CHARACTERIZING PRMT BIOLOGY DURING MYOGENESIS

CHARACTERIZING PROTEIN ARGININE METHYLTRANSFERASE EXPRESSION AND ACTIVITY DURING MYOGENESIS

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TITLE: Characterizing the expression and activity of protein arginine methyltransferases during myogenesis

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Lay Abstract

Protein arginine methyltransferases (PRMTs) are responsible for many important functions in skeletal muscle. However, significant knowledge gaps exist with respect to PRMT expression and activity during conditions of muscle remodeling. Therefore, the purpose of this Thesis was to investigate PRMT biology throughout skeletal muscle development. Mouse muscle cells were employed to examine characteristics of PRMT1, -4, and -5 at numerous timepoints during myogenesis. PRMTs exhibited distinct patterns of gene expression and activity during muscle maturation. A PRMT1 inhibitor (TC-E) was utilized to investigate the role of this enzyme during myogenesis. Muscle differentiation was impaired in TC-E-treated cells, which coincided with reduced mitochondrial biogenesis and respiratory function. Altogether, these results suggest a PRMT-specific pattern of expression and activity during myogenesis. Furthermore, PRMT1 plays a crucial role in skeletal muscle differentiation via a mitochondrially-mediated mechanism. Our study provides a more comprehensive view on the role of PRMTs in governing skeletal muscle plasticity.

Abstract

Despite the emerging importance of protein arginine methyltransferases (PRMTs) in regulating skeletal muscle plasticity, the biology of these enzymes during muscle development remains poorly understood. Therefore, our purpose was to investigate PRMT1, -4, and -5 expression and function in skeletal muscle cells during the phenotypic remodeling elicited by myogenesis. C_2C_{12} muscle cell maturation, assessed during the myoblast stage, and during days 1, 3, 5, and 7 of differentiation, was employed as an in vitro model of myogenesis. We observed PRMT-specific patterns of expression and activity during myogenesis. PRMT4 and -5 gene expression was unchanged, while PRMT1 mRNA and protein content were significantly induced. Cellular monomethylarginines and symmetric dimethylarginines, indicative of global and type II PRMT activities, respectively, remained steady during development, while type I PRMT activity indicator asymmetric dimethylarginines increased through myogenesis. Histone 4 arginine 3 (H4R3) and H3R17 contents were elevated coincident with the myonuclear accumulation of PRMT1 and -4. Collectively, this suggests that PRMTs are methyl donors throughout myogenesis and demonstrate specificity for their protein targets. Cells were then treated with TC-E 5003 (TC-E), a selective inhibitor of PRMT1 in order to specifically examine the enzymes role during myogenic differentiation. TC-E treated cells exhibited decrements in muscle differentiation, which were consistent with attenuated mitochondrial biogenesis and respiratory function. In

summary, this study increases our understanding of PRMT1, -4, and -5 biology during the plasticity of skeletal muscle development. Our results provide evidence for a role of PRMT1, via a mitochondrially-mediated mechanism, in driving the muscle differentiation program.

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TABLE OF CONTENTS

List of Figures/Tables

Figures from Review of the Literature

Figures from Manuscript

List of Abbreviations

18S	18S ribosomal RNA
ADMA	asymmetric dimethylarginine
AMPK	AMP-activated protein kinase
ATP	adenosine triphosphate
ADP	adenosine diphosphate
ATF2	activating transcription factor2
BCA	bicinchoninic acid
BSA	bovine serum albumin
CARM1	coactivator-associated arginine methyltransferase-1
c-myc	cellular myelocytomatosis oncogene
COPR5	cooperator of PRMT5
Cyto-c	cytochrome c
DAPI	4',6-diamidino-2-phenylindole
DM	differentiation media
DMD	Duchenne muscular dystrophy
DMEM	Dulbecco's modified Eagle's medium
dpc	day postcoitum
DMSO	dimethyl sulfoxide
eMHC	embryonic myosin heavy chain
ERRα	estrogen-related receptor alpha

GAPDH	glyceraldehyde 3-phosphate dehydrogenase
Glut +Mal	glutamate and malate
GM	growth media
GRIP1	glucocorticoid receptor-interacting protein 1
H2B	histone 2B
H3	histone 3
H3R2	histone 3 arginine 3
H3R8	histone 3 arginine 8
H3R17	histone 3 arginine 17
H4	histone 4
H4R3	histone 4 arginine 3
H3R26	histone 3 arginine 26
kDa	kilodalton
MB	myoblast
MDH1	malate dehydrogenase 1
MEF2C	myocyte enhancer factor 2C
MiR05	mitochondrial respiration medium
MMA	monomethylarginine
MRFs	myogenic regulatory factors
MSC	muscle stem cell
MT	myotube

mtDNA	mitochondrial deoxyribonucleic acid
Myf5	myogenic factor 5
mRNA	messenger ribonucleic acid
MyoD	myogenic differentiation 1
NRF-1	nuclear respiratory factor-1
OXPHOS	oxidative phosphorylation
PBS	phosphate buffered saline
PGC-1α	(PPARγ) coactivator-1α
PRMT	protein arginine methyltransferase
Pyr	pyruvate
ROS	reactive oxygen species
RPS11	40 S ribosomal protein S11
SAM	S-adenosylmethionine
SAH	S-adenosylhomocysteine
SDMA	symmetric dimethylarginine
snRNP	small nuclear ribonucleoprotein
Succ	succinate
TBST	tris-buffered saline-tween 20
TC-E	TC-E 5003
Tfam	mitochondrial transcription factor A
VEH	vehicle

Declaration of Academic Achievement

NS was the principal contributor. SN measured cellular respiration. ST visualized and analyzed immunofluorescent cells as well as performed some immunoblotting. VL assisted with conceiving and designing the study, as well as with writing the manuscript.

Review of the Literature

1. Introduction to Protein Arginine Methyltransferases

The epigenetic and post-translational modifications of proteins are critical to the proper functioning of mammalian cells. In particular, methylation of protein arginine residues play a crucial role in numerous cellular processes, such as cell signalling, as well as in many steps of the gene expression pathway, including transcriptional activation, mRNA processing, and post-translational control (Bedford & Clarke, 2009). Indeed, the occurrence of methylation is on par with that of phosphorylation and ubiquitylation (Larsen et al., 2016), which emphasizes the ubiquity and importance of the methyl mark. Protein arginine methyltransferases (PRMTs) are a family of enzymes that catalyze the transfer of a methyl group from S-adenosyl-L-methionine to a guanidino nitrogen atom of arginine residues of target proteins. These enzymes deposit key activating or repressive marks on their protein targets, which for example, alter the behaviour of downstream signalling cascades. Nine PRMTs have been identified, generally termed PRMT1-9, although recently other enzymes have been suggested to be included in the family (Blanc & Richard, 2017). All PRMTs catalyze the formation of monomethylarginine (MMA) residues on their protein targets (Fig. 1). Type I PRMTs subsequently deposit a second methyl group onto the same terminal guanidino nitrogen atom of arginine, thus producing the asymmetric dimethylarginine (ADMA) mark, while the type II enzymes synthesize symmetric dimethylarginines (SDMAs) by depositing the second methyl group onto the other terminal guanidino nitrogen atom of arginine.

PRMT1, -2, -3, -4, -6, and -8 are classified as type I enzymes, whereas PRMT5, -7, and -9 are considered type II. PRMTs are dysregulated in the most prevalent diseases of Western society, including cardiovascular disease, cancers, and diabetes (Yang & Bedford, 2012; Wei et al., 2014). It is therefore critical to increase our understanding of the tissue expression and functions of this enzyme family in order to beneficially impact the health and disease status of Canadians.

By utilizing genetic and pharmacological technologies, such as PRMT knockout mice and specific enzyme agonists and antagonists, advances have been made to better understand the functions of PRMTs. In mammals, PRMT1 is the predominant PRMT as it performs over 80% of methyltransferase activity within the cell (Wei et al., 2014). As such, PRMT1 is the primary enzyme responsible for generating MMA and ADMA. Full body deletion of PRMT1 is embryonic lethal, as a loss of this enzyme induces genomic instability. There are at least seven different isoforms of PRMT1, which are formed through complex alternative splicing in the 5′-end of the pre-mRNA (Goulet et al., 2007). PRMT1 is found ubiquitously throughout the body (Wei et al., 2014), including the cellular level where this enzyme can be situated at the plasma membrane, within the cytosol, as well as in nuclei (Iwasaki & Yada, 2007).

PRMT2 and -3 are not as well-defined within the literature as the other PRMT members. PRMT2 expression is shown to increase in hypoxic conditions,

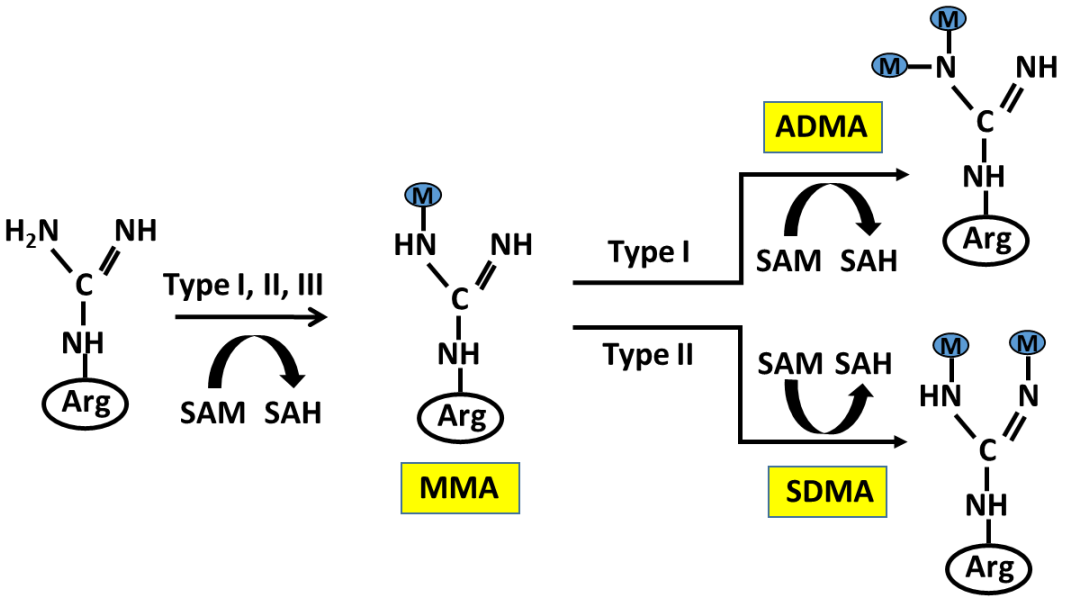

Figure 1. Classification of methylated arginines. The protein arginine methyltransferase (PRMT) family utilize S-adenosyl-L-methionine (SAM), a methyl donor, to deposit one or more methyl groups onto L-Arginine (Arg with guanidino group as displayed on the left). Three types of methylated arginine species exist in mammalian cells – monomethylarginine (MMA), asymmetric dimethylarginine (ADMA), and symmetric dimethylarginine (SDMA). PRMTs generate methylated arginine residues by the conversion of SAM to S-adenosylhomocysteine (SAH). The formation of MMA refers to a single methyl group that is placed on the terminal nitrogen atom and is catalyzed by type I, II, or III PRMTs. Type I PRMTs include: PRMT1, 3, 4, 6, 8. Type II PRMTs include PRMT5, 7, and 9. PRMT7 is the sole type III PRMT, which only generates MMA marks. Interestingly, PRMT7 can function as either a type II or type III enzyme depending on its target substrate. ADMA refers to two methyl groups being placed on the same terminal nitrogen atom of the guanidine group and is catalyzed by type I PRMTs. SDMA refers to two methyl groups placed on each of the terminal nitrogen atoms and is catalyzed by type II PRMTs.

while its expression is observed to decrease in high glucose conditions (Yildirim et al., 2006).Notably, PRMT2 functions as a transcriptional repressor and promotes apoptosis by blocking the IkappaB-alpha nuclear export. Moreover, mice lacking PRMT2 exhibit reduced serum leptin levels and are resistant to dietary-induced obesity (Iwasaki et al., 2010). Among this family of enzymes, PRMT3 is unique for its zinc-finger domain (Frankel & Clarke, 2000). This enzyme is known primarily to interact with ribosomal proteins such as the S2 protein of the small ribosomal subunit which is involved in formation of the translation initiation complex (Swiercz et al., 2007).

