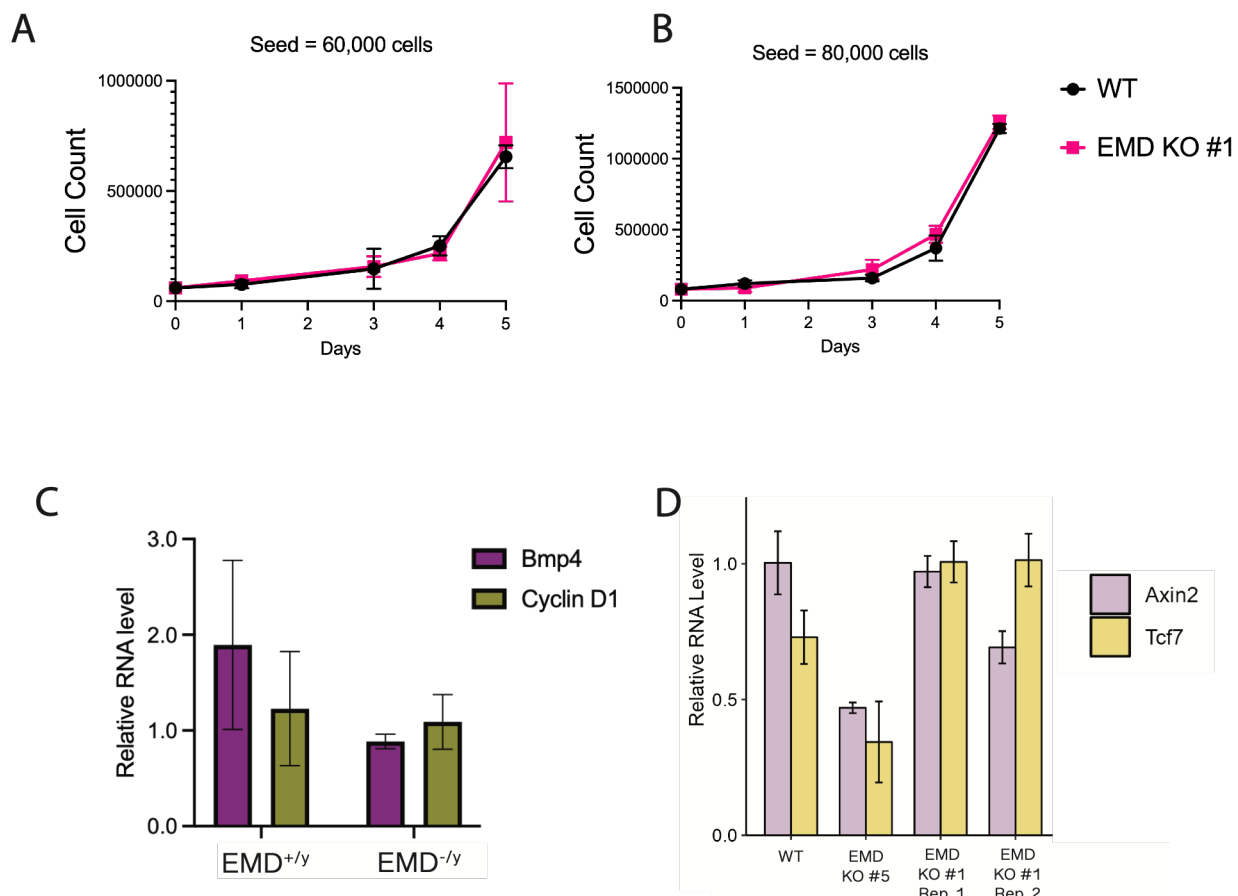


Figure 3.6. Verification of additional Wnt inhibition phenotypes.

A-B) Quantification of proliferation of wildtype and EMD KO clone #1 from Day 0 to Day 5. Seeded at the indicated density at Day 0. N=3 technical replicates per genotype. C-D) RT-qPCR of common β -catenin transcriptional target genes in MEF (C) and hiPSC (D) wildtypes and EMD KOs. For MEFs, n=5 technical replicates per genotype. For hiPSCs, n=3 technical replicates per genotype.

Chapter 4: Technical development of a combinatorial screen of LEM domain proteins using multiAsCas12a

Abstract

This chapter briefly summarizes attempts to identify specific LEM domain protein necessity and redundancy through a combinatorial CRISPR-Cas12a screen.

