Inhibiting histone acetyltransferase activity rescues differentiation of emerin-null myogenic progenitors

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Running Title: HAT inhibition rescues emerin-null differentiation

Abbreviations:

BAF: Barrier-to-Autointegration Factor
BSA: Bovine Serum Albumin
bHLH: Basic Helix-Loop-Helix
DAPI: 4',6-diamidino-2-phenylindole
DMEM: Dulbecco's Modified Eagle's medium
DMSO: Dimethyl Sulfoxide
EDMD: Emery-Dreifuss Muscular Dystrophy
EdU: 5-Ethynyl-2'-deoxyuridine
H4K5: Histone 4 lysine 5
H4K5ac: Histone 4 acetylated on lysine 5
HAT: Histone Acetyltransferase
HATi: Histone Acetyltransferase inhibitor
HDAC: Histone Deacetylase
HDACi: Histone Deacetylase Inhibitor

LAD: Lamina Associated Domain
LAP: Lamina Associated Polypeptide
NCoR: Nuclear Co-Repressor
MEF: Myocyte Enhancer Factor
MyHC: Myosin Heavy Chain
PBST: Phosphate Buffered Saline with Tween
SIRT1: Sirtuin 1

Key Words
Cell signaling, Emerin, Emery-Dreifuss Muscular Dystrophy, Myogenic differentiation

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SUMMARY STATEMENT

Emerin regulation of H4K5 acetylation dynamics are important in the later stages of myogenic differentiation.

ABSTRACT

Emery-Dreifuss Muscular Dystrophy (EDMD) is a disease characterized by skeletal muscle wasting, contractures of the major tendons, and cardiac conduction defects. Compromised skeletal muscle regeneration is predicted to result from impaired muscle stem cell differentiation. Mutations in the gene encoding emerin cause EDMD. We previously showed emerin-null myogenic progenitors fail to properly exit the cell cycle, fail to exhibit decreased myosin heavy chain (MyHC) expression and have decreased myotube formation. Treatments with theophylline, a HDAC3 activator, rescued myotube formation in differentiating emerin-null myogenic progenitors, suggesting emerin activation of HDAC3 activity is important for myogenic differentiation. Pharmacological inhibitors of H4K5 histone acetyltransferases (HATs) were used to test if the deacetylation function of HDAC3 by theophylline treatment was responsible for rescuing emerin-null myogenic differentiation. Nu9056 and L002 were added to differentiating wildtype and emerin-null myogenic progenitors and differentiation was assessed. HAT inhibition rescued emerin-null myogenic progenitor differentiation. In contrast to treatment with these HAT inhibitors, emerin-null myogenic progenitors treated with SRT1720, which targets SIRT1, a NAD^+-dependent deacetylase, showed no significant change in myotube formation. Thus, we conclude emerin regulation of HDAC3 activity is important for myogenic differentiation. Therefore, pharmacological targeting of H4K5 acetylation is a promising therapeutic approach for rescuing muscle regeneration in EDMD.

INTRODUCTION

The nuclear envelope is composed of two lipid bilayers, the outer nuclear membrane, which is contiguous with the endoplasmic reticulum, and the inner nuclear membrane (Holaska, 2016). Although the outer and inner nuclear membranes arise from a common membrane, they are functionally distinct membranes containing proteins localizing specifically to either the outer or the inner nuclear membrane. Underlying the inner nuclear membrane is a network of Type V intermediate filament proteins named lamins that provide nuclear rigidity and elasticity (Tatli and Medalia, 2018). The inner nuclear membrane contains a large number of integral inner nuclear membrane proteins (Gruenbaum and Foisner, 2015), many of which show cell-type-specific expression (de Las Heras et al., 2013; Gonzalez et al., 2012; Korfali et al., 2010; Korfali et al., 2012; Malik et al., 2010; Schirmer et al., 2003; Wilkie et al., 2011; Worman and Schirmer, 2015). Inner nuclear membrane proteins function in diverse roles, including nuclear structure, genomic organization, chromatin architecture, gene expression, cell cycle regulation, and cytoskeletal organization (Barton et al., 2015; Holaska, 2016). The nuclear lamins and its associated inner nuclear membrane proteins define the nuclear lamina.

Emerin is a lamin-binding integral inner nuclear membrane protein. Mutations in the gene encoding emerin cause X-linked Emery-Dreifuss muscular dystrophy (EDMD), an inherited disorder causing progressive skeletal muscle wasting, irregular heart rhythms, and contractures of major tendons (Bione et al., 1994; Mendez-Lopez and Worman, 2012; Vlcek and Foisner, 2007; Worman, 2012). Evidence suggests the skeletal muscle wasting seen in EDMD is caused by impaired differentiation of skeletal muscle stem cells and inefficient skeletal muscle regeneration. For example, skeletal muscle necrosis and increased skeletal muscle fiber permeability is rarely seen in EDMD patients (Bonne et al., 1993). Further supporting this hypothesis, emerin-null mice exhibit delayed skeletal muscle regeneration and repair, motor coordination defects, and mild atrioventricular conduction defects (Melcon et al., 2006; Ozawa et al., 2006). Skeletal muscle from both EDMD patients and emerin-null mice showed altered expression of muscle regeneration pathway components (Bakay et al., 2006; Melcon et al., 2006) (Bakay et al., 2006; Melcon et al., 2006). Emerin-null myogenic progenitors and emerin-downregulated C2C12 myoblasts exhibit impaired differentiation and myotube formation (Collins et al., 2017; Frock et al., 2006; Huber et al., 2009) due to aberrant temporal

activation of myogenic differentiation genes (Dedeic et al., 2011) and disruption of key signaling pathways (Koch and Holaska, 2012), suggesting defective muscle regeneration contributes to the EDMD skeletal muscle phenotype (Frock et al., 2006; Huber et al., 2009; Melcon et al., 2006). The coordinated temporal expression of MyoD, Myf5, Pax3 and Pax7 was also disrupted in emerin-null myogenic progenitors (Demmerle et al., 2013) due to the inability of the genome to properly reorganize during differentiation (Bakay et al., 2006; Koch and Holaska, 2012; Melcon et al., 2006). The failure of the genome to properly reorganize during emerin-null myogenic differentiation supports the hypothesis that emerin-null myogenic progenitors fail to undergo the transcriptional reprogramming required for myogenic differentiation of their transcriptome.