PRMT4 and -5 function are extensively studied within the literature. PRMT4, also known as coactivator-associated arginine methyltransferase (CARM1), is known for its transcriptional co-activator function (Blanc & Richard, 2017), as one of its roles is to activate steroid receptors within the nucleus. This type I enzyme also methylates histone 3 arginine 17 (H3R17) and histone 3 arginine 26 (H3R26). Interestingly, PRMT4-deficient mice are smaller in size when compared to their wild-type littermates and die shortly after birth (Yadav et al., 2003). Moreover, a knockout of PRMT5 in embryonic stem cells is embryonic lethal (Tee et al., 2010). PRMT5 binds to cooperator of PRMT5 (COPR5) in the nucleus and preferentially methylates histone 4 arginine 3 (H4R3) and histone 3 arginine 8 (H3R8). This enzyme is known to be involved in methylating a number of Sm proteins which mediate catalysis of pre-mRNA splicing (Neuenkirchen et al.,

2008). In the nucleus, both PRMT4 and -5 are known to form a complex with SWI/SNF ATP-dependent chromatin remodeling proteins (Dacwag et al., 2009). This complex is involved in producing alterations to the nucleosome structure, which is one way of regulating transcription.

PRMT6 is predominantly found in the nucleus and is responsible for methylating histone 3 arginine 2 (H3R2) (Bedford & Clarke, 2009). PRMT6-mediated methylation plays a role in regulating embryonic stem cell fate by maintaining a balance between pluripotency and differentiation (Blanc & Richard 2017). PRMT7 plays a role in transcriptional regulation, as well as in splicing. More recently, it was shown that PRMT7 expression decreased with age and obesity (Jeong et al., 2017). A lack of PRMT7 in mice caused decreased oxidative metabolism and exacerbated age-related obesity. PRMT8 is a membrane-bound protein (Blanc & Richard, 2017). This distinct enzyme is found in limited types of tissues and is distributed mainly in the brain (Bedford & Clarke, 2009). PRMT9 has been identified as a non-histone methyltransferase (Yang et al., 2015). This type II enzyme regulates alternative splicing by methylating spliceosome-associated proteins. On balance, PRMTs have the capacity to modify histone and non-histone proteins to activate, repress, and/or alter cellular pathways. As such, this family of enzymes is crucial at influencing gene expression and thus pose to be potential targets for therapeutic applications.

2. Myogenesis

Skeletal muscle is one of the largest organs in the human body that serves in a multitude of functions such as maintaining posture and producing movement. (Shiozu et al., 2015). As a major site of glucose storage and consumption, this tissue is also among the most metabolically important tissues (Meyer et al., 2002). It is an intricately organized tissue composed of long multinucleated myofibers that have matured from either myotubes or secondary fibers (Bentzinger et al., 2012). Skeletal muscle contains myogenic stem cells called satellite cells which confer the robust regenerative potential necessary for an adaptive response to muscle damage (Hawke & Garry, 2001; Grounds et al., 2002). As such, skeletal muscle is a highly plastic organ as it possesses the ability to remodel in response to various physiological and pathological traumas such as injury or intense exercise.

The production of new muscle as a regenerative process or during development is defined as myogenesis. Myoblasts derived from embryonic stem cells or satellite cells traverse the stages of proliferation, differentiation and finally terminal fusion to form or repair multinucleated myofibers (Fig. 2A). The host of intracellular and extracellular signalling cascades that modulate myogenesis affects the expression and activity of transcriptional regulators, regulatory RNAs, and chromatin-remodeling factors. A timeline for many of these signalling and expression changes has been well-established and will be discussed in this review. Furthermore, the myogenic process is similar in the developmental (embryogenic)

and regenerative contexts, but exhibits notable incongruencies that may make different models of myogenesis more or less optimal depending on research demands. The merits of regenerative and embryogenic animal models as well as in vitro manipulation of C_2C_{12} immortalized cells will also be addressed in this section.

The myogenic regulatory factors

The myogenic regulatory factors (MRFs) are muscle regulatory genes that are vital to the formation and maintenance of skeletal muscle. MRFs belong to the basic helix-loop-helix of transcription factors that are tightly regulated in a spatial and temporal fashion during myogenesis (Fig. 2B). Myf5 (Braun et al., 1989), myogenin (Braun et al., 1989; Edmondson & Olson, 1989), MRF4 (Kassar-Duchossoy et al., 2004), and MyoD (Davis et al., 1987), all of which belong to the MRF family, are exclusively expressed in muscle cells. The expression of each MRF differs in the timing and stage of myogenesis, which reflects the underlying differences in the roles of each enzyme in muscle cell commitment and differentiation (Bentzinger et al., 2012). MyoD and Myf5 are the earliest MRFs to

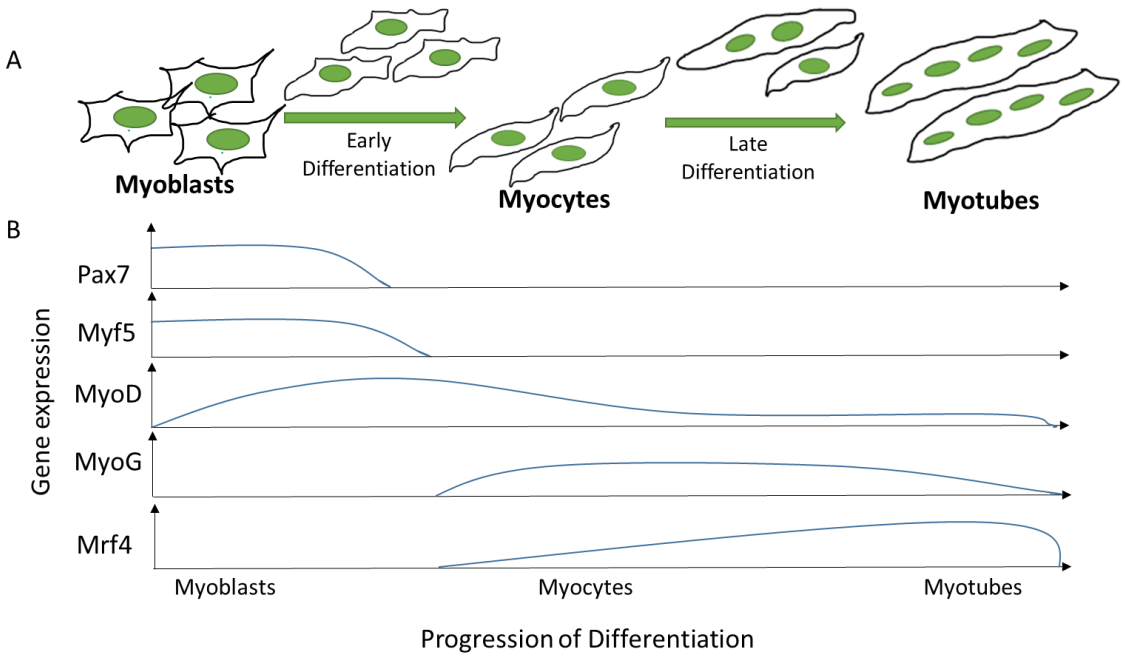

Figure 2. Molecular characterization of myogenesis. A. Schematic illustration of muscle differentiation from myoblasts to terminally differentiated myotubes. First, mononucleated myoblasts align and begin to fuse as myocytes during early differentiation. Next, myocytes fuse into multinucleated myotubes during late differentiation. B. Graphical depiction of the sequential expression of transcription factors that regulate the progression of myogenesis. Expression of paired box 7 (Pax7), myogenic factor 5 (Myf5), and myogenic differentiation (MyoD) is at its highest during the myoblast stage. As myoblasts transition to become myocytes, Pax7 and Myf5 expression is down-regulated. Next, myocytes begin to fuse and form myotubes as the expression of myogenin (MyoG) and myogenic regulatory factor 4 (MRF4) is upregulated, all while MyoD expression progressively decreases.

be expressed during myogenesis and induce satellite cells to commit to the myogenic program. Moreover, these MRFs have the ability to transform a selection of cell types, such as fibroblasts, into muscle phenotypic cells that are capable of fusing into myotubes (Davis et al., 1987). Myogenin and MRF4 are more directly involved in the differentiation process and trigger the expression of myotube-specific genes (Bentzinger et al., 2012). Our broadened understanding about the regulation of MRFs are due, in part, to the ability to analyse robust in vitro and in vivo models of myogenesis, which have permitted a time-dependent examination of the complexities of this process.

Models of myogenesis

Mouse Embryogenesis. This system has been extensively utilized to examine the mechanisms of skeletal muscle gene regulation for over many decades (Buckingham & Rigby, 2014). Myf5 expression is first activated in the dorsomedial portion of the somite at day 8 postcoitum (dpc) (Ott et al., 1991). Approximately 12 hours later, transcription of the myogenin gene is turned on and remains detectable throughout fetal development (Sassoon et al., 1989). The importance of myogenin is highlighted by myogenin knockout mice that exhibit perinatal lethality due to a failure to form myofibers as a result of a disruption of myoblast differentiation (Hasty et al., 1993). MyoD expression is first detected approximately 9.75 dpc in the hypaxial somitic domain and continues to be expressed throughout development (Sassoon et al., 1989). In the limb bud, the

temporal appearance of these transcription factors is slightly different. Although Myf5 expression is again detected first, it is followed very quickly by MyoD and myogenin, which are detected from day 10.5 onward. MRF4 expression is detected transiently between days 10 and 11 and from day 16 onward, becoming the predominant MRF expressed in mature muscle (Hinterberger et al., 1991).

Utilizing embryonic models allows for observation of very early morphogenetic cues that are unique to developing muscle and are not recapitulated in adult myogenesis (Wang & Conboy, 2010). Furthermore, an advantage of studying this model is its rapid development and its accessibility for visualization throughout the entire developmental process. Live optical imaging constitutes an effective tool for tracking cell movements and cell fates in real time (Vergara & Canto-Soler, 2012). However, technology for manipulation of gene activity via targeted mutagenesis 'knock-out' or gene replacement 'knock-in' in embryo is less sophisticated than other models of myogenesis and require more research to develop these transgenic technologies (Yen et al., 2014).

Skeletal Muscle Regeneration. Skeletal muscle regeneration is initiated by satellite cells that are located beneath the basal lamina of myofibers and are required for the growth and regeneration of skeletal muscle (Bischoff & Heintz, 1994; Chargé & Rudnicki, 2004). Muscle tissue repair following damage consists of two separate phases - degeneration followed by regeneration (Karalaki et al., 2009).

Degeneration can be induced by local physical or chemical trauma, resulting in necrosis of myofibers. In response to the damage and degeneration, quiescent satellite cells become activated and re-enter the cell cycle in order to generate myoblasts that will participate in myofiber repair. New muscle fibers are then formed when these myogenic cells differentiate via the induction of MRF4 and fuse to existing damaged fibers. This model of myogenesis is commonly used to examine cellular and molecular responses activated in response to muscle damage. It permits targeted manipulation of gene expression with high spatiotemporal resolution and over a broad range of developmental stages (Karalaki et al., 2009). A drawback to utilizing this model is the translatability of the initial muscle damage in animal models, and the extrapolation of the effects to human muscle (Cibelli et al., 2013).

C_2C_{12} immortalized cell line. The C_2C_{12} muscle cell line, derived from thigh muscle of C3H mice (Yaffe & Saxel 1977), is also commonly used to study myogenesis. This model of myogenesis is a highly reproducible and relatively inexpensive model for investigating skeletal muscle biology in the absence of confounding influences. Some advantages of using this model of myogenesis include effective delivery of exogenous oligonucleotides via transfection or transduction, permissive conditions for pharmacological treatments, as well as time-efficiency in comparison to in vivo studies of skeletal muscle (Cornall et al., 2012).

C_2C_{12} cells have an indefinite myogenic potential (immortalised), as the cells continuously undergo mitotic divisions and expand rapidly when cultured in ideal conditions (Cornall et al., 2012). As a result, these cells do not readily respond to apoptotic signals and continue to replicate and divide beyond the life span of healthy cells typically found in vivo. Therefore, on balance, C_2C_{12} cells are an effective tool to examine mechanisms of skeletal muscle plasticity under highly controlled and reproducible conditions.

3. The role of protein arginine methyltransferases in skeletal muscle

Our knowledge of the roles that PRMTs play in skeletal muscle development and regeneration is largely undefined. The literature examining arginine methyltransferase function and activity in myogenesis primarily concern PRMT1, -4, -5, and -7 (Chen et al., 2002; Iwasaki and Yada 2007; Kim et al, 2011; Paul et al., 2012; Blanc et al., 2017). Most studies in this area have utilized in vitro models of myogenesis to show that the mRNA and protein levels of these enzymes are expressed in proliferating myoblasts, as well as at all stages of differentiation (Chen et al., 2002; Kim et al., 2011; Paul et al. 2012; Wang et al., 2012; Blanc et al., 2017). However, additional research must be done to clarify unknown, and in some cases conflicting, temporal and spatial aspects of PRMT expression during myogenesis.