Introduction

Proteins of the nuclear periphery exhibit nontrivial redundancies. The 7 proteins of the LEM family, in particular (*EMD*, *LEMD1*, *LEMD2*, *LEMD3* encoding MAN1, *ANKLE1*, *ANKLE2*, and *TMPO* encoding LAP2 and its isoforms), are known to play compensatory roles for shared functions.^{30,146} Only double LEM knockouts or knockdowns have been reported so far,^{146,147} but this method cannot identify all possible combinations leading to exacerbated or rescued phenotypes. Additionally, it would require 127 individual cell lines to engineer these combinations manually. In light of these considerations, a more practical solution is needed to probe functions of the entire LEM protein family.

Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) is a powerful genetic tool used to alter specific gene sequences and expression. CRISPR interference (CRISPRi) is a version of this technique in which a catalytically dead Cas enzyme linked to repressor domain is paired with a targeting guide RNA (sgRNA) that targets a sequence immediately following a protospacer adjacent motif (PAM) to silence or decrease expression without cutting DNA.¹⁴⁸ Cas12a is frequently used for CRISPRi because of its small size, auto-cleaving capabilities of

multiple sgRNAs, and limited off-target effects due to a stricter PAM sequence requirement ('TTTV' vs. 'NGG' for Cas9). Due to recent advances in CRISPRi technology, it is now possible to target up to 10 gene sequences using a single arrayed vector for expression knockdown.¹⁴⁹ The multiAsCas12a variant demonstrates stabilized target sequence binding, and thus improved knockdown, when targeting multiple guides within one cell. Altogether, this multiplexing system allows for pooled genetic screening, where a library of vectors each harboring guide RNAs against different combinations of LEM proteins can be generated. By incorporating these arrays into cells of interest, allowing them to proliferate, then sorting and sequencing remaining cells, we can determine which combinations of LEM proteins are required for cell survival. This technique can also be employed to perform arrayed or image-based screens to observe nuclear shape and/or rupture upon LEM perturbation.

Results

multiAsCas12a demonstrates ~50% knockdown efficiency in HEK 293T cells

We first needed to verify the functionality of the newly engineered Cas12a variant in our own hands. We received an optimized multiAsCas12a-mCherry fusion construct and corresponding guide vector backbone expressing BFP from Adam Litterman's lab at the Laboratory for Genomics Research. Additional materials and guides were gifted by Luke Gilbert's lab at the Arc Institute. We first checked Cas12a efficiency using a guide targeting CD55, which encodes a widely expressed cell surface marker. Human embryonic kidney (HEK) 293T cells were infected with multiAsCas12a fusion vector lentivirus to generate stable cell lines, which were subsequently infected with CD55 guide RNA lentivirus. Cells were stained with an anti-CD55-APC antibody and quantified by flow cytometry. Compared to the Cas12a-only controls, Cas12a

+ sgCD55 cells demonstrated a knockdown efficiency of ~50-60%, seen in two separate cell lines (Fig. 4.1A). There was also a net left shift in both sgCD55 cell lines compared to the Cas12a only control, indicating a global decrease in surface CD55 signal even in CD55-positive cells. We additionally generated stable lines expressing two guides against BAF at once in Cas12a parental cells. BAF is required for cell survival,¹⁵⁰ so we expected to see notable underrepresentation of Cas12a + sgBAF cells. Indeed, when compared with unsorted sgCD55-positive cells, there were ~50% fewer sgBAF-positive cells (Fig. 4.1B). Both findings are consistent with a ~50% knockdown efficiency of this Cas12a construct in HEK 293T cells.

Characterization of LEM sgRNA efficiency

Encouraged by these preliminary results, we next designed guides targeting all 7 LEM proteins. Using the CRISPick tool with previously published parameters,^{149,151,152} we generated a list of all possible sgRNA sequences that satisfied the strict Cas12a PAM requirement. Two LEM genes (LEMD2 and ANKLE2) did not contain such sequences, however, and suboptimal PAM sites had to be used for their sgRNA design.¹⁵³ The top predicted guides were each cloned into the backbone vector and Cas12a cells were infected with the corresponding lentivirus. We performed RT-PCR to determine general knockdown efficiency of these guides in HCT-116 cells. Transcript abundance of some LEM genes was notably decreased (e.g. *EMD*, *LEMD3*, *ANKLE1*). However, abundance of other transcripts appeared minimally affected (e.g. *TMPO*, *ANKLE2*) (Fig. 4.2A).