Emerin is a ubiquitously expressed integral inner nuclear membrane protein (Manilal et al., 1996; Nagano et al., 1996; Tunnah et al., 2005) with reported roles in nuclear structure, chromatin architecture, genomic organization, cell signaling, and gene expression (Collins et al., 2017; Dedeic et al., 2011; Demmerle et al., 2012; Demmerle et al., 2013; Haraguchi et al., 2004; Holaska et al., 2004; Holaska et al., 2006; Holaska and Wilson, 2006; Holaska and Wilson, 2007; Koch and Holaska, 2012; Markiewicz et al., 2006). Growing evidence supports roles for emerin in regulating genomic architecture and gene expression (Koch and Holaska, 2013). Emerin-null nuclei have more relaxed chromatin (Meaburn et al., 2007; Mewborn et al., 2010; Ognibene et al., 1999). Further, we showed emerin binds directly to HDAC3 and activates its deacetylase activity (Demmerle et al., 2012). Skeletal muscle from humans and mice lacking emerin show altered expression of muscle regeneration components (Bakay et al., 2006; Koch and Holaska, 2012; Melcon et al., 2006). Thus, the relaxed chromatin architecture in emerin-null cells directly effects the expression of genes important for muscle regeneration. We posit emerin assists in regulating the temporal association of repressive chromatin at the nuclear lamina to regulate the coordinated expression of muscle stem cell differentiation genes.

Muscle regeneration is activated by muscle injury and stimulates myogenic progenitors to proliferate, differentiate into committed myoblasts, formation of myocytes and fusion to the myofiber to repair muscle (Kuang and Rudnicki, 2008). Coordinated temporal expression of differentiation factors (e.g., MyoD, Pax3, Pax7, Myf5) is required for proper

differentiation and skeletal muscle regeneration. Myoblasts lacking emerin exhibit impaired differentiation (Frock et al., 2006; Huber et al., 2009) due to the aberrant temporal activation of differentiation genes (Dedeic et al., 2011) and disruption of key signaling pathways (Koch and Holaska, 2012); stimulating HDAC3 activity rescued emerin-null differentiation (Collins et al., 2017).

Here we independently tested if H4K5 acetylation dynamics were important for myogenic differentiation using histone acetyltransferase (HAT) inhibitors that targeted HATs mediating H4K5 acetylation. HAT inhibition rescued emerin-null myogenic differentiation showing that increased H4K5 acetylation contributes to the impaired differentiation of emerin-null myogenic progenitors.

RESULTS

Histone acetyltransferase (HAT) inhibition rescues emerin-null myogenic differentiation

We previously showed emerin-null myogenic progenitors had impaired differentiation, which could be rescued by activation of HDAC3 (Collins et al., 2017). Histone acetyltransferase inhibitors (HATi) were used to independently test if the altered H4K5 acetylation dynamics was responsible for the impaired differentiation of emerin-null progenitors. HATi used for these studies were chosen because they preferentially inhibit acetylation of lysine residues targeted by HDAC3 (e.g., H4K5; Anamika et al., 2010). Cell cycle withdrawal, myosin heavy chain (MyHC) expression and the differentiation index were analyzed 36 hours post-differentiation induction within the same cell population during differentiation. Emerin-null and wildtype myogenic progenitors were treated with 0.5 µM L002 upon differentiation induction to test whether inhibition of H4K5 acetylation rescued myogenic differentiation of emerin-null progenitors, (Figure 1A). L002 preferentially affects H4K5 and H4K8 acetylation (Anamika et al., 2010). L002-treated wildtype progenitors exited the cell cycle normally (Figure 1C',J). 2.7% of emerin-null progenitors failed to exit the cell cycle after 36 hours, as expected (Figure 1G,J). Emerin-null progenitors treated with L002 showed a trend toward reducing the number of emerin-null cells in the cell cycle (2.1%; p=0.06; Figure 1G',J). L002 treatment resulted in a small, but significant increase in the percentage of differentiating emerin-null progenitors expressing MyHC, as 40% of control emerin-null cells were MyHC-positive and 46% of L002-treated emerin-null cells were MyHC-positive (Figure 1H, K; p=0.015).

The number of MyHC-positive L002-treated differentiating emerin-null progenitors is statistically similar to untreated wildtype progenitors (47.8% in wildtype, p=0.35; Figure 1D,H',K). L002 treatment increased myotube formation in differentiating emerin-null progenitors (6.9% L002-treated emerin-null cells vs. 3.9% DMSO-treated emerin-null cells; p=0.042; Figure 1 I, L) and rescued myotube formation (differentiation index) to wildtype levels (6.9% L002-treated emerin-null cells vs. 7.7% wildtype cells; p=0.97; Figure 1E, I', L). Unexpectedly, L002 treatment of wildtype progenitors caused a 20% reduction in myotube formation (Figure 1 E, L; 6.1%, p=0.004).