The first evidence associating the PRMT family with skeletal muscle plasticity, specifically myogenesis, arose from the identification of PRMT4 as a

glucocorticoid receptor-interacting protein 1 (GRIP1) binding protein (Chen et al., 2000). Indeed, work in George Muscat's laboratory demonstrated that PRMT4 and GRIP1 together formed part of a functional complex that coactivated myogenic transcription factors. These initial findings led to an investigation that revealed that this methyltransferase was responsible for coactivating the transcriptional activity of myocyte enhancer factor-2C (MEF2C) via GRIP1 (Chen et al., 2002). Subsequent work has demonstrated that PRMT4 is required for the late stages of myogenesis, as it is necessary for the binding of SWI/SNF Brg1 ATPase chromatin remodeling enzymes and myogenin to the myogenin promoter (Dacwag et al., 2009; Mallappa et al., 2011). However, it should be pointed out that there are some conflicting data that have alluded to low PRMT4 expression throughout myogenesis (Kim et al., 2010). Interestingly, recent in vivo data from Michael Rudnicki's laboratory indicate that muscle progenitor cells deficient in PRMT4 proliferate poorly (Kawabe et al., 2012). Furthermore, mice with a targeted ablation of PRMT4 in satellite cells display deficiencies during muscle regeneration in response to Tamoxifen treatment. This is the result of an absence in PRMT4-mediated methylation of Pax7, since the interaction between PRMT4 and Pax7 appears to be required for the activation of the myogenic determination gene Myf5 and the regulation of asymmetric cell division (Kawabe et al., 2012). PRMT4 may also play an important role in metabolic disease, as its expression and methyltransferase activity may regulate the gene program involved in glycogen

metabolism in skeletal muscle (Wang et al., 2012). In addition, PRMT4 protein levels appear to be elevated in dystrophic skeletal muscle in vivo, and its expression can be altered via acute exercise (Ljubicic et al., 2012).

PRMT5 has multiple binding partners, and similar to PRMT4, the enzyme functions as a transcriptional coactivator for gene activation and repression events. Interestingly, these two PRMT members have been shown to have both cooperative and differential functions during the muscle differentiation program. PRMT4 and-5 modify the transcriptional activity of myogenic genes through association with the Brg1 ATPase subunit of SWI/SNF chromatin-remodeling enzymes (Dacwag et al. 2009). While PRMT5 is required for early gene expression, it is dispensable for late gene expression (Dacwag 2009). PRMT5 symmetrically dimethylates histone 3 arginine 8 (H3R8) at the myogenin promoter (Dacwag et al, 2006) and is essential for cell proliferation (Paul 2012). After demonstrating that cooperator of PRMT5 (COPR5) binds to PRMT5 and histone 4, Paul and colleagues showed that C_2C_{12} cells that lack COPR5 expressed very low levels of myosin heavy chain 1 and failed to form differentiated myotubes (2012). As such, the PRMT5-associated protein COPR5 is responsible for coordinating the expression of cell cycle regulators in order for differentiation to proceed.

More recent studies from Thomas Braun's group have examined PRMT5 function in vivo. The authors found that PRMT5 has a prominent role in murine adult muscle stem cell (MSC) proliferation in response to muscle injury (Zhang et

al., 2015). Indeed, this enzyme is required for satellite cell expansion, and is also needed to replenish the MSC niche during aging (Zhang et al., 2015). While the mechanisms by which these functions are carried out remain elusive, the authors provide evidence suggesting that co-repression of the cell cycle repressor p21 by PRMT5 is involved (Zhang et al., 2015). Notably, in contrast to its importance during mature muscle regeneration, PRMT5 does not appear to have a role during mouse embryonic myogenesis (Zhang et al., 2015). Interestingly, this does not seem to be the case for embryonic myogenesis in zebrafish, where the loss of PRMT5 leads to abrogation of MyoD and Myf5 expression and abnormal somite phenotypes (Batut et al., 2011). Thus, regardless of some species-specificity in PRMT5 function, PRMT5 has a well-established role early in the myogenic process. Most notable among these are its involvement in the proliferation of activated MSCs and the induction of myogenic determination (Batut et al., 2011; Dacwag 2007; Zhang 2015).

The role of PRMT1 during myogenesis is only beginning to emerge. The general physiological importance of this family member is highlighted by the fact that PRMT1-null mice die prenatally (Pawlak et al., 2000; Yu et al., 2009). PGC-1α, known as the master regulator of energy metabolism, is methylated by PRMT1 at three C-terminal arginine residues. This modification is necessary for PGC-1α to induce endogenous target genes important for mitochondrial biogenesis (Teyssier et al., 2005). The ablation of PRMT1-mediated PGC-1α methylation disrupts its

ability to cooperatively enhance transcriptional activation by nuclear receptors, and to induce genes encoding mitochondrial proteins (Teyssier et al., 2005). The interaction between PRMT1 and PGC-1α provided early evidence for a potential role of this enzyme in muscle plasticity, since PGC-1α is a master regulator of muscle phenotype maintenance and remodeling.

The first report of PRMT1 biology in muscle revealed that the enzyme regulates the IR/IRS-1/PI3-K pathway involved in glucose transport in L6 skeletal muscle cells (Iwasaki & Yada, 2007). The discovery that PRMT1 transcript levels are induced by acute exercise, and that PRMT1 protein content is elevated in dystrophic skeletal muscle further hinted at some involvement of this molecule in muscle plasticity (Ljubicic et al., 2012). Moreover, PRMT1 can be found in the cytoplasm and myonuclei of myoblasts before, during, and after fusion (Kim et al., 2011; Blanc et al, 2017), perhaps suggesting its multifunctional role in various sub-compartments of the cell. Blanc and colleagues (2017) recently provided insight on target substrates and functions of PRMT1 in muscle. These researchers reported that a loss of PRMT1 in MSCs disrupted the myogenic differentiation program, which led to an impaired ability for the muscle to regenerate in vivo (Blanc et al., 2017). Mechanistically, PRMT1 induces the transcriptional co-activator Eya1, which promotes the activity of Six1, a transcription factor responsible for regulating muscle stem cell fate, and required for MyoD expression (Blanc et al., 2017).

More recently, PRMT7 was added to the list of PRMT family members involved in myogenesis. Blanc et al. (2016) demonstrated that PRMT7-deficient MSCs display premature senescence and a delay in differentiation, coincident with a reduction in the size of the MSC pool. Mechanistically, PRMT7 along with PRMT5, regulates the presence of Cdkn1a at the DNMT3b locus, which is critical for MSC expansion and regeneration (Blanc et al., 2016). Therefore, PRMT7 is ultimately required to preserve MSC regenerative and self-renewing capacity (Blanc et al., 2016). PRMT7 has also been found to regulate PGC-1α expression and PGC-1α transcriptional coactivator activities in skeletal muscle. Jeong and colleagues (2016) observed that whole body PRMT7-null mice had a reduced oxidative capacity and a decreased energy expenditure that the authors suggest may have contributed to the exacerbated age-related obesity seen in the *PRMT7*$^{-/-}$ mice.

In summary, efforts have been made in recent years to elucidate the molecular mechanisms by which PRMTs regulate skeletal muscle plasticity, particularly how these enzymes mediate myogenesis. A better understanding of the PRMT family during muscle development and regeneration will provide insight on what factors determine, maintain, and remodel skeletal muscle biology.

4. Mitochondrial biogenesis during muscle differentiation

Mitochondria are well known for their role in the regulation of energy metabolism, but are also critical to the regulation of cell growth and differentiation. The conversion of myoblasts to mature muscle fibers requires adaptations to the

structure of the mitochondrial network and mitochondrial content during differentiation (Fortini et al., 2016; Sin et al., 2016). Indeed, mitochondrial mass/volume, mtDNA copy number, and mitochondrial respiration markedly increase as muscle differentiation proceeds (Remels et al., 2010; Wagatsuma & Sakuma, 2013). Mitochondrial enzyme activity is also elevated, which accompanies a metabolic shift from glycolysis to oxidative phosphorylation as the major source of energy at later stages of myogenic development (Remels et al. 2011; Wagatsuma & Sakuma, 2012). This shift towards a more oxidative status occurs coincident with the increased expression of peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α), a key upstream regulator of mitochondrial biogenesis and the oxidative phenotype (Calvo et al., 2008). PGC-1α interacts with a variety of transcription factors and nuclear receptors in the promoter region of genes such as nuclear respiratory factor-1 (NRF-1), which controls the transcription of mitochondrial genes involved in encoding the complexes found in the electron transport chain (Kelly & Scarpulla, 2004). Possibly due to increased energetic demands, AMP-activated protein kinase (AMPK) is also upregulated during differentiation (Fortini et al., 2016). The activities of PGC-1α, myogenin, MEF2, and MyoD are all potentiated by AMPK directly or indirectly, and contribute to the expression of a more oxidative metabolic profile (Ljubicic et al., 2012; Fortini et al., 2016; Hood et al., 2016).

Inhibitor studies have demonstrated the importance of mitochondrial function and activity for myogenesis (Rochard et al., 2000; Seyer et al., 2006). In particular, inhibition of mitochondrial activity by selectively blocking protein synthesis in the organelle has been associated with decreased proliferation and impaired fusion of different myogenic cell lines (Rochard et al., 2000; Seyer et al., 2006). To characterize the regulatory pathways involved in the inhibition of cell differentiation, Rochard and colleagues (2000) treated an avian myoblast cell line (QM7) with chloramphenicol, a drug that inhibits the translation of 4 out of 5 mitochondrial respiratory chain complexes. They reported that myogenin expression was downregulated when mitochondrial activity was impaired. Further work revealed cellular myelocytomatosis oncogene (c-myc) as a target of mitochondrial activity which is also involved in the regulation of myogenin expression (Seyer et al. 2006).

Recent in vivo data have extended our knowledge of the potential role of mitochondria in myogenesis. Not surprisingly, muscle regeneration is impaired when mitochondrial protein synthesis is inhibited with chloramphenicol (Wagatsuma et al., 2011). In the presence of chloramphenicol, regenerating skeletal muscle exhibited poor repair with small myofibers and an increased amount of connective tissue. Interestingly, mitochondrial biogenesis-related genes such as mitochondrial transcription factor A (Tfam) and NRF-1 follow a similar pattern of expression with that of the early MRF genes such as MyoD and myogenin during

muscle regeneration. Therefore, these data suggest that mitochondrial content and metabolism may play a role in regulating the onset of differentiation in vivo. In line with these findings, Freyssenet's laboratory showed that skeletal muscle regeneration was accompanied by a robust stimulation of mitochondrial biogenesis. This was evident by elevated levels of citrate synthase activity, PGC-1 mRNA and Tfam protein (Duguez et al., 2002). Not long after, the same laboratory further demonstrated that mitochondrial biogenesis is tightly regulated during cell cycle progression, namely upon the induction of differentiation. They proposed that control of mitochondrial biogenesis during myogenesis might be regulated by the production of hydrogen peroxide and calcium via a mitochondria-to-nucleus retrograde signaling pathway (Jahnke et al., 2009).

Recently, Fukamizu's laboratory demonstrated that PRMT1-null worms had compromised oxidative phosphorylation (OXPHOS) function (2017). These PRMT1 mutants exhibited lower ATP production and reduced basal respiration and respiratory capacity, indicating a dysfunction of the electron transport chain. In line with their findings, they also reported that PRMT1-null worms had higher reactive oxygen species (ROS) production in mitochondria. The authors also speculated that the mitochondrial defects were due to low levels of asymmetric arginine dimethylation by PRMT1 on mitochondrial proteins. Though this study provides valuable insight on the involvement of PRMT1 in mitochondria, the physiological significance of the data is at the whole-body level and much remains elusive with

respect to the role of PRMT1 in mitochondria specifically in skeletal muscle. Along these lines, in COS-7 cells, PRMT1 has been shown to methylate PGC-1α at the C-terminal region and modulate the expression of cytochrome c and estrogen-related receptor alpha (ERRα) genes, which are important for driving mitochondrial biogenesis (Teyssier et al., 2005).

Other PRMT family members such as PRMT4 and -7 have also been implicated in mitochondrial regulation. Wang and colleagues (2016) demonstrated that malate dehydrogenase 1 (MDH1) was a substrate of PRMT4, which is responsible for regulating mitochondrial respiration and redox balance. Interestingly, they found that PRMT4 activity was inhibited by high levels of ROS, which suggests that PRMT4 may function as a ROS sensor to modulate MDH1 activity. PRMT7 has also been shown to regulate PGC-1α expression by activating p38/ATF2, which subsequently augments mitochondrial biogenesis and oxidative muscle metabolism (Jeong et al., 2016). Furthermore, the blunted expression of succinate dehydrogenase subunit b and Mtco1 in PRMT7-deficient C_2C_{12} cells illustrate the role that PRMT7 plays in the transcription of mitochondrial genes. Notably, the muscle of whole-body PRMT7 deficient mice exhibited a reduced endurance capacity and less oxidative fibers. Overall, the evidence suggest that the PRMT family has integral functions in regulating mitochondrial biogenesis and function, but more research must be done in skeletal muscle to fully appreciate the role of these enzymes in mitochondrial biology.

Study Objectives

Several PRMT members have been implicated as influential factors in regulating skeletal muscle plasticity. Despite their emerging importance however, knowledge gaps exist with respect to PRMT expression and activity during the conditions of skeletal muscle remodeling evoked during myogenesis. Further investigation of PRMT biology in skeletal muscle would contribute to our understanding of the molecular mechanisms that mediate muscle phenotype maintenance and remodelling. Thus, the purpose of this Thesis was to: 1) examine PRMT1, -4, and -5 expression and activity during skeletal muscle development; and 2) determine the role of PRMT1 during myogenesis. We hypothesized that each PRMT would exhibit distinct patterns of expression and activity, and that these would be dynamic during myogenesis. Furthermore, we anticipate that PRMT1 is essential for optimal development to occur during muscle maturation.