Given that this screen depended on efficient and even knockdown of each LEM, we carefully tested all 4 predicted guides generated by CRISPick for *TMPO*, a well-expressed and critical LEM gene that displayed minimal knockdown. No single guide was able to elicit any appreciable knockdown of *TMPO* in HCT-116 cells, shown by both RT-PCR and immunostaining (Fig. 4.2B-

C). In the latter, we observed limited nuclei expressing both the sgRNA-BFP construct and the Cas12a-mCherry fusion, suggesting that expression of both had a deleterious effect ; however, BFP+/mCherry+ cells still did not exhibit lower LAP2 levels than control cells (yellow arrowheads denote examples of this). It should also be noted that, RT-PCR primers targeted a sequence which favors the LAP2b isoform. The inability of the system to knock down several LEMs, including one of significant interest, led us to conclude that a combinatorial screen of the LEM protein family was not feasible at that time.

Discussion

The LEM protein family is ideally suited for a multiplexed CRISPRi toolkit; with only 7 discrete genes, all possible combinations of sgRNA targets are easily within the range of a standard genome-wide screen (127 arrays plus controls vs. tens of thousands of sgRNAs). A combinatorial screen could be extremely effective in disentangling redundant LEM functions. However, technical limitations of the technique prevent execution at this current stage. Until an alternative Cas12a can be engineered exhibiting both efficient multiplexed knockdown as well as more permissive PAM sequence requirements, other techniques will need to be employed to investigate LEM redundancies.

Methods

Cell Culture

HEK 293T cells were cultured in high glucose DMEM (GenClone 25-500) + 10% fetal bovine serum + 1X penicillin/streptomycin (Gibco 15070-063). HCT-116 cells were cultured in

McCoy's 5A (Gibco 16600-082) + 10% fetal bovine serum + 1X penicillin/streptomycin. All cell lines were incubated at 37C in 5% CO₂.

Lentivirus Production and Infection

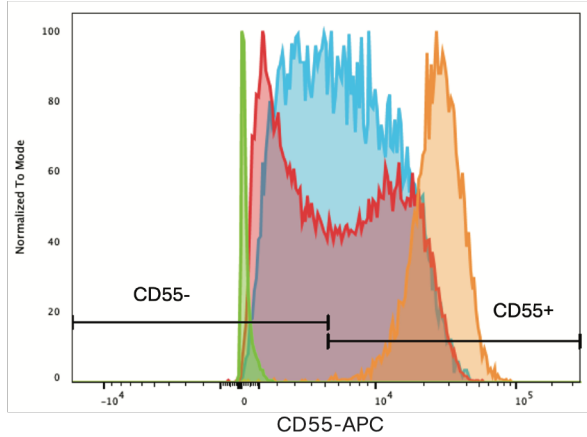
HEK 293Ts were transfected with the vector of interest as well as 2nd generation lentiviral vectors pSPAX2 and pMD2.G using the jetPrime reagent kit (Polyplus 101000046). Media was collected after 48-72 hours, depending on cell survival, filtered through a 0.45um filter to remove cell debris, and aliquoted. Cells were infected by seeding 1x10⁶ cells in one well of a 6well plate. After 24 hours, lentivirus was added along with 8ug/mL polybrene. After another 24 hours, lentivirus was replaced with fresh media. Cells were expanded into 10cm plates the following day.

FACS Staining/Sorting

Cells were collected and blocked for 10min at room temperature using Fc block (BioLegend 422302) diluted in FACS buffer (0.5% BSA in 1X PBS). Anti-CD55-APC antibody diluted in FACS buffer (1:50) was added to cells after removing blocking buffer, incubated on ice for 20min, and washed twice in 1X PBS. FACS analysis was performed on cells resuspended in FACS buffer using the Sony SH800 cell sorter.

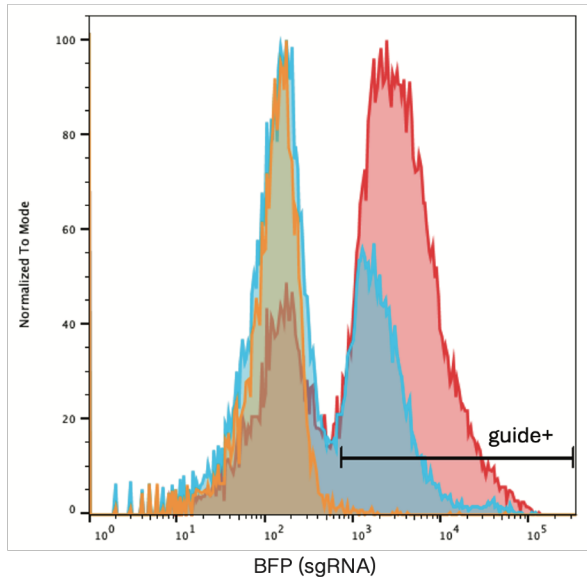
Figures

A



Cell line	Antibody	Count	% CD55+
Cas12a only	IgG	5,448	0.055
Cas12a only	CD55-APC	5,360	99.8
Cas12a + sgCD55 #1	CD55-APC	21,648	52.9
Cas12a + sgCD55 #2	CD55-APC	8,141	57.7