Wildtype and emerin-null myogenic progenitors were differentiated for 36 hours in the presence of the HAT inhibitor Nu9056 to independently confirm HAT inhibition rescued emerin-null myogenic differentiation. Nu9056 preferentially affects H4K5 and H4K16 acetylation (Anamika et al., 2010). 0.5 µM Nu9056 in DMSO or DMSO alone were incubated with wildtype or emerin-null myogenic progenitors upon differentiation induction (Figure 2A). Nu9056 treatment had no effect on cell cycle withdrawal of wildtype or emerin-null myogenic progenitors, (Figure 2 C,G J). The number of MyHC-expressing cells was similar in Nu9056-treated (51.0%) and untreated emerin-null myogenic progenitors (50.3%; Figure 2H, K), retaining their 19% reduction in committed myoblasts. Myotube formation in emerin-null progenitors was rescued by Nu9056 treatment, as 15.1% of Nu9056-treated emerin-null progenitors fused to form myotubes, compared to 10.8% of DMSO-treated emerin-null progenitors (Figure 2I,L). Myotube formation in Nu9056-treated emerin-null progenitors was statistically similar to wildtype progenitors (p=0.11). Treatment of wildtype progenitors with Nu9056 had no effect on myotube formation (p=0.09; Figure 2E,L).

Western blotting using antibodies against H4 and H4 acetylated on lysine 5 (H4K5ac) was used to confirm L002 and Nu9056 inhibited H4K5 acetylation during differentiation of emerin-null myogenic progenitors. We previously showed emerin-null cells increased H4K5ac by 1.9-fold (Collins et al., 2017). Treatment of emerin-null progenitors with L002 during differentiation reduced H4K5ac 2.4-fold (Figure 3), comparable to H4K5ac levels in wildtype progenitors. Nu9056 treatment decreased H4K5ac 3.2-fold in emerin-null myogenic progenitors (Figure 3). Decreased H4K5ac seen in emerin-null myogenic progenitors treated with Nu9056 is similar to the H4K5ac levels seen in differentiating wildtype progenitors.

Sirtuin 1 (SIRT1) activation has no effect on emerin-null myogenic differentiation

A SIRT1 activator was used to confirm rescue of emerin-null progenitor differentiation by L002 and Nu9056 acted specifically by blocking H4 acetylation. SIRT1 is an NAD+-dependent protein deacetylase that has been shown to deacetylate a number of protein targets within the cell, including p53 and PGC1α (Bao and Sack, 2010) and thus its mechanism of action and targets are distinct from HDAC3. 1.5 µM SRT1720 was added to wildtype or emerin-null myogenic progenitors upon differentiation induction and differentiation was analyzed 36 hours post-induction (Figure 4A). SRT1720 failed to rescue cell cycle withdrawal of differentiating emerin-null progenitors, as 7.0% of DMSO-treated and 5.4% of SRT1720-treated cells were cycling (Figure 4G, J; p=0.09). 41.1% of SRT1720-treated differentiating emerin-null progenitors expressed MyHC compared to 42.7% of DMSO-treated emerin-null progenitors (Figure 4H, K; p=0.49). SRT1720 treatment also failed to rescue myotube formation in emerin-null progenitors, as 5.9% of DMSO-treated and 7.0% of SRT1720-treated emerin-null myogenic progenitors formed myotubes (Figure 4I, L; p=0.44). SIRT1 activation inhibited wildtype progenitor exit from the cell cycle, as SRT1720-treated progenitors had 2.1% of cells still cycling (Figure 4 C,J). SRT1720 had no effect on myoblast commitment (MyHC expression) or myotube formation (Figure 4D,E,K,L). Western blotting confirmed the levels of H4K5ac were unchanged by treatment with SRT1720 (Figure 5).

DISCUSSION

The studies presented here used a cell-based system to follow differentiation, in which myotubes are formed by myoblast-to-myoblast fusion or myoblast-to-myotube fusion. This system recapitulates what occurs in primary emerin-null or downregulated myoblasts, which exhibit impaired differentiation (Frock et al., 2006; Huber et al., 2009). Further, the cell-based studies show altered expression of similar genes to those from skeletal muscle biopsies of EDMD patients and emerin-null mice, which are enriched for skeletal muscle regeneration genes (Bakay et al., 2006; Iyer et al., 2017; Melcon et al., 2006). Upon stimulation of myogenic progenitors to differentiate, transcriptional reprogramming is initiated, leading to cell cycle exit. This reprogramming activates the myogenic differentiation program and represses the proliferative program. Transcriptional reprogramming is compromised in emerin-null progenitors (Iyer et al., 2017). The failure of emerin-null progenitors to coordinate the temporal reorganization of

their genome during differentiation is predicted to cause this defective transcriptional reprogramming. This failure in genomic reorganization would explain the large number of transcripts differentially expressed in differentiating emerin-null progenitors (Iyer et al., 2017).

Emerin is proposed to play an important role in regulating myogenic differentiation by regulating chromatin architecture. We previously showed activation of HDAC3 rescues emerin-null myotube formation (Collins et al., 2017; Demmerle et al., 2013). Here we confirm the dynamics of H4K5 acetylation are important for myogenic differentiation by showing emerin-null myogenic progenitor differentiation is rescued by inhibiting HATs targeting H4K5. Thus, the H4K5 acetylation cycle is predicted to be important for ensuring proper transcriptional reprogramming upon differentiation induction (Figure 6).