References

Batut, J., Duboé, C., & Vandel, L. (2011). The methyltransferases PRMT4/CARM1 and PRMT5 control differentially myogenesis in zebrafish. *PLoS ONE*, *6*(10). https://doi.org/10.1371/journal.pone.0025427

Bedford, M. T., & Clarke, S. G. (2009). Protein arginine methylation in mammals: who, what, and why. *Molecular Cell*, *33*(1), 1–13. https://doi.org/10.1016/j.molcel.2008.12.013

Bentzinger, C. F., Wang, Y. X., & Rudnicki, M. A. (2012). Building muscle: molecular regulation of myogenesis. *Cold Spring Harbor Perspectives in Biology*, *4*(2), a008342. https://doi.org/10.1101/cshperspect.a008342

Bischoff, R., & Heintz, C. (1994). Enhancement of skeletal muscle regeneration. *Developmental Dynamics*, *201*(1), 41–54. https://doi.org/10.1002/aja.1002010105

Bissinger, E.-M., Heinke, R., Spannhoff, A., Eberlin, A., Metzger, E., Cura, V., … Jung, M. (2011). Acyl derivatives of p-aminosulfonamides and dapsone as new inhibitors of the arginine methyltransferase hPRMT1. *Bioorganic & Medicinal Chemistry*, *19*(12), 3717–3731.

https://doi.org/10.1016/j.bmc.2011.02.032

Blanc, R. S., & Richard, S. (2017). Regenerating muscle with arginine methylation. *Transcription*, e1291083. https://doi.org/10.1080/21541264.2017.1291083

Blanc, R. S., Vogel, G., Li, X., Yu, Z., Li, S., & Richard, S. (2016). Arginine methylation by PRMT1 regulates muscle stem cell fate. *Molecular and Cellular Biology*, *37*(3), MCB.00457-16. https://doi.org/10.1128/MCB.00457-16

Blanc, R. S., Vogel, G., Li, X., Yu, Z., Li, S., & Richard, S. (2017). Arginine Methylation by PRMT1 Regulates Muscle Stem Cell Fate. *Molecular and Cellular Biology*, *37*(3), e00457-16. https://doi.org/10.1128/MCB.00457-16

Braun, T., Bober, E., Buschhausen-Denker, G., Kohtz, S., Grzeschik, K. H., Arnold, H. H., & Kotz, S. (1989). Differential expression of myogenic determination genes in muscle cells: possible autoactivation by the Myf gene products. *The EMBO Journal*, *8*(12), 3617–25. http://www.ncbi.nlm.nih.gov/pubmed/2583111

Braun, T., Buschhausen-Denker, G., Bober, E., Tannich, E., & Arnold, H. H. (1989). A novel human muscle factor related to but distinct from MyoD1 induces myogenic conversion in 10T1/2 fibroblasts. *The EMBO Journal*, *8*(3), 701–9. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/2721498

Buckingham, M., & Rigby, P. W. J. (2014). Gene Regulatory Networks and Transcriptional Mechanisms that Control Myogenesis. *Developmental Cell*, *28*(3), 225–238. https://doi.org/10.1016/j.devcel.2013.12.020

Calvo, J. A., Daniels, T. G., Wang, X., Paul, A., Lin, J., Spiegelman, B. M., … Rangwala, S. M. (2008). Muscle-specific expression of PPARgamma coactivator-1alpha improves exercise performance and increases peak oxygen uptake. *Journal of Applied Physiology (Bethesda, Md. : 1985)*, *104*(5), 1304–1312. https://doi.org/10.1152/japplphysiol.01231.2007

Cha, B., & Jho, E.-H. (2012). Protein arginine methyltransferases (PRMTs) as therapeutic targets. *Expert Opinion on Therapeutic Targets*, *16*(7), 651–664. https://doi.org/10.1517/14728222.2012.688030

Charge, S. B. P., & Rudnicki, M. A. (2004). Cellular and Molecular Regulation of Muscle Regeneration. *Physiological Reviews*, *84*(1).

http://physrev.physiology.org/content/84/1/209.long

Chen, S. L., Dowhan, D. H., Hosking, B. M., & Muscat, G. E. (2000). The steroid receptor coactivator, GRIP-1, is necessary for MEF-2C-dependent gene expression and skeletal muscle differentiation. *Genes & Development*, *14*(10), 1209–28. https://doi.org/10.1101/gad.14.10.1209

Chen, S. L., Loffler, K. A., Chen, D., Stallcup, M. R., & Muscat, G. E. O. (2002). The coactivator-associated arginine methyltransferase is necessary for muscle differentiation: CARM1 coactivates myocyte enhancer factor-2. *Journal of Biological Chemistry*, *277*(6), 4324–4333. https://doi.org/10.1074/jbc.M109835200

Cibelli, J., Emborg, M. E., Prockop, D. J., Roberts, M., Schatten, G., Rao, M., … Mirochnitchenko, O. (2013). Strategies for improving animal models for regenerative medicine. *Cell Stem Cell*, *12*(3), 271–4. https://doi.org/10.1016/j.stem.2013.01.004

Collu-Marchese, M., Shuen, M., Pauly, M., Saleem, A., & Hood, D. A. (2015). The regulation of mitochondrial transcription factor A (Tfam) expression during skeletal muscle cell differentiation. *Bioscience Reports*, *35*(3).

https://doi.org/10.1042/BSR20150073

Cornall, L., Hryciw, D., Mathai, M., & Mcainch, A. (2012). Generation and Use of Cultured Human Primary Myotubes. http://cdn.intechopen.com/pdfs/25595/InTech-Generation_and_use_of_cultured_human_primary_myotubes.pdf

Dacwag, C. S., Bedford, M. T., Sif, S., & Imbalzano, A. N. (2009). Distinct Protein Arginine Methyltransferases Promote ATP-Dependent Chromatin Remodeling Function at Different Stages of Skeletal Muscle Differentiation. *Molecular and Cellular Biology*, *29*(7), 1909–1921. https://doi.org/10.1128/MCB.00742-08

Dacwag, C. S., Ohkawa, Y., Pal, S., Sif, S., & Imbalzano, A. N. (2007). The protein arginine methyltransferase Prmt5 is required for myogenesis because it facilitates ATP-dependent chromatin remodeling. *Molecular and Cellular Biology*, *27*(1), 384–94. https://doi.org/10.1128/MCB.01528-06

Davis, R. L., Weintraub, H., & Lassar, A. B. (1987). Expression of a single transfected cDNA converts fibroblasts to myoblasts. *Cell*, *51*(6), 987–1000. https://doi.org/10.1016/0092-8674(87)90585

Dhar, S., Vemulapalli, V., Patananan, A. N., Huang, G. L., Lorenzo, A. Di, Richard, S., … Bedford, M. T. (2013). Loss of the major Type I arginine methyltransferase PRMT1 causes substrate scavenging by other PRMTs. https://doi.org/10.1038/srep01311

Di Lorenzo, A., & Bedford, M. T. (2011). Histone arginine methylation. *FEBS Letters*, *585*(13), 2024–31. https://doi.org/10.1016/j.febslet.2010.11.010

Dimauro, I., Pearson, T., Caporossi, D., & Jackson, M. J. (2012). A simple protocol for the subcellular fractionation of skeletal muscle cells and tissue. *BMC Res. Notes*, *5*, 513. https://doi.org/10.1186/1756-0500-5-513

Duguez, S., Féasson, L., Denis, C., & Freyssenet, D. (2002). Mitochondrial biogenesis during skeletal muscle regeneration. *American Journal of Physiology - Endocrinology and Metabolism*, *282*(4). Retrieved from http://ajpendo.physiology.org/content/282/4/E802.long

Edmondson, D. G., & Olson, E. N. (1989). A gene with homology to the myc imilarity region of MyoDl is expressed uring myogenesis and is sufficient to activate the muscle differentiation program. http://genesdev.cshlp.org/content/3/5/628.full.pdf

Fortini, P., Ferretti, C., Iorio, E., Cagnin, M., Garribba, L., Pietraforte, D., … Dogliotti, E. (2016). The fine tuning of metabolism, autophagy and differentiation during in vitro myogenesis. *Cell Death and Disease*, *7*(3), e2168. https://doi.org/10.1038/cddis.2016.50

Frankel, A., & Clarke, S. (2000). PRMT3 is a distinct member of the protein arginine N-methyltransferase family. Conferral of substrate specificity by a zinc-finger domain. *The Journal of Biological Chemistry*, *275*(42), 32974–82. https://doi.org/10.1074/jbc.M006445200

Goulet, I., Gauvin, G., Boisvenue, S., & Côté, J. (2007). Alternative splicing yields protein arginine methyltransferase 1 isoforms with distinct activity, substrate specificity, and subcellular localization. *The Journal of Biological Chemistry*, *282*(45), 33009–21. https://doi.org/10.1074/jbc.M704349200

Grounds, M. D., White, J. D., Rosenthal, N., & Bogoyevitch, M. A. (2002). The Role of Stem Cells in Skeletal and Cardiac Muscle Repair. *Journal of Histochemistry & Cytochemistry*, *50*(5), 589–610. https://doi.org/10.1177/002215540205000501

Handschin, C., Rhee, J., Lin, J., Tarr, P. T., & Spiegelman, B. M. (2003). An

autoregulatory loop controls peroxisome proliferator-activated receptor gamma coactivator 1alpha expression in muscle. *Proceedings of the National Academy of Sciences of the United States of America*, *100*(12), 7111–6. https://doi.org/10.1073/pnas.1232352100

Hasty, P., Bradley, A., Morris, J. H., Edmondson, D. G., Venuti, J. M., Olson, E. N., & Klein, W. H. (1993). Muscle deficiency and neonatal death in mice with a targeted mutation in the myogenin gene. *Nature*, *364*(6437), 501–506. https://doi.org/10.1038/364501a0

Hawke, T. J., & Garry, D. J. (2001). Myogenic satellite cells: physiology to molecular biology. *Journal of Applied Physiology*, *91*(2). http://jap.physiology.org/content/91/2/534.long

Herzberg, N. H., Zwart, R., Wolterman, R. A., Ruiter, J. P. N., Wanders, R. J. A., Bolhuis, P. A., & van den Bogert, C. (1993). Differentiation and proliferation of respiration-deficient human myoblasts. *Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease*, *1181*(1), 63–67. https://doi.org/10.1016/0925-4439(93)90091-E

Hinterberger, T. J., Sassoon, D. A., Rhodes, S. J., & Konieczny, S. F. (1991).

Expression of the muscle regulatory factor MRF4 during somite and skeletal myofiber development. *Developmental Biology*, *147*(1), 144–156. https://doi.org/10.1016/S0012-1606(05)80014-4

Hood, D. A., Tryon, L. D., Carter, H. N., Kim, Y., & Chen, C. C. W. (2016). Unravelling the mechanisms regulating muscle mitochondrial biogenesis. *Biochemical Journal*, *473*(15). http://www.biochemj.org/content/473/15/2295.long#sec-4

Iwasaki, H., Kovacic, J. C., Olive, M., Beers, J. K., Yoshimoto, T., Crook, M. F., … Nabel, E. G. (2010). Disruption of protein arginine N-methyltransferase 2 regulates leptin signaling and produces leanness in vivo through loss of STAT3 methylation. *Circulation Research*, *107*(8), 992–1001. https://doi.org/10.1161/CIRCRESAHA.110.225326

Iwasaki, H., & Yada, T. (2007). Protein arginine methylation regulates insulin signaling in L6 skeletal muscle cells. *Biochemical and Biophysical Research Communications*, *364*(4), 1015–1021. https://doi.org/10.1016/j.bbrc.2007.10.113

Jahnke, V. E., Sabido, O., & Freyssenet, D. (2009). Control of mitochondrial

biogenesis, ROS level, and cytosolic Ca2+ concentration during the cell cycle and the onset of differentiation in L6E9 myoblasts. *AJP - Cell Physiology*, *296*(5), C1185--94. https://doi.org/10.1152/ajpcell.00377.2008

Jeong, H.-J., Lee, H.-J., Vuong, T. A., Choi, K.-S., Choi, D., Koo, S.-H., … Moraes, C. (2016). Prmt7 Deficiency Causes Reduced Skeletal Muscle Oxidative Metabolism and Age-Related Obesity. *Diabetes*, *65*(7), 1868–82. https://doi.org/10.2337/db15-1500

Jornayvaz, F. R., & Shulman, G. I. (2010). Regulation of mitochondrial biogenesis. *Essays in Biochemistry*, *47*, 69–84. https://doi.org/10.1042/bse0470069