B



Cell line	Count	% BFP+
Cas12a only	5,360	0.78
Cas12a + sgBAF	25,814	32.8
Cas12a + sgCD55 #1	31,699	68.3

Figure 4.1. Analysis of multiAsCas12a knockdown efficiency

A) FACS plot of relative abundance of CD55 in each noted condition, normalized to the mode. The Cas12a only cell line stained with either IgG or anti-CD55-APC was used to establish gates and denote lower and upper limits, respectively. B) FACS plot of relative BFP signal in each condition, normalized to the mode. The Cas12a only cell line was used to establish the gate for sgRNA-BFP-positive cells.

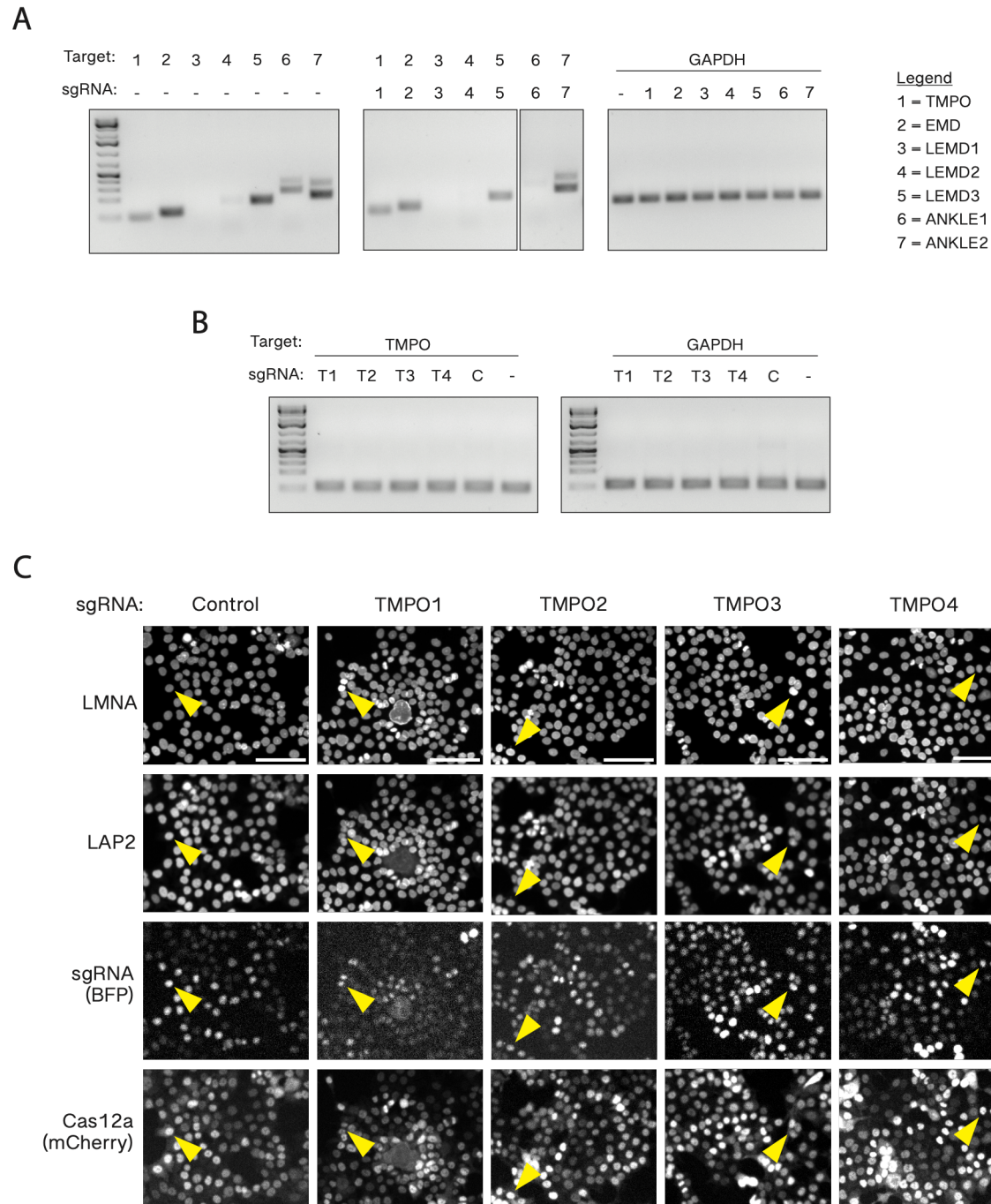


Figure 4.2. Determining the efficacy of all predicted *TMPO* sgRNA sequences

A) RT-PCR of HCT-116 cells probing for transcription of the indicated genes (target). All cells stably express Cas12a and either no sgRNA (-) or the indicated sgRNA. B) RT-PCR of HCT-116 cells expressing Cas12a and each of the 4 *TMPO* sgRNAs (T1-4) plus a control guide (C). C) Immunofluorescence of corresponding cells from (B). Yellow arrowheads indicate BFP+/mCherry+ nuclei. All scale bars = 100µm.

Chapter 5: Conclusion and Future Directions

Throughout this thesis, we have investigated the function and molecular mechanism of emerin in multiple aspects. In Chapter 2, we highlighted the necessity of emerin in human iPSC-derived, late-stage skeletal muscle differentiation. Establishing iSMs as a model for emerin loss in humans, we observed multiple defects in the absence of emerin, including cell cycle exit failure, loss of p21 expression, and a significant number of undifferentiated cells. Globally, we observed differential expression of PRC2 targets in EMD KO cells. While this was positively correlated with expected H3K27me3 chromatin marks in myotubes, differentially expressed genes were only moderately enriched within LADs. Chapter 3 summarized attempts to observe emerin's reported antagonistic relationship with the Wnt effector β -catenin. While we observed potential sensitization to Wnt activation in EMD null MEFs and co-immunoprecipitation of β -catenin and emerin in iPSCs, all other cell types and assays were unable to recapitulate published phenotypes. The combinatorial screen proposed in Chapter 4 has the potential to inform overlap in the function of emerin and other LEM proteins.

Though we show enrichment of emerin-sensitive genes within predicted PRC2 targets, it is currently unknown how or under what conditions this occurs. Emerin's ability to influence chromatin modification has been previously reported.^{29,48,154} Of laminopathies in general, the seemingly random collection of symptoms and molecular changes can best be explained by alterations in chromatin state. The limited enrichment of differentially expressed genes in predicted LADs is also surprising given emerin's association with the nuclear lamina. Moreover, the correlation between expected H3K27me3-positive regions and LADs implies that there is a subset of emerin-sensitive, H3K27me3⁺ genes not associated with the lamina. It remains unclear