Emerin and LAP2β interact with chromatin regulatory complexes containing BAF or histone deacetylases (HDACs; Demmerle et al., 2012; Holaska and Wilson, 2007; Somech et al., 2005). Emerin binds directly to HDAC3, the catalytic component of the Nuclear Co-Repressor (NCoR) complex (Demmerle et al., 2012; Holaska and Wilson, 2007) and activates its activity. Emerin-binding also recruits HDAC3 to the nuclear envelope. The functional interaction between emerin and HDAC3 coordinates the spatiotemporal nuclear envelope localization of genomic regions containing *Myf5*, *MyoD*, and *Pax7* to ensure differentiation proceeds normally (Demmerle et al., 2012; Demmerle et al., 2013). Loss of emerin disrupts this genomic reorganization resulting in impaired myogenic differentiation. Recent evidence showed the localization of HDAC3 at the nuclear periphery was also required for lineage restriction during cardiomyocyte differentiation (Poleshko et al., 2017). In the absence of HDAC3 or nuclear envelope-localized HDAC3, repressed genomic loci were aberrantly localized to the nuclear interior resulting in precocious differentiation. Thus, this is an important regulatory mechanism used to regulate differentiation.

Treatment with theophylline, an HDAC3-specific activator rescued myogenic differentiation in emerin-null cells (Collins et al., 2017; Demmerle et al., 2013). LAP2β also interacts with HDAC3 and induces H4 deacetylation (Somech et al., 2005) to contribute to LAD formation (Zullo et al., 2012). Thus, we presume emerin loss caused more HDAC3 to associate with LAP2β at the nuclear envelope and theophylline

activated this pool of HDAC3 to rescue the coordinated temporal sequestration and silencing of promoters to regulate the differentiation transcriptional program. Therefore, H4K5 acetylation is predicted to play a crucial role in the transcriptional reprogramming required for differentiation in multiple cell types. Treatment with HAT inhibitors independently confirmed this result.

HAT inhibition and HDAC3 activation rescued the latter steps of myogenic differentiation, as there was no statistically significant difference in cell cycle withdrawal, although there was a trend. This suggests emerin regulation of H4K5ac dynamics during transcriptional reprogramming may impair complete reprograming during differentiation to effect genes that act at later stages of differentiation. Alternatively, emerin regulation of H4K5ac levels, and thus transcriptional activity, may specifically regulate genes important for cell fusion or myotube maturation. Consistent with these results, HDAC3 inhibition by RGFP966 blocked MyHC expression and fusion in both differentiating wildtype and emerin-null myogenic progenitors (Collins et al., 2017). We propose H4K5 acetylation levels are tightly regulated and that increases or decreases in H4K5 acetylation levels impairs the transition from proliferating myogenic progenitors to differentiating myoblasts by altering transcription reprogramming upon differentiation induction.

HDAC3 is among the first HDACs to function during these two stages of myogenic differentiation (Collins et al., 2017; this study). Class I HDACs primarily function during satellite cell proliferation and activation of the early stage of differentiation (Marroncelli et al., 2018), whereas Class II HDACs act at later differentiation steps, including commitment, myotube formation and maturation (Marroncelli et al., 2018). HDAC4 was recently shown to also regulate both cell proliferation and satellite cell differentiation (Marroncelli et al., 2018). The interaction of HDAC4 with two important regulatory proteins acting in distinct pathways is responsible for this duality. HDAC4 activity increases satellite cell proliferation by blocking $p21^{Cip1/Waf1}$, a potent cell cycle inhibitor (Marroncelli et al., 2018). HDAC4 activates myogenic differentiation through its interaction with and inhibition of Sharp1. Sharp1 is a bHLH transcription factor that interacts with G9a, a histone methyltransferase, to inhibit MyoD activity and the expression of MEF2C and MyHC (Azmi et al., 2004; Fujimoto et al., 2007; Gulbagci et al., 2009; Marroncelli et al., 2018; Yamada and Miyamoto, 2005). HDAC4 thus allows for optimal satellite cell activation and proliferation by blocking $p21^{Cip1/Waf1}$, while enhancing

myogenic differentiation by activating MyoD in later stages of differentiation. In contrast, our data shows HDAC3 activity inhibits proliferation, as emerin-null myogenic progenitors proliferate more rapidly (Collins et al., 2017) and have reduced $p21^{Cip1/Waf1}$ expression (Iyer et al., 2017). HDAC3 activity is significantly compromised in emerin-null progenitors, suggesting reduced HDAC3 is responsible for changes in $p21^{Cip1/Waf1}$ expression. Differentiation is also delayed in these cells, as they fail to exit the cell cycle due to decreased $p21^{Cip1/Waf1}$ expression. Rescuing H4K5 acetylation state by HAT inhibition or HDAC3 activation rescued cell-cycle withdrawal and emerin-null myogenic differentiation, supporting our model that H4K5 acetylation regulates both cell cycle withdrawal and later stages of myogenic differentiation, likely by two distinct mechanism, as seen for HDAC4. Future studies are needed to elucidate these distinct mechanisms.

MATERIALS AND METHODS

Pharmacological treatments

A 1.0 mM stock solution of L002 in DMSO was added to a final concentration of 0.5 µM in differentiation medium. A 1.0 mM stock solution of Nu9056 in DMSO was added to a final concentration of 0.5 µM in differentiation medium. A 3.0 mM stock solution of SRT1720 in DMSO was added to a final concentration of 1.5 µM in differentiation medium. Differentiation media containing each inhibitor or DMSO was added to induce differentiation of wildtype or emerin-null myogenic progenitors.

Cell culture

Cell culture of proliferation and differentiation of H2Ks were done as previously described (Collins et al., 2017). Briefly, for proliferation, wildtype and emerin-null H2K myogenic progenitors were seeded onto tissue culture plates (Falcon cat no. 353046 and 3530003) and maintained at 33℃ and 10% CO_2 in proliferation medium (high glucose DMEM supplemented with 20% heat-inactivated fetal bovine serum, 2% L-glutamine, 2% chick embryo extract, 1% penicillin/streptomycin, sodium pyruvate, 20 units/ml γ-interferon, ThermoFisher Scientific). The plates were coated with 0.01% gelatin (Sigma-Aldrich) prior to seeding.