Karalaki, M., Fili, S., Philippou, A., & Koutsilieris, M. (2009). Muscle regeneration: cellular and molecular events. *In Vivo (Athens, Greece)*, *23*(5), 779–96. https://doi.org/23/5/779 [pii]

Kassar-Duchossoy, L., Gayraud-Morel, B., Gomès, D., Rocancourt, D., Buckingham, M., Shinin, V., & Tajbakhsh, S. (2004). Mrf4 determines skeletal muscle identity in Myf5:Myod double-mutant mice. *Nature*, *431*(7007), 466–471. https://doi.org/10.1038/nature02876

Kawabe, Y. I., Wang, Y. X., McKinnell, I. W., Bedford, M. T., & Rudnicki, M. A. (2012a). Carm1 regulates Pax7 transcriptional activity through MLL1/2 recruitment during asymmetric satellite stem cell divisions. *Cell Stem Cell*, *11*(3), 333–345. https://doi.org/10.1016/j.stem.2012.07.001

Kawabe, Y. I., Wang, Y. X., McKinnell, I. W., Bedford, M. T., & Rudnicki, M. A. (2012b). Carm1 regulates Pax7 transcriptional activity through MLL1/2 recruitment during asymmetric satellite stem cell divisions. *Cell Stem Cell*, *11*(3), 333–345. https://doi.org/10.1016/j.stem.2012.07.001

Kelly, D. P., & Scarpulla, R. C. (2004). Transcriptional regulatory circuits controlling mitochondrial biogenesis and function. *Genes and Development*. https://doi.org/10.1101/gad.1177604

Kim, S. J., Yoo, B. C., Uhm, C. S., & Lee, S. W. (2011). Posttranslational arginine methylation of lamin A/C during myoblast fusion. *Biochimica et Biophysica Acta - Proteins and Proteomics*, *1814*(2), 308–317. https://doi.org/10.1016/j.bbapap.2010.11.006

Lacroix, M., El Messaoudi, S., Rodier, G., Le Cam, A., Sardet, C., & Fabbrizio, E. (2008). The histone-binding protein COPR5 is required for nuclear

functions of the protein arginine methyltransferase PRMT5. *EMBO Reports*, *9*(5), 452–8. https://doi.org/10.1038/embor.2008.45

Larsen, S. C., Sylvestersen, K. B., Mund, A., Lyon, D., Mullari, M., Madsen, M. V., … Nielsen, M. L. (2016). Proteome-wide analysis of arginine monomethylation reveals widespread occurrence in human cells. *Science Signaling*, *9*(443). http://stke.sciencemag.org/content/9/443/rs9.long

Larsson, N.-G., Wang, J., Wilhelmsson, H., Oldfors, A., Rustin, P., Lewandoski, M., … Clayton, D. A. (1998). Mitochondrial transcription factor A is necessary for mtDNA maintance and embryogenesis in mice. *Nature Genetics*, *18*(3), 231–236. https://doi.org/10.1038/ng0398-231

Le Grand, F., Grifone, R., Mourikis, P., Houbron, C., Gigaud, C., Pujol, J., … Maire, P. (2012). Six1 regulates stem cell repair potential and self-renewal during skeletal muscle regeneration. *The Journal of Cell Biology*, *198*(5). http://jcb.rupress.org/content/198/5/815?ijkey=197a484fa738a98000c477a3a5451d1e0288ee9f&keytype2=tf_ipsecsha

Ljubicic, V., Khogali, S., Renaud, J.-M., & Jasmin, B. J. (2012). Chronic AMPK stimulation attenuates adaptive signaling in dystrophic skeletal muscle. *AJP:*

Cell Physiology, *302*(1), C110–C121. https://doi.org/10.1152/ajpcell.00183.2011

Mallappa, C., Hu, Y. J., Shamulailatpam, P., Tae, S., Sif, S., & Imbalzano, A. N. (2011). The expression of myogenic microRNAs indirectly requires protein arginine methyltransferase (Prmt)5 but directly requires Prmt4. *Nucleic Acids Research*, *39*(4), 1243–1255. https://doi.org/10.1093/nar/gkq896

Meyer, C., Dostou, J. M., Welle, S. L., & Gerich, J. E. (2002). Role of human liver, kidney, and skeletal muscle in postprandial glucose homeostasis. *American Journal of Physiology. Endocrinology and Metabolism*, *282*(2), E419–E427. https://doi.org/10.1152/ajpendo.00032.2001

Neuenkirchen, N., Chari, A., & Fischer, U. (2008). Deciphering the assembly pathway of Sm-class U snRNPs. *FEBS Letters*, *582*(14), 1997–2003. https://doi.org/10.1016/j.febslet.2008.03.009

Ott, M. O., Bober, E., Lyons, G., Arnold, H., & Buckingham, M. (1991). Early expression of the myogenic regulatory gene, myf-5, in precursor cells of skeletal muscle in the mouse embryo. *Development*, *111*(4). Retrieved from http://dev.biologists.org/content/111/4/1097.long

Paul, C., Sardet, C., & Fabbrizio, E. (2012). The histone- and PRMT5-associated protein COPR5 is required for myogenic differentiation. *Cell Death and Differentiation*, *19*(5), 900–908. https://doi.org/10.1038/cdd.2011.193

Pawlak, M. R., Scherer, C. A., Chen, J., Roshon, M. J., & Ruley, H. E. (2000). Arginine N-methyltransferase 1 Is Required for Early Postimplantation Mouse Development, but Cells Deficient in the Enzyme Are Viable. *Molecular and Cellular Biology*, *20*(13), 4859–4869. https://doi.org/10.1128/MCB.20.13.4859-4869.2000

Remels, A. H. V., Langen, R. C. J., Schrauwen, P., Schaart, G., Schols, A. M. W. J., & Gosker, H. R. (2010). Regulation of mitochondrial biogenesis during myogenesis. *Molecular and Cellular Endocrinology*, *315*(1), 113–120. https://doi.org/10.1016/j.mce.2009.09.029

Reporter, M., & Corbin, J. L. (1971). NG, NG-Dimethylarginine in myosin during muscle development. *Biochemical and Biophysical Research Communications*, *43*(3), 644–650. https://doi.org/10.1016/0006-291X(71)90663-2

Rhein, V. F., Carroll, J., Ding, S., Fearnley, I. M., & Walker, J. E. (2013).

NDUFAF7 Methylates Arginine 85 in the NDUFS2 Subunit of Human Complex I *. https://doi.org/10.1074/jbc.M113.518803

Rochard, P., Rodier, A., Casas, F., Cassar-Malek, I., Marchal-Victorion, S., Daury, L., … Cabello, G. (2000). Mitochondrial activity is involved in the regulation of myoblast differentiation through myogenin expression and activity of myogenic factors. *Journal of Biological Chemistry*, *275*(4), 2733–2744. https://doi.org/10.1074/jbc.275.4.2733

Rutter, J., Winge, D. R., & Schiffman, J. D. (2010). Succinate dehydrogenase - Assembly, regulation and role in human disease. *Mitochondrion*, *10*(4), 393–401. https://doi.org/10.1016/j.mito.2010.03.001

Saleem, A., Carter, H. N., & Hood, D. A. (2014). p53 is necessary for the adaptive changes in the cellular milieu subsequent to an acute bout of endurance exercise. *American Journal of Physiology. Cell Physiology*, *306*(3), 241–249. https://doi.org/10.1152/ajpcell.00270.2013

Sassoon, D., Lyons, G., Wright, W. E., Lin, V., Lassar, A., Weintraub, H., & Buckingham, M. (1989). Expression of two myogenic regulatory factors myogenin and MyoDl during mouse embryogenesis. *Nature*, *341*(6240),

303–307. https://doi.org/10.1038/341303a0

Scarpulla, R. C. (2008). Transcriptional Paradigms in Mammalian Mitochondrial Biogenesis and Function. *Physiological Reviews*, *88*(2). Retrieved from http://physrev.physiology.org/content/88/2/611

Seyer, P., Grandemange, S., Busson, M., Carazo, A., Gamaléri, F., Pessemesse, L., … Wrutniak-Cabello, C. (2006). Mitochondrial activity regulates myoblast differentiation by control of c-Myc expression. *Journal of Cellular Physiology*, *207*(1), 75–86. https://doi.org/10.1002/jcp.20539

Sha, L., Daitoku, H., Araoi, S., Kaneko, Y., Takahashi, Y., Kako, K., & Fukamizu, A. (2017). Asymmetric Arginine Dimethylation Modulates Mitochondrial Energy Metabolism and Homeostasis in Caenorhabditis elegans 2 3. *Mol. Cell. Biol*. https://doi.org/10.1128/MCB.00504-16

Shiozu, H., Higashijima, M., & Koga, T. (2015). Association of sarcopenia with swallowing problems, related to nutrition and activities of daily living of elderly individuals. *Journal of Physical Therapy Science*, *27*(2), 393–6. https://doi.org/10.1589/jpts.27.393

Sin, J., Andres, A. M., Taylo R, D. J. R., Weston, T., Hiraumi, Y., Stotland, A., … Gottlieb, R. A. (2016). Mitophagy is required for mitochondrial biogenesis and myogenic differentiation of C2C12 myoblasts. *Autophagy*, *12*(2), 369–380. https://doi.org/10.1080/15548627.2015.1115172

Swiercz, R., Cheng, D., Kim, D., & Bedford, M. T. (2007). Ribosomal protein rpS2 is hypomethylated in PRMT3-deficient mice. *The Journal of Biological Chemistry*, *282*(23), 16917–23. https://doi.org/10.1074/jbc.M609778200

Tang, J., Frankel, A., Cook, R. J., Kim, S., Paik, W. K., Williams, K. R., … Herschman, H. R. (2000). PRMT1 is the predominant type I protein arginine methyltransferase in mammalian cells. *The Journal of Biological Chemistry*, *275*(11), 7723–30. https://doi.org/10.1074/JBC.275.11.7723

Tee, W.-W., Pardo, M., Theunissen, T. W., Yu, L., Choudhary, J. S., Hajkova, P., & Surani, M. A. (2010). Prmt5 is essential for early mouse development and acts in the cytoplasm to maintain ES cell pluripotency. *Genes & Development*, *24*(24), 2772–7. https://doi.org/10.1101/gad.606110

Teyssier, C., Ma, H., Emter, R., Kralli, A., & Stallcup, M. R. (2005). Activation of nuclear receptor coactivator PGC-1alpha by arginine methylation. *Genes*

& Development, *19*(12), 1466–73. https://doi.org/10.1101/gad.1295005

Vergara, M. N., & Canto-Soler, M. V. (2012). Rediscovering the chick embryo as a model to study retinal development. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3541172/pdf/1749-8104-7-22.pdf

Wagatsuma, A., Kotake, N., & Yamada, S. (2011). Muscle regeneration occurs to coincide with mitochondrial biogenesis. *Molecular and Cellular Biochemistry*, *349*(1–2), 139–147. https://doi.org/10.1007/s11010-010-0668

Wagatsuma, A., & Sakuma, K. (2013). Mitochondria as a potential regulator of myogenesis. *The Scientific World Journal*. https://doi.org/10.1155/2013/593267

Wang, J., & Conboy, I. (2010). Embryonic vs. Adult Myogenesis: Challenging the "Regeneration Recapitulates Development" Paradigm. *Journal of Molecular Cell Biology*, *2*(1), 1–4. https://doi.org/10.1093/jmcb/mjp027

Wang, S.-C. M., & Muscat, G. E. O. (2013). Nuclear receptors and epigenetic signaling: novel regulators of glycogen metabolism in skeletal muscle. *IUBMB Life*, *65*(8), 657–664. https://doi.org/10.1002/iub.1181

Wang, S. M., Dowhan, D. H., Eriksson, N. A., & Muscat, G. E. O. (2012). CARM1/PRMT4 is necessary for the glycogen gene expression programme in skeletal muscle cells. *Biochemical Journal*, *444*(2), 323–331. https://doi.org/10.1042/BJ20112033

Wang, Y. P., Zhou, W., Wang, J., Huang, X., Zuo, Y., Wang, T. S., … Lei, Q. Y. (2016). Arginine Methylation of MDH1 by CARM1 Inhibits Glutamine Metabolism and Suppresses Pancreatic Cancer. *Molecular Cell*, *64*(4), 673–687. https://doi.org/10.1016/j.molcel.2016.09.028

Wei, H., Mundade, R., Lange, K. C., & Lu, T. (2014). Protein arginine methylation of non-histone proteins and its role in diseases. *Cell Cycle (Georgetown, Tex.)*, *13*(1), 32–41. https://doi.org/10.4161/cc.27353

Wu, Z., Puigserver, P., Andersson, U., Zhang, C., Adelmant, G., Mootha, V., … Spiegelman, B. M. (1999). Mechanisms Controlling Mitochondrial Biogenesis and Respiration through the Thermogenic Coactivator PGC-1. *Cell*, *98*(1), 115–124. https://doi.org/10.1016/S0092-8674(00)80611-X