how membrane-bound emerin influences gene expression of loci localized to the nuclear interior. However, given emerin's proposed binding of multiple factors within the nucleus, including EZH2 in some contexts,¹⁵⁴ one could speculate that it functions to sequester and/or stabilize transcriptional regulators.

Emerin's inhibition of the Wnt pathway is likely not mutually exclusive to its function in cardiac and skeletal muscle differentiation. Emerin may actually influence a number of signaling pathways, either directly or indirectly. The presence of the APC-L domain within emerin's unstructured lamin-binding domain is suggestive of its binding capabilities, and it is tempting to infer a direct interaction between emerin and β -catenin. However, it is surprising that a single, unstructured, membrane-bound, 18 amino acid sequence is able to actively compete with β -catenin cofactors like TCF/LEF which bind β -catenin with $\sim 20\text{nM}$ affinity.¹⁵⁵ Additional structural and biochemical data could shed light on emerin- β -catenin interactions relative to the surrounding β -catenin interactome.

There are additional considerations outside of the nucleus when investigating emerin's regulatory capabilities. β -catenin's alternate function as a key regulator of cell adhesion, particularly in the adherens junctions of cardiomyocyte intercalated discs,¹³⁸ opens a new line of inquiry into its relationship with the nuclear periphery. The transduction of mechanical forces from the plasma membrane to the nucleus is conducted via the LINC complex, of which emerin is a member. While endogenous emerin likely does not functionally exit the nuclear periphery,¹¹⁰ it could be interesting to investigate emerin- β -catenin interactions as a means of mechanical regulation rather than only transcriptional.

Altogether, this thesis has illustrated the necessity of emerin during later stages of myogenesis in human cells. We have established the use of iSMs as a valuable model for understanding emerin's function in a human context without the need for patient samples. While this work highlighted new potential mechanisms for emerin, many questions still remain surrounding its interactions with various nuclear factors. Future experiments will be needed to fully elucidate the function of emerin in human tissues.

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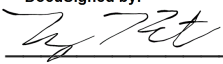
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