Wildtype and emerin-null H2K myogenic progenitors were seeded onto 12 well tissue culture plates coated with 0.01% gelatin (Sigma-Aldrich) for differentiation induction. Cells were seeded at 23,500 cells/cm2 in proliferation media for 24h at 33℃ and 10%

CO_2. Differentiation was stimulated by replacing the proliferation medium with differentiation medium (high glucose DMEM with sodium pyruvate, 5% horse serum, 2% L-glutamine, ThermoFisher Scientific). The cells were maintained at 37℃ and 5% CO_2 throughout differentiation.

Immunofluorescence/EdU

Cells were treated with 10 µM EdU (ThermoFisher Scientific) in DMSO 2h prior to fixing, while incubating at 37℃ and 5% CO_2. Cells were then fixed with 3.7% formaldehyde for 15 min and washed three times with PBS. Fixed cells were then stored at 4°C with 0.1% sodium azide in PBS. The cells were permeabilized with 0.5% triton X-100 in PBS for 20 minutes, washed twice with 3% BSA in PBS for five minutes per wash and treated with the Click-IT EdU reaction cocktail for 25 minutes. Cells were washed with PBS and blocked for 1 h at room temperature with 3% BSA with 0.1% Triton X-100. MyHC antibodies (1:20, Santa Cruz Biotechnologies, H-300 for L002 and Nu506 experiments; 1:50, Santa Cruz Biotechnologies, B-5 for SRT1720 treatments) were added and the cells were incubated at room temperature for 1 h. The cells were washed with PBS three times and treated with Alexa Fluor 594 secondary antibodies (1:200, C10637; A11032, ThermoFisher Scientific) at room temperature for 1 hour, washed with PBS and incubated with DAPI for 5 minutes.

Immunofluorescence Microscopy

Images were taken using the EVOS-FL imaging system (ThermoFisher LifeSciences) for experiments with L002. The remainder of the images were taken with the EVOS-FL Auto (ThermoFisher LifeSciences). All images were obtained using a long working distance 40x objective. At least three replicates, with each replicate containing three culture wells per group, were done for each drug treatment. Images from five different sections from each well were taken, with each section containing approximately 50-200 cells. The total number of cells analyzed for each experiment ranged between 500-1500.

The cell counter plugin on ImageJ was used to count proliferating cells. The percent of cells still in the cycle was determined by dividing the number of EdU positive nuclei by the total number of nuclei. The DAPI and MyHC images were superimposed to calculate the percentage of cells expressing MyHC. Myotube formation was determined by superimposing the phase contrast image, which allowed for monitoring nuclei within a

shared cytoplasm, with DAPI and MyHC images. Myotube formation, or the differentiation index, was determined by counting the number of nuclei in MyHC positive cells that contained three or more nuclei divided by the total number of nuclei in the field.

Western Blotting

Differentiated H2K cells were resuspended directly in sample buffer and 50,000 to 100,000 cell equivalents were separated by SDS-PAGE and transferred to a nitrocellulose membrane. The membranes were blocked either at room temperature for 2 h or overnight at 4°C in 3% BSA in PBST(PBS with 0.1% Tween 20). Antibodies against H4 (1:50,000; Millipore, 05-858) and H4K5-ac (1:1,000; Millipore, 07-327), were then incubated either at room temperature for 2 h or overnight at 4°C. The membranes were washed three times in PBS and incubated with Goat Anti-Rabbit HRP secondary antibody (1:10,000; ThermoFisher Scientific) in PBST either at room temperature for 2 h or overnight at 4°C. The membranes were treated with ECL chemiluminescence detection reagent (GE healthcare, product # RPN2106V1 and RPN2106V20 and imaged using the Bio-Rad Chemidoc system (Bio-Rad Laboratories). Densitometry was done using ImageLab software (Bio-Rad Laboratories) as per the manufacturer's instructions.

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COMPETING INTERESTS

The authors declare no competing or financial interests.

AUTHOR CONTRIBUTIONS

Conceptualization, methodology, and validation: J.M.H.; Formal analysis: J.M.H. and K.A.B.; Investigation: K.A.B. and J.A.E.; Resources: J.M.H.; Data curation: J.M.H.,

K.A.B., and J.A.E.; Writing- original draft: J.M.H.; Writing- reviewing and editing: J.M.H., K.A.B., and J.A.E.; Visualization: K.A.B. and J.M.H.; Supervision: J.M.H.; Project administration: J.M.H., K.A.B., and J.A.E. Funding acquisition: J.M.H.