Yadav, N., Lee, J., Kim, J., Shen, J., Hu, M. C.-T., Aldaz, C. M., & Bedford, M. T. (2003). Specific protein methylation defects and gene expression

perturbations in coactivator-associated arginine methyltransferase 1-deficient mice. *Proceedings of the National Academy of Sciences of the United States of America*, *100*(11), 6464–8. https://doi.org/10.1073/pnas.1232272100

Yang, Y., & Bedford, M. T. (2012). Protein arginine methyltransferases and cancer. *Nature Reviews Cancer*. https://doi.org/10.1038/nrc3409

Yang, Y., Hadjikyriacou, A., Xia, Z., Gayatri, S., Kim, D., Zurita-Lopez, C., … Bedford, M. T. (2015). ARTICLE PRMT9 is a Type II methyltransferase that methylates the splicing factor SAP145. https://doi.org/10.1038/ncomms7428

Yen, J., White, R. M., & Stemple, D. L. (2014). Zebrafish models of cancer: progress and future challenges. *Current Opinion in Genetics & Development*, *24*(100), 38–45. https://doi.org/10.1016/j.gde.2013.11.003

Yildirim, A. O., Bulau, P., Zakrzewicz, D., Kitowska, K. E., Weissmann, N., Grimminger, F., … Eickelberg, O. (2006). Increased protein arginine methylation in chronic hypoxia: Role of protein arginine methyltransferases. *American Journal of Respiratory Cell and Molecular Biology*, *35*(4), 436–443. https://doi.org/10.1165/rcmb.2006-0097OC

Yin, H., Price, F., & Rudnicki, M. a. (2013). Satellite cells and the muscle stem cell niche. *Physiological Reviews*, *93*(1), 23–67. https://doi.org/10.1152/physrev.00043.2011

Zhang, T., Günther, S., Looso, M., Künne, C., Krüger, M., Kim, J., … Braun, T. (2015). Prmt5 is a regulator of muscle stem cell expansion in adult mice. *Nature Communications*, *6*, 7140. https://doi.org/10.1038/ncomms8140

Characterizing Protein Arginine Methyltransferase Expression and Activity During Myogenesis

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Abstract

Protein arginine methyltransferases mediate skeletal muscle glucose metabolism, oxidative capacity, and regeneration in response to cytotoxic injury. Despite the emerging importance of PRMTs in regulating skeletal muscle plasticity, the biology of these enzymes during muscle development remains poorly understood. Therefore, the purpose of this study was to investigate PRMT1, -4, and -5 expression and function in skeletal muscle cells during the phenotypic remodeling elicited by myogenesis. C_2C_{12} muscle cell maturation, assessed during the myoblast stage, and during days 1, 3, 5, and 7 of differentiation, was employed as an in vitro model of myogenesis. We observed PRMT-specific patterns of expression and activity during myogenesis. PRMT4 and -5 gene expression was unchanged, while PRMT1 mRNA and protein content were significantly induced. Cellular methylarginine species levels, indicative of PRMT activities, increased during development. Targeted histone arginine methylation was elevated in a PRMT-specific manner, coincident with the myonuclear accumulation of PRMT proteins. Collectively, this suggests that PRMTs are methyl donors throughout myogenesis and demonstrate specificity for their histone and non-histone targets. Cells were then treated with TC-E 5003 (TC-E), a selective inhibitor of PRMT1 in order to specifically examine the enzymes role during myogenic differentiation. TC-E treated cells exhibited decrements in muscle differentiation, which were consistent with attenuated mitochondrial biogenesis and respiratory function. In

summary, this study increases our understanding of PRMT1, -4, and -5 biology during the plasticity of skeletal muscle development. Our results provide additional evidence for a role of PRMT1, via a mitochondrially-mediated mechanism, in driving the myogenic program.

Introduction

Protein arginine methyltransferases (PRMTs) are key regulators of important cellular events such as signal transduction, as well as transcriptional activation and repression (Bedford & Clarke, 2009; Cha & Jho, 2012). These enzymes methylate arginine residues by transferring methyl groups from S-adenosyl-L-methionine to terminal guanidino nitrogen atoms of targeted proteins (Kim et al., 2011). As a result of PRMT activity, three different methylarginine species are generated, including monomethylarginine (MMA), symmetric dimethylarginine (SDMA), and asymmetric dimethylarginine (ADMA) marks on target proteins (Wei et al., 2014). It has recently been demonstrated that the occurrence of arginine methylation is comparable to phosphorylation and ubiquitylation (Larsen et al., 2016), demonstrating the importance of this relatively less understood modification. PRMTs are classified in two groups based on their methylated arginine products: type I PRMTs (i.e., PRMT1, -2, -3, -4, -6, and -8) produce MMA and ADMA, while type II PRMTs (i.e., PRMT5, -7, and -9) generate MMA and SDMA. The importance of PRMT1, -4, and -5 has been demonstrated

through mice knockout models where ablation of these enzymes is incompatible with life (Pawlak et al., 2000; Yadav et al., 2003; Tee et al., 2010). Furthermore, dysfunction of these PRMTs has been implicated in the most prevalent diseases of Western society, namely cardiovascular disease, diabetes, and cancer (Bedford & Clarke, 2009; Yang & Bedford, 2012). For example, overexpression of PRMT1 is linked to lung cancer, while aberrant expression levels PRMT4 and -5 are observed in breast tumors (Wei et al., 2014). Therefore, expanding our understanding of these enzymes will likely have critically important health implications.

Though the presence of arginine methylation in skeletal muscle was first reported almost five decades ago (Reporter & Corbin, 1971), only recently have studies emerged implicating roles for PRMTs in muscle biology. The expression and activities of PRMTs are altered throughout myogenesis, which is a robust example of the skeletal muscle remodeling process. However, when these adaptations occur, and to what extent they occur, remain unclear. Reports of PRMT1, -4, -5, and -7 levels during skeletal muscle development have varied from no detected expression (Kim et al., 2011), to constitutively expressed (Chen et al., 2002; Kim et al., 2011; Wang et al., 2012), to unchanged (Blanc et al., 2017), to increased (Blanc et al., 2016). Along these lines, the activities of these enzymes throughout myogenesis have also differed between studies. For example, while work from Anthony Imbalzano's laboratory demonstrated that PRMT4 was only required for late myogenic gene expression (Dacwag et al., 2009), recent data from

Kawabe et al. (2012) showed that PRMT4 methylation regulated Pax7 transcriptional activity, which was necessary for the induction of the early gene Myf5. Furthermore, reconciling conflicting reports of PRMT5 function during distinct phases of the myogenic program presents additional challenges to achieving clarity with respect to PRMT activity during skeletal muscle development (Dacwag et al., 2007; Dacwag et al., 2009; Batut et al, 2011; Kim et al. 2011; Zhang et al., 2015). Altogether, critical aspects of PRMT biology during myogenesis, such as gene expression and function, are still undefined.

On balance, the conflicting literature on PRMT expression and activity during myogenesis may be due to differences in the PRMTs examined, experimental timecourse utilized, as well as models of muscle differentiation employed in these studies. These inconsistencies make it difficult to elucidate PRMT biology in skeletal muscle. A comprehensive examination of PRMT1, -4, and -5 expression and function throughout a complete timecourse of myogenesis is required in order to clarify and expand our knowledge of the roles of PRMTs in skeletal muscle plasticity. Thus, the purpose of this study was to examine PRMT1, -4, and -5 expression and function during the conditions of skeletal muscle remodeling evoked during myogenesis. We hypothesized that PRMT expression and activity would be dynamic during muscle development, and that these alterations would exhibit enzyme-specific patterns. A secondary objective, designed to complement a study identifying a critical role for PRMT1 in muscle

regeneration that was published during the preparation of this manuscript (Blanc et al., 2017), was to test the requirement of PRMT1 for the progression of skeletal muscle differentiation. We anticipated that the function of PRMT1 during muscle maturation would indeed be essential for optimal muscle development to occur.

Methods

C_2C_{12} muscle cell culture. Commercially available C_2C_{12} mouse myoblasts (American Type Culture Collection, Manassas, USA) were used in this study. C_2C_{12} cells were grown in the presence of growth media (GM), which was comprised of Dulbecco's modified Eagle's medium (DMEM; Invitrogen Life Technologies, Burlington, Canada) supplemented with 10% fetal bovine serum (Invitrogen Life Technologies), and 1% penicillin/streptomycin (Invitrogen Life Technologies). The cultured dishes were maintained in a 5% CO_2 atmosphere at 37 °C. Myogenic differentiation was induced when ~90% cell confluence was attained, after which GM was replaced with differentiation media (DM; DMEM supplemented with 2% horse serum and 1% penicillin/streptomycin; Invitrogen Life Technologies). Cells were grown in DM for 7 days, with DM changed every 48 hrs. Five experimental timepoints were employed to characterize myogenesis in vitro. The first was the myoblast (MB) stage, which was when cells reached ~90% confluence. The second to fifth timepoints occurred throughout the fusion and growth of myotubes (MT), including day 1 (D1) MT (24 hrs after the transition from GM to DM), day 3 (D3;

72 hrs after the GM to DM switch), day 5 (D5; 120 hrs), and day 7 (D7; 168 hrs). At the specified timepoints, C_2C_{12} cells were washed three times with Dulbecco's Phosphate Buffered Saline (PBS; Hyclone, South Lake, Utah) and processed for subsequent analyses.

Drug treatments. C_2C_{12} myoblasts were cultured until reaching ~90% confluence and then induced to differentiate in media treated with vehicle (VEH; DM supplemented with dimethyl sulfoxide (DMSO); Invitrogen Life Technologies) or treated with TC-E 5003 (TC-E; DM supplemented with (volume; final concentration) 2.1µL; 0.1 µM TC-E 5003; Tocris, Bristol, United Kingdom), a PRMT1-specific antagonist (Bissinger et al., 2011). Four experimental timepoints were employed to examine skeletal muscle differentiation, including D1, D3, D5, and D7, after which cells were washed three times with PBS and prepared for analyses.

Whole cell protein extraction. Cells were scraped in ice-cold RIPA buffer (Sigma-Aldrich, Oakville, Canada) supplemented with cOmplete Mini Protease Inhibitor Cocktail (Roche, Laval, Canada) and PhosSTOP (Roche). Samples were sonicated (Fisher Scientific) 5 X 3 sec on ice at 100% power. The samples were spun at 20,000 x g for 15 min. The protein concentrations of the supernates were determined using the Pierce bicinchoninic acid (BCA) Protein Assay kit (Thermo-Scientific, Rockford, USA) using bovine serum albumin (BSA) as the standard.

Cell fractionation. Nuclear fractions were isolated from C_2C_{12} cells at the MB stage as well as at D1, D3, D5, and D7 of muscle differentiation according to procedures as described previously (Dimauro et al., 2012), with modifications. Briefly, 500 µL of STM buffer was added to each sample tube and the solution was then homogenized on ice using sonication at 100% power for 10 X 2 sec, with 30 sec in between each bout. An additional 200 µL of STM buffer was added before the samples were spun. After centrifuging at 800 x g for 15 min, the supernatant and pellet (P0) were separated. The pellet (P0) was resuspended in 1000 µL of STM buffer, vortexed for 15 sec and then centrifuged at 500 x g for 15 min. The nuclear pellet (P1) was then suspended in 250-400 µL mL of STM buffer and spun at 100 x g for an additional 15 min while the supernatant was discarded. The sample was again separated and the pellet (P5) was kept. 400 µL of NET buffer was added to the tube before vortexing and then placed on ice for 30 min. The sample was then sonicated at 100% power for 10 X 2 sec with 30 sec in between bouts. The sample was spun at 9000 x g for 30 minutes before being separated and the supernatant was kept and labelled as the nuclear fraction. Protein concentrations of the cellular fractions were determined using the BCA Protein Assay kit.

Immunoblotting. For whole cell lysates, 20-50 µg of cellular protein was loaded into each lane of 10% or 12.5% polyacrylamide gels and resolved by SDS-PAGE. For nuclear fractions, 40 µg of protein was loaded into each lane. Gels were transferred onto nitrocellulose membranes (Bio-Rad, Mississauga, Canada) and

stained with either Ponceau S (Sigma-Aldrich, Oakville, Canada) or Amido black (Sigma-Aldrich, Oakville, Canada) to confirm equal loading across samples (Fortes et al., 2016). Membranes were washed with Tris-buffered Saline-Tween 20 [(TBST) (25 mM Tris·HCl (pH 7.5), 1 mM NaCl, and 0.1% Tween 20)] and blocked with 5% skim milk in TBST solution for 1 hr. Membranes were subsequently incubated in 5% milk-TBST with primary antibodies overnight at 4 °C on a compact digital rocker (Thermo-Scientific). The antibodies used were: myogenin (M3559; Dako, Santa Clara, USA), PRMT1 (P1620; Sigma-Aldrich), PRMT4 (A300–421A; Bethyl, Montgomery, TX), PRMT5 (07-405; EMD Millipore, Massachusetts, USA), histone 2B (H2B; 8135, Cell Signaling Technology, Massachusetts, USA), glyceraldehyde 3-phosphate dehydrogenase (GAPDH; ab9483; Abcam, Toronto, Canada), histone 4 arginine 3 (H4R3; 39705, Active Motif, Carlsbad, USA), H3R8 (ab130740, Abcam), H3R17 (ab8284, Abcam), H4 (ab10158, Abcam), H3 (ab1791, Abcam), MMA (8015, Cell Signaling Technology), ADMA (13522, Cell Signaling Technology), SDMA (13222, Cell Signaling Technology), PGC-1α (AB3242; EMD Millipore), and total oxidative phosphorylation (OXPHOS) cocktail (ab110413; Abcam). After incubation, the blots were washed 3 X 5 min in TBST, and appropriate horseradish peroxidise-linked secondary antibodies were applied. The bound antibodies were visualized by enhanced chemiluminesence (Bio-Rad, Mississauga, Canada) and the membrane

was imaged with Alpha Innotech imaging equipment (Alpha Innotech, San Leandro, USA). ImageJ (NIH) was employed for densitometry.