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FIGURES

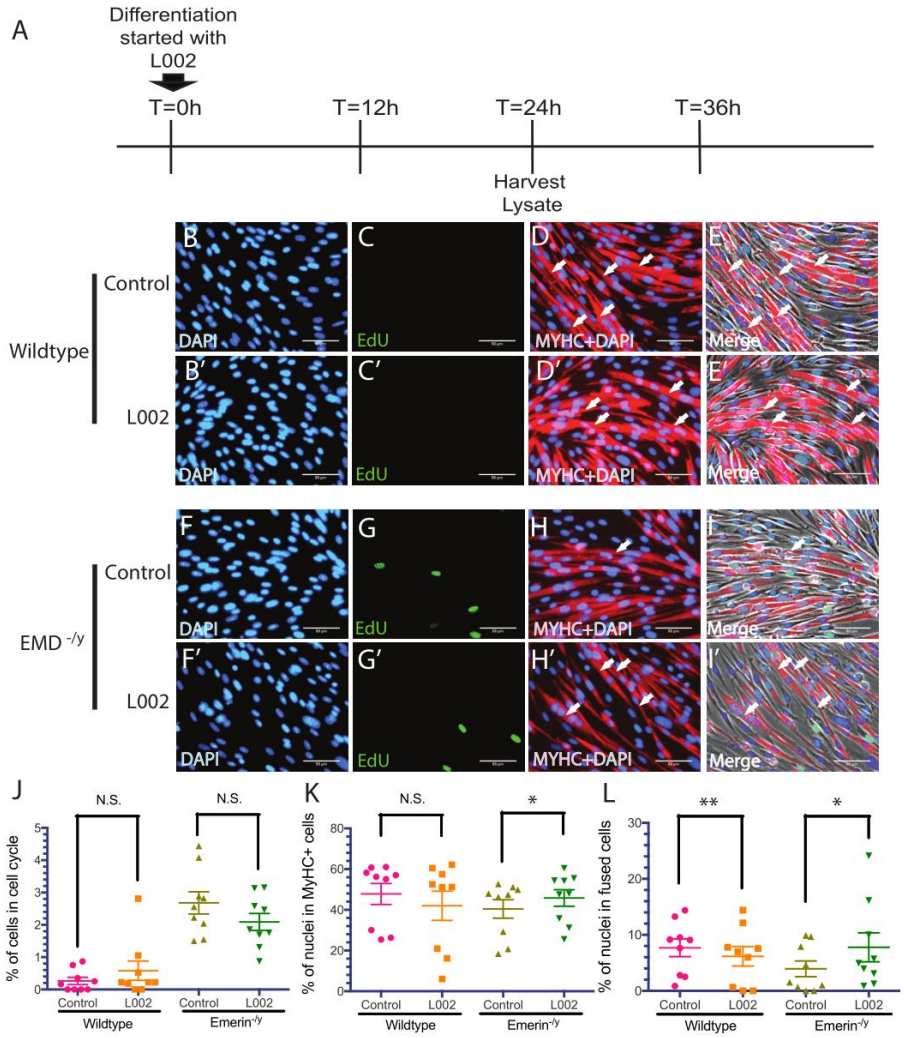

Figure 1. Inhibition of HAT activity with L002 treatment rescues myotube formation and myosin heavy chain expression in emerin-null myogenic progenitors. (A) Timelines showing the time point L002 was added and whole cell lysate collection for western blot analysis. Representative images at 40X magnification of vehicle-treated wildtype (B-E) or emerin-null (F-I) and L002-treated wildtype (B′-E′) or emerin-null (F′-I′) cells 36 h after initiating differentiation. Arrows mark myotubes. (J-L) Quantification of >500 nuclei for each experimental treatment (n=3) was carried out to determine the percentage of myogenic progenitors in the cell cycle (J), expressing MyHC (K) and formed tubes (L) 36 h after inducing differentiation. Results are mean±s.d. of n=3; N.S., not significant; *P<0.05, **P<0.01 using paired, two-tailed t-tests.

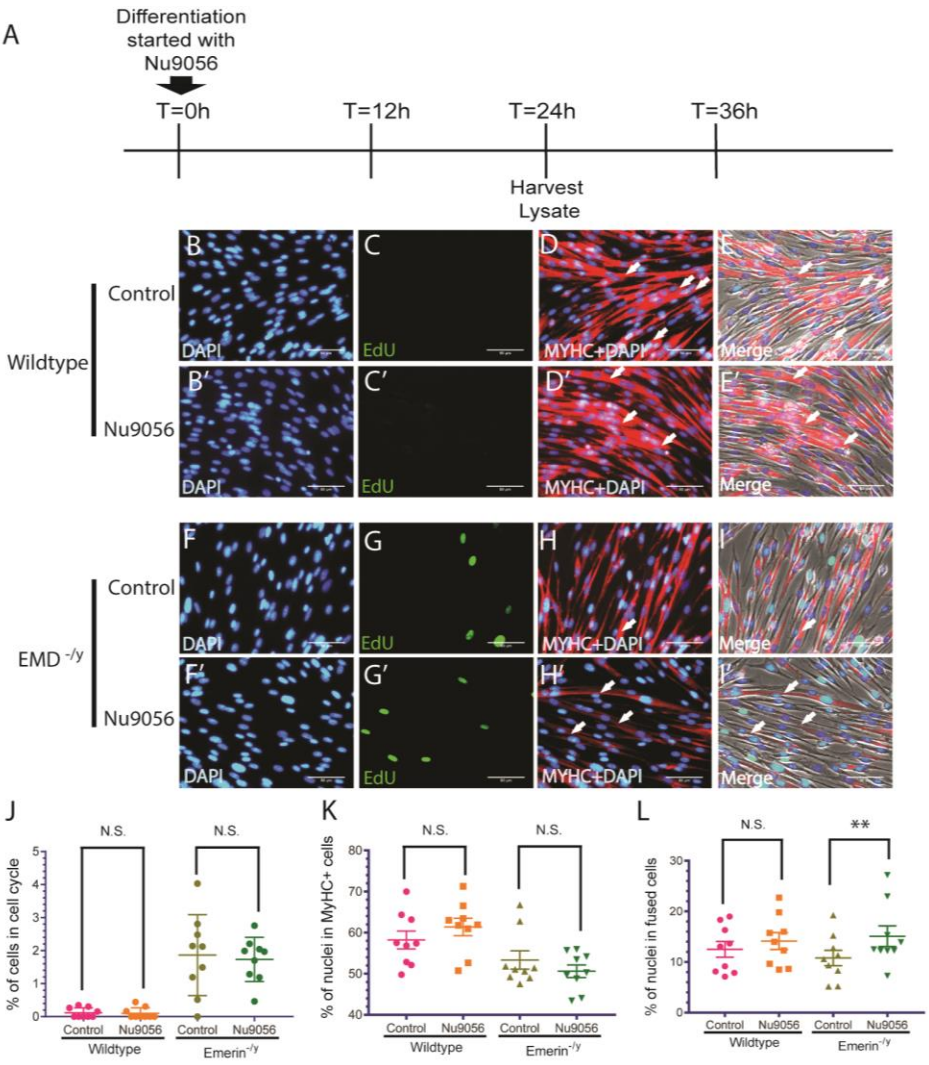

Figure 2. Inhibition of HAT activity with Nu9056 treatment rescues myotube formation in emerin-null myogenic progenitors. (A) Timelines showing the time point Nu9056 was added and whole cell lysate collection for western blot analysis. Representative images at 40X magnification of vehicle-treated wildtype (B-E) or emerin-null (F-I) and Nu9056-treated wildtype (B′-E′) or emerin-null (F′-I′) cells 36 h after initiating differentiation. Arrows mark myotubes. (J-L) Quantification of >500 nuclei for each experimental treatment (n=3) was carried out to determine the percentage of myogenic progenitors in the cell cycle (J), expressing MyHC (K) and formed tubes (L) 36 h after inducing differentiation. Results are mean±s.d. of n=3; N.S., not significant; **P<0.01 using paired, two-tailed t-tests.

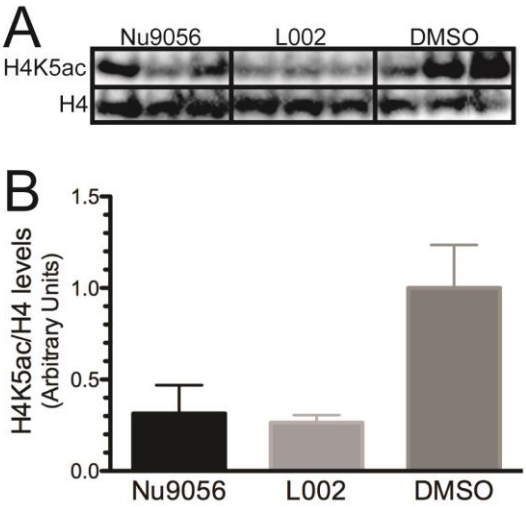

Figure 3. H4K5 acetylation is decreased by treatment with L002 and Nu9056. (A) Western blotting of whole cell lysates treated with Nu9056 or L002 to analyze H4K5 acetylation during differentiation of emerin-null progenitors. DMSO-only treatment was the control. Three biological replicates are shown for each treatment. (B) Densitometry was performed and acetylated H4K5 in each sample was normalized to total H4 protein in each sample. Levels of acetylated H4K5 for each condition were normalized to DMSO-treated cells. Results are mean±s.d. of n=3 for each condition.