Immunofluorescence imaging. Cells were cultured on three 15 mm glass cover slips that were inserted at the bottom of individual 35 x 10 mm^2 dishes. At each experimental time point, cells were washed three times with PBS and fixed with 4% paraformaldehyde for 30 min. After fixation, cells were washed again in PBS and incubated in 0.25% triton X in PBS for 5 min. Following the incubation, blocking was performed with 10% goat serum for 60 min. Subsequently, cells were incubated with embryonic myosin heavy chain (eMHC) primary antibody (Developmental Studies Hybridoma Bank, Iowa, USA) overnight at 4 °C. The next morning, cells were washed in PBS and incubated in secondary antibody (Alexa fluor goat anti-mouse 594, 1:500 dilution prepared in 1% BSA) for 60 min. Following incubation, the cells were washed in PBS and 4',6-diamidino-2-phenylindole (DAPI; 1:20,000) was applied and incubated for 5 min. Cells were then washed and the coverslips were carefully removed from the plate. Coverslips were mounted using DAKO fluorescence mounting media (Agilent Technologies, USA). Three images were taken from each coverslip. The images were viewed using a fluorescence microscope (Nikon Instruments Canada, Mississauga, ON) at 20X magnification. Myotube length and width were determined by calculating the average of the five longest and widest myotubes using 4x4 eMHC stained immunofluorescence images. The fusion index was calculated by identifying the

percentage of eMHC positive cells that had two or more myonuclei. The area fraction was calculated by determining the percentage of eMHC positive cells relative to the total surface area of the image. Cell metrics were determined using Nikon Eclipse Ti-E software (ver. 4.4.2).

RNA isolation and reverse transcription real-time polymerase chain reaction (RT-qPCR). Total RNA was extracted from C_2C_{12} cells using TRIzol reagent (Invitrogen Life Technologies) according to the manufacturer's protocol. After treating with Turbo DNaseI (Invitrogen Life Technologies) for 30 min, RNA was further purified with RNeasy columns (Qiagen, Toronto, Canada). RNA concentration and integrity (i.e., A260/A230) was measured using a Nanodrop instrument (Thermo-Scientific) before being reverse transcribed into cDNA. Superscript III (Invitrogen Life Technologies) was used to synthesize cDNA from total RNA, according to the manufacturer's instructions. Target cDNA levels were compared by qPCR in reactions containing either SYBR green (Roche Diagnostics, Meylan, France) or GoTaq qPCR Mastermix (Promega, Wisconsin, USA), forward (F) and reverse (R) primers, and cDNA. qPCR was conducted over 45 cycles of 95 °C for 15 sec and 60 °C for 1 min, preceded by an initial 95 °C for 10 min. The ΔΔCT method was used to calculate the expression of the genes of interest with the average of 18S ribosomal RNA (18S), glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and 40 S ribosomal protein S11 (RPS11) utilized as the internal control (Manzano et al., 2011; Hildyard and Wells, 2014). Primers utilized in this study

were as follows: myogenin forward (F) 5'-GCAAGGTGTGTAAGAGGAAG-3', reverse (R) 5'-TGTGGGAGTTGCATTCACTG-3'; PRMT1 F 5'-GCCTGCAAGTGAAGAGGAAC-3', R 5'-CTCAGGACTGGTGGAGAAGC-3'; PRMT4 F 5'-ACCACACGGACTTCAAGGAC-3', R 5'-CTCTTCACCAGGACCTCTGC-3'; PRMT5 F 5'-TCTCCCCACCAGCATTTTCC-3', R 5'-TGGAGGGCGATTTTGGCTTA-3'; GAPDH F 5'- AACACTGAGCATCTCCCTCA-3', R 5'-GTGGGTGCAGCGAACTTTAT-3'; 18S F 5'- GTAACCCGTTGAACCCCATT-3', R 5'- CCATCCAATCGGTAGTAGCG-3'; RPS11 5'-CGTGACGAACATGAAGATGC-3', R 5'- GCACATTGAATCGCACAGTC-3'.

Mitochondrial Respiration. At each timepoint of interest, C_2C_{12} cells were washed with PBS and removed from the 100 mm x 20 mm culture plate using trypsin-EDTA. Two culture plates were combined together and the sample was centrifuged at 100 x g at room temperature for 3 min. The supernatant was discarded and the pellet was resuspended in mitochondrial respiration medium (MiR05) which contains: 0.5 mM EGTA, 10 mM KH_2PO_4, 3 mM $MgCl_2 \cdot 6H2O$, 60 mM potassium lactobionate, 20 mM HEPES, 20 mM taurine, 110 mM sucrose, and 1 g/L BSA. Cells were subsequently counted using trypan blue and a hemocytometer (Invitrogen). Samples were treated with 3 μg/10^6 cells/ml digitonin for 5 min at 37 °C on a digital rocker. Following permeabilization, samples were centrifuged at 800 x g for 3 min. The permeabilized cells were resuspended in MiR05 buffer and

were used for determination of mitochondrial oxygen (O_2) consumption at 37 ºC using the Oroboros Oxygraph-2 K (Oroboros Instruments Corp., Innsbruck, Austria). The cells (1 x 10^6 cells/chamber) were placed into separate sealed chambers and the following substrates were added (volume; final concentration): glutamate (5µl; 5mM), followed by malate (4µl; 2mM), ADP (20µl; 5mM), cytochrome c (5µl; 10µM), pyruvate (5µl; 5mM), and succinate (20µl; 20mM). The rate of O_2 consumption was recorded and expressed as picomoles/second/million cells using DatLab software (Oroboros Instruments Corp.).

Statistical Analyses. A one-way ANOVA with Bonferroni post-hoc test and student t-tests were used to identify differences between means during the myogenesis timecourse experiments. A two-way ANOVA with Bonferroni post-hoc tests was used for analysis of the PRMT1 inhibition experiments. Statistical analyses were performed on the raw data sets prior to the conversion to -fold differences. Sample sizes for all experiments were n = 3-6, with each n being the mean of two or three independent observations. Statistical significance was accepted at $p < 0.05$. Data in graphical summaries are presented as mean ± SEM.

Results

C_2C_{12} myogenesis. To begin our characterization of PRMT expression and function during myogenesis, we first sought to confirm the progression of muscle development throughout the experimental timecourse. To this end, we examined

cell morphology, eMHC expression, as well as myogenin transcript and protein levels, which all represent established histological and molecular markers of myogenesis (Hawke & Garry, 2001; Yin et al., 2013). Qualitative assessment of light microscopy images indicates that the C_2C_{12} cells progressed morphologically from mononucleated MBs to robust MTs as expected throughout the differentiation timecourse (Fig. 1A, left column panels). In situ immunofluorescence detection of eMHC also demonstrated the advancement of the myogenic program (Fig. 1A, right column panels), as expression of the enzyme increased coincident with the fusion and growth of MTs throughout the 7-day timecourse. Furthermore, mRNA and protein levels of the myogenic regulatory factor myogenin increased as expected during the differentiation protocol (Fig. 1B, C), which is indicative of typically progressing myogenesis. Myogenin mRNA content was ~1.5-fold higher after D3 relative to the MB stage ($p < 0.05$). Myogenin protein content was significantly greater at all differentiating timepoints in comparison to the MB stage.

PRMT gene expression throughout myogenesis. We next endeavoured to examine the gene expression of PRMT1, PRMT4, and PRMT5 during the differentiation timecourse by measuring mRNA and protein levels of the enzymes. PRMT1 transcript levels were ~2-fold higher ($p < 0.05$) at D5 compared to all preceding timepoints, and returned to baseline by D7 (Fig. 2A). Both PRMT4 and PRMT5 mRNA levels remained unchanged throughout myogenesis (Fig. 2A). The protein content of these enzymes followed their mRNA patterns of expression and

were constitutively expressed across the timecourse (Fig. 2B, C). In contrast, PRMT1 protein expression was significantly higher by D3 of differentiation, as compared to earlier timepoints, and remained elevated 2-2.2-fold until D7 (Fig. 2B, C).

PRMT activity during skeletal muscle development. To determine the global activity of PRMTs during myogenesis, we utilized immunoblotting to probe for MMA, ADMA, and SDMA levels, which are established markers of PRMT activity, type I PRMT activity, and type II PRMT activity, respectively (Bedford & Clarke, 2009; Blanc & Richard, 2017). ADMA content was increased by ~1.7-fold ($p < 0.05$) at D3, as compared to the MB stage, and remained elevated (Fig. 3A, B). In contrast, cellular MMA and SDMA levels did not change throughout myogenesis (Fig. 3A, B).

To examine PRMT-specific methyltransferase activities, we measured the methylated arginine levels of their histone targets. Asymmetric arginine dimethylation of H4R3 and H3R17, as well as symmetric arginine dimethylation of H3R8, are specific and exclusive methylation targets of PRMT1, PRMT4, and PRMT5, respectively (Di Lorenzo & Bedford, 2011; Blanc & Richard, 2017). The myonuclear subfractions employed in this analysis were isolated with a high level of purity, as indicated by the presence of the nuclear protein H2B, and the absence of the cytosolic molecule GAPDH (Fig. 4A). We first assessed myonuclear PRMT content during myogenesis since specific methyltransferase functions are

dependent, in part, on the subcellular localization of the enzymes. Myonuclear PRMT1 and -4 accumulated throughout differentiation, demonstrating levels that were ~1.5-2.1-fold higher ($p < 0.05$) at D5 and D7 compared to MB (Fig. 4A, B). Nuclear PRMT5 content remained unchanged. Total H4 and H3 levels did not change over the course of muscle development (Fig. 4C). Histone methylation status, expressed as the methylated form of the histone relative to the total amount of the protein, significantly increased during myogenesis for H4R3 and H3R17, reaching levels that were ~1.9-fold greater at D7 compared to MB (Fig. 4D). In contrast, H3R8 methylation status remained unchanged across myogenic development.

PRMT1 function in myogenesis. In an effort to elucidate the role of PRMT1 in myogenesis, we employed the specific PRMT1 antagonist TC-E 5003 (TC-E; Bissinger et al., 2011) to inhibit PRMT1 methyltransferase function during muscle differentiation. TC-E treatment did not affect cellular H4 content, which remained unchanged throughout myogenesis (Fig. 5A). TC-E completely blocked the increase in H4R3 methylation status that occurred with myogenic development (Fig. 5A, B). H4R3 methylation levels were significantly blunted by ~25% at D3-D7, thereby confirming the efficacy of the inhibitor.

Light microscopy images revealed smaller and less robust myotubes in the TC-E treated condition as compared to the VEH-treated cells (Fig. 6A). To further provide evidence of the role of PRMT1 on muscle development, cellular

morphology was quantitatively examined between the VEH and TC-E-treated conditions using eMHC and DAPI fluorescence analyses. As expected, there was a progressive increase in myotube fusion, length, width, and surface coverage (i.e., area fraction) across the experimental timecourse in the VEH-treated cells (Fig. 6B-F). This was also observed in the TC-E condition. However, TC-E treatment resulted in significant ~20-40% attenuations in all morphology metrics, which were observed at timepoints ranging from D3 to D7 of differentiation.

Effects of PRMT1 inhibition on mitochondrial biogenesis and function. To understand the potential mechanisms underlying the differentiation defects observed in PRMT1-inhibited cells, we examined the effects of TC-E on mitochondrial biogenesis and oxygen consumption. Indeed, mitochondrial content and function are required for optimal myogenic progression (Larsson et al., 1998; Collu-Marchese et al., 2015). Protein levels of representative subunits of mitochondrial electron transport chain complex I (CI), CIII, and CV were attenuated by ~20-40% ($p < 0.05$) in D3-D7 cells in response to PRMT1 inhibition (Fig. 7A, B, D, E). In contrast, CII expression was similar between VEH and TC-E treatment conditions throughout myogenesis (Fig. 7A, C). PRMT1 inhibition also resulted in the significant reduction by 25-40% in the protein content of PGC-1α, a master regulator of muscle mitochondrial biogenesis (Jornayvaz & Shulman, 2010), during D3-D7 of differentiation (Fig. 7A, F).