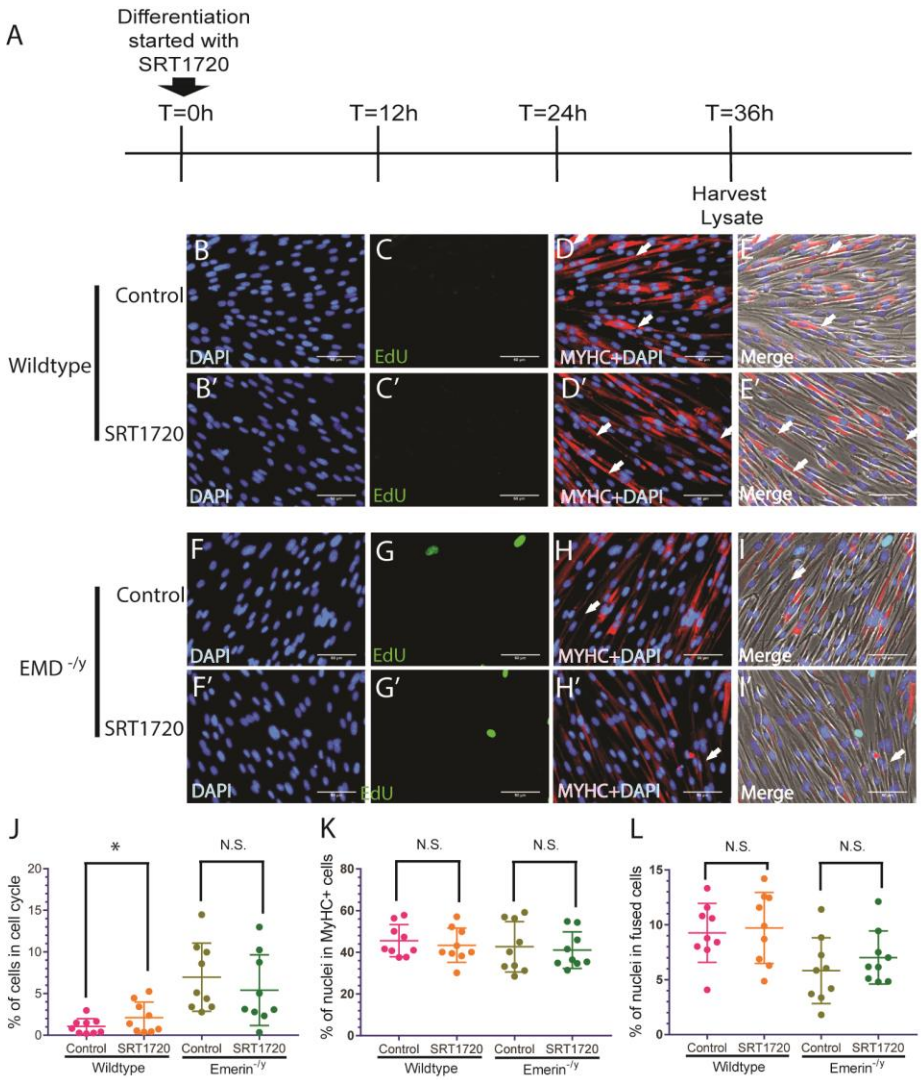

Figure 4. SIRT1 activation with SRT1720 treatment does not change cell cycle exit, myosin heavy chain expression, or myotube formation in emerin-null myogenic progenitors. (A) Timelines showing the time point SRT1720 was added and whole cell lysate collection for western blot analysis. Representative images at 40X magnification of vehicle-treated wildtype (B-E) or emerin-null (F-I) and SRT1720-treated wildtype (B′-E′) or emerin-null (F′-I′) cells 36 h after initiating differentiation. Arrows mark myotubes. (J-L) Quantification of >500 nuclei for each experimental treatment (n=3) was carried out to determine the percentage of myogenic progenitors in the cell cycle (J), expressing MyHC (K) and formed tubes (L) 36 h after inducing differentiation. Results are mean±s.d. of n=3; N.S., not significant; *P<0.05 using paired, two-tailed t-tests.

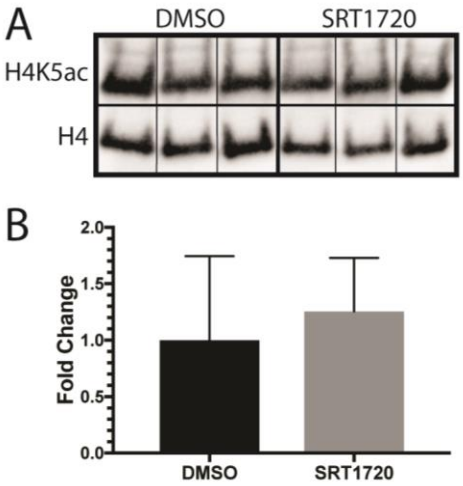

Figure 5. H4K5 acetylation remains unchanged SRT1720 treatment. (A) Western blotting of whole cell lysates treated with DMSO or SRT1720 to analyze changes in H4K5 acetylation. DMSO was the treatment control. Each treatment was done in triplicate. (B) Quantification of western blots was done by densitometry. H4K5 levels were normalized to H4 levels for each sample. SRT1720-treated emerin-null cells were normalized to DMSO-treated emerin-null cells. Results are mean±s.d. of n=3 for each condition.

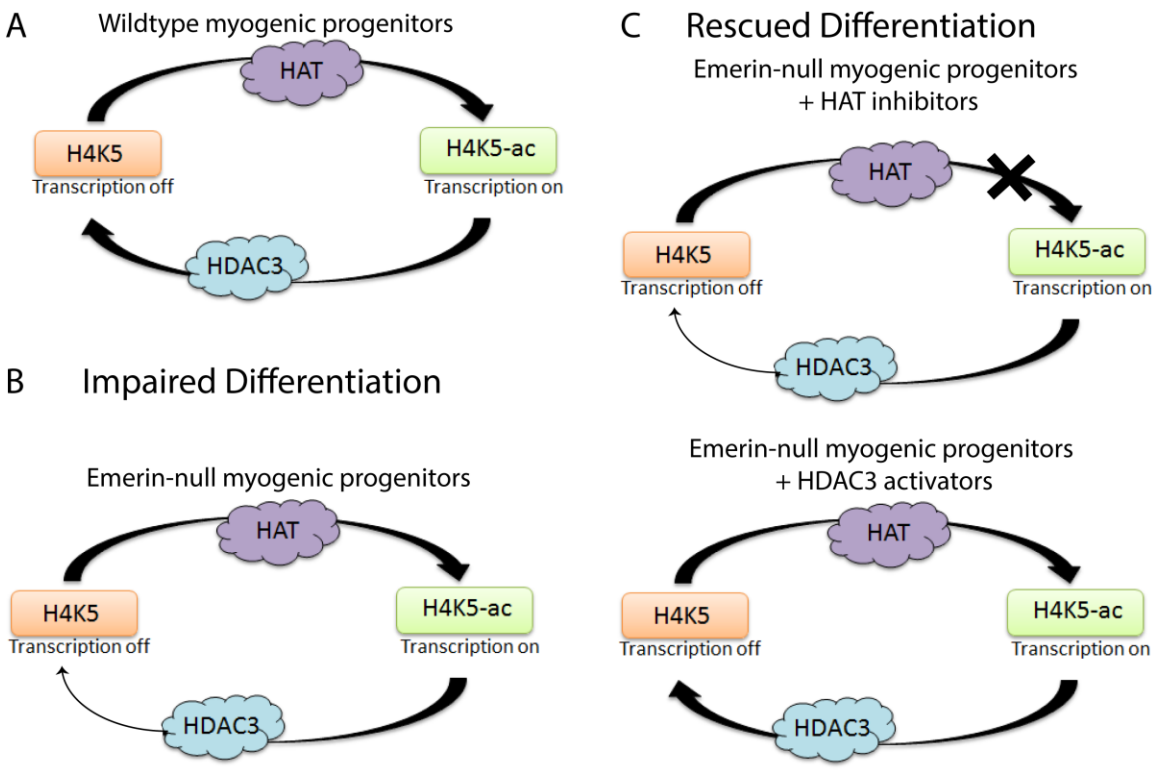

Figure 6. Model for H4K5 acetylation dynamics regulating myogenic differentiation. (A) Wildtype myogenic differentiation. (B) Emerin-null myogenic differentiation is impaired due to increased H4K5 acetylation. (C) Emerin-null myogenic differentiation is rescued by treatment with HAT inhibitors or an HDAC3 activator to restore the H4K5 and H4K5ac equilibrium.