Finally, to further investigate the role of PRMT1 in mitochondrial biology, organelle respiration was measured during the timecourse of muscle development in the presence or absence of the PRMT1-specific antagonist. TC-E treatment did not affect CI- or CI+CII-driven mitochondrial oxygen consumption in D1 differentiated cells (Fig. 8A, E, F). However, there was a ~20-35% reduction ($p < 0.05$) in oxygen consumption in D3-D7 TC-E-treated cells following the addition of the CI substrate pyruvate, as compared to VEH-treated cells (Fig. 8B-E). PRMT1 inhibition also significantly attenuated succinate/CI+CII supported mitochondrial respiration at D5 and D7 of myogenesis (Fig. 8D, F).

Discussion

The purpose of the current study was to provide a more comprehensive characterization of PRMT biology throughout the process of skeletal muscle differentiation. Our data revealed PRMT-specific patterns of expression and activity during myogenesis, which suggest individualized contributions for each enzyme to the muscle development process. Moreover, complementary assessments of PRMT1, -4, and -5 function indicate that PRMT methyltransferase activity in muscle is substrate specific, depending in part, on the subcellular location of the protein target. Since PRMT1 expression and activity were particularly responsive to myogenic cues, we inhibited its methyltransferase activity in order to elucidate its role in skeletal muscle differentiation. Muscle cells in which PRMT1

activity was knocked down exhibited differentiation defects that were associated with attenuated mitochondrial biogenesis and respiratory function. Thus, extending recent work implicating the requirement of PRMT1 in skeletal muscle regeneration in vivo (Blanc et al., 2017), our results demonstrate that PRMT1 is necessary during differentiation in order to evoke complete myogenic development. Moreover, the negative effects of PRMT1 inhibition on myogenesis are at least partially mitochondrially-mediated. This study enhances our understanding of PRMT1, -4, and -5 biology during the plasticity of skeletal muscle development, as well as provides additional mechanistic evidence for a role of PRMT1 in driving the myogenic program.

Previous studies have demonstrated that several members of the PRMT family are expressed during skeletal muscle cell development, including PRMT1, -4, -5, and -7 (Chen et al, 2002; Wang et al., 2012; Paul et al., 2012; Blanc et al., 2017). Unfortunately however, a coherent understanding of PRMT transcript and protein levels during myogenesis has been difficult to achieve due, in part, to the disparate models and timing utilized thus far. Therefore, we sought to address this knowledge gap by clarifying PRMT gene expression via the employment of a standardized, comprehensive timecourse of C_2C_{12} skeletal muscle cell differentiation. Our data revealed a measure of PRMT-specificity with respect to gene expression during myogenesis. PRMT1 was induced at the mRNA and protein levels, while PRMT4 and -5 remained unchanged. PRMT1 followed a similar

pattern of expression as the myogenic regulatory factor myogenin, which suggests a common upstream regulator. A candidate may be Eya1, which affects myogenin expression (Le Grand et al., 2012), and was recently demonstrated to be part of a myogenic pathway involving PRMT1 (Blanc et al., 2017). However, our data contradict this recent study by Blanc and colleagues (2017), where the authors observed that PRMT1 mRNA and protein content were similar between proliferating and differentiating isolated primary muscle stem cells. The discrepancy between their results and those of the current study may be attributed to differences in cell type and/or duration of the myogenic timecourse utilized. In line with our findings, previous studies reported that PRMT4 and -5 transcript and protein levels were constitutively expressed during early and later points of skeletal muscle development (Chen et al., 2002; Kim et al., 2011; Wang et al, 2012). Constitutive expression of PRMT5 appears to be consistent with its roles in both the proliferative and differentiation phases of skeletal muscle development (Mallappa et al., 2011; Zhang et al., 2015). The differential expression pattern that we observed between PRMTs during myogenesis is likely due, in part, to unique, PRMT-specific upstream regulatory mechanisms. Little has been documented regarding for example, the transcriptional control of PRMTs, particularly in skeletal muscle. Thus, future work that more clearly defines the events governing PRMT gene expression in muscle is warranted.

We utilized two complementary approaches to investigate PRMT function during myogenesis. We first examined global PRMT activity by assessing total cellular MMA, ADMA, and SDMA levels. The MMA mark is a measure of non-specific PRMT activity, while the accumulation of ADMA and SDMA marks are indicative of type I and type II PRMT function, respectively (Bedford & Clarke, 2009; Dhar et al., 2013). Furthermore, since PRMT1 and PRMT5 catalyze the majority of ADMA and SDMA producing reactions, respectively (Tang et al., 2000; Di Lorenzo & Bedford, 2011; Dhar et al., 2013), the appearance of these marks generally reflects the activities of these enzymes. A notable caveat is that relative PRMT activities have not yet been elucidated in skeletal muscle. We observed an increase in ADMA levels coincident with unchanged amounts of MMA and SDMA methylarginine species during the progression of muscle differentiation. The significant upregulation in ADMA content was consistent with the rise in PRMT1 protein content. Previous evidence supports the idea that enzymatic activities of PRMTs are altered during muscle development (Kim et al., 2011; Kawabe et al., 2012; Blanc et al., 2017). For example, Kim and colleagues (2011) showed that hnRNP A1, an established target of PRMT1, was methylated to a greater degree during and after myoblast fusion, as compared to before fusion. It is interesting to note here that, similar to our PRMT4 results, others have also reported alterations in PRMT activity during myogenesis that are independent from any lack of change in PRMT content (Kim et al., 2011). Certainly, activation of PRMTs may be

facilitated by a number of mechanisms in this scenario, including the subcellular translocation of existing enzymes to the appropriate compartment, as well as stimulatory protein-protein interactions or posttranslational modifications (Chen et al., 2002; Iwasaki & Yada, 2007; Kawabe et al., 2012).

We isolated myonuclei and assessed the targeted methyltransferase activities of PRMT1, -4, and -5 throughout the experimental myogenic timecourse. The ADMA marks on H4R3 and H3R17, as well as the SDMA deposited on H3R8, are specifically and exclusively catalyzed by PRMT, -4, and -5, respectively (Bedford & Clarke, 2009; Di Lorenzo & Bedford, 2011). We found that the elevation in targeted PRMT activities generally reflected the increased nuclear accumulation of the enzymes. Moreover, the myonuclear accretion of PRMT1 along with the elevation in H4R3 methylation status was consistent with the increased cellular PRMT1 and ADMA levels during muscle development. Nuclear PRMT5 levels mirrored its histone methyltransferase activity during differentiation, which remained unchanged. These data are consistent with cellular SDMA levels, which are most likely driven by PRMT5. (Di Lorenzo & Bedford, 2011). Interestingly, while cellular PRMT4 content remained unchanged during differentiation, we observed a specific subcellular redistribution of PRMT4 within myonuclei, which corresponded with increased H3R17 methylation status. Previous studies have identified histone arginine methylation, such as the modifications of H3R8 and H4R3, as an important step in the activation of genes that are permissive for

myogenesis (Dacwag et al., 2007; Blanc et al., 2016). Furthermore, numerous non-histone nuclear PRMT targets, including GRIP-1, Pax7, and p21, serve to facilitate the myogenic program (Chen et al., 2002; Kawabe et al., 2012; Zhang et al., 2015). It must also be highlighted that each PRMT has many putative arginine methylation targets, and each member can have differential preferences for its targets (Di Lorenzo & Bedford, 2011). For instance, the interaction between PRMT5 and COPR5 causes PRMT5 to alter its specificity to preferentially methylate H4R3 over H3R8 (Lacroix et al., 2008). Altogether, our data suggest that PRMTs are active methyl donors throughout myogenesis and demonstrate specificity for their histone and non-histone targets in skeletal muscle.

Relative to other PRMTs, PRMT4 and -5 expression and function during myogenesis have been extensively studied (Bentzinger et al., 2012; Chen et al., 2002; Dacwag et al., 2009; Dacwag, et al., 2007; Paul et al., 2012; Wang et al., 2012). Work from George Muscat's laboratory was the first to demonstrate a role for PRMTs, specifically PRMT4, in potentiating myogenesis, clearly supporting a positive function of arginine methylation in mammalian differentiation (Chen et al., 2002). By contrast, there are few reports that have directly assessed the necessity of PRMT1 throughout skeletal muscle development. Current evidence supports the involvement of the enzyme in 1) mediating glucose uptake into skeletal muscle cells (Iwasaki & Yada, 2007), 2) methylating lamin C2, an intermediate filament that forms the essential structure of myonuclear lamina (Kim et al., 2011), and 3)

regulating muscle regenerative capacity (Blanc et al., 2016). In an effort to expand our understanding of the myogenic functions of PRMT1, the enzyme that accounts for the majority (>80%) of arginine methyltransferase activity (Wei et al., 2014), we pharmacologically inhibited PRMT1 and evaluated its impact on myogenesis. TC-E possesses high specificity for PRMT1, as compared to other arginine and lysine methyltransferases (Bissinger et al., 2011). Furthermore, the compound demonstrated potent anticancer activity and inhibition of androgen-dependent transcription in MCF7a and LNCaP cells, indicative of attenuated PRMT1 function. In skeletal muscle cells, TC-E treatment was able to completely block the differentiation-associated increase in PRMT1 activity, as evidenced by H4R3 methylation status, indicating that the drug successfully inhibited PRMT1. Our data demonstrating that TC-E treatment significantly attenuated various morphological metrics of C_2C_{12} differentiation reveals that PRMT1 activity is required for the optimal progression of the myogenic program. These results confirm recent work by Blanc and colleagues (2017) who showed that PRMT1 is essential to successful muscle regeneration in vivo in response to cytotoxic injury. Our work also builds on, and extends these in vivo data by demonstrating that inhibition of PRMT1 exclusively during and after the onset of differentiation, as compared to initiating the knockout prior to the myogenic stimulus (i.e., cardiotoxin injury), reveals a role for the enzyme specifically during muscle differentiation.

Mitochondrial biogenesis is necessary for the myogenic program to proceed under both in vitro and in vivo conditions. For example, myoblasts that lack mitochondrial DNA fail to differentiate into myotubes (Herzberg et al., 1993). Moreover, skeletal muscle regeneration is characterized by a nearly 5-fold increase in mitochondrial content during the onset of muscle differentiation (Duguez et al., 2002). We suspected that a potential mechanism linking PRMT1 inhibition to the observed differentiation defects was mitochondrially-mediated. Our rationale was based on previous studies implicating PRMT1 (Sha et al., 2017; Teyssier et al., 2005) and arginine methyltransferase activity (Rhein et al., 2013) in mitochondrial biogenesis and function. Indeed, Teyssier et al. (2005) very elegantly demonstrated more than a decade ago that PRMT1 methylates PGC-1α, a master regulator of muscle plasticity and mitochondrial biogenesis, which directly stimulates the transcriptional function of the coactivator. More recent work from Sha and colleagues (2017) showed that PRMT1 is almost entirely responsible for depositing the ADMA mark on mitochondrial proteins, and that PRMT1 knockdown resulted in reduced mitochondrial respiratory activity, ATP synthesis, as well as a significant elevation in oxidant production. Consistent with these reports, we observed that PRMT1 inhibition led to attenuated mitochondrial biogenesis and function in skeletal muscle cells. Although we were unable to resolve the OXPHOS complex IV subunit with a reliable degree of confidence, representative protein subunits from complexes I, III, and V were significantly lower in the TC-E-treated

cells during days 3-7 of differentiation. Interestingly, the rise in complex II content during myogenesis was unaffected by PRMT1 inhibition. The OXPHOS protein content data were reflected by results from the mitochondrial oxygen consumption trials, which demonstrated attenuated organelle respiratory function when PRMT1 activity was blunted. It is likely that complex II was largely spared due, in part, to its composition being solely dependent on nuclear DNA-encoded subunits, as well as to its dedicated assembly apparatus (Rutter et al., 2010).

PGC-1α levels were also blunted as a function of PRMT1 inhibition in skeletal muscle. The coactivator stimulates the transcription of mitochondrial genes located in nuclear and mitochondrial genomes by for example, interacting with nuclear respiratory factor 1 and mitochondrial transcription factor A (Tfam), respectively (Hood et al., 2016; Scarpulla, 2008). Notably, PGC-1α also participates in an autoregulatory positive feedback loop driving its own expression (Handschin et al., 2003), as well as contributes to the transcriptional activation of Tfam (Wu et al., 1999). It is therefore reasonable to posit that the attenuated PGC-1α expression in response to PRMT1 inhibition caused a dysregulation in Tfam expression and/or function. The expression of mitochondrial DNA (mtDNA) genes encoding OXPHOS subunits is critical for maintaining proper function of the organelle, as evidenced by the considerable impairments caused by mtDNA mutations (Scarpulla, 2008). Thus, although speculative, our results suggest that PRMT1 inhibition likely affects mitochondrial content and function via decrements

in PGC-1α content and/or activity, with particular consequence on events occurring within the organelle.

In summary, our results demonstrate that the expression and activities of PRMT1, -4, and -5 display differential responses during skeletal muscle development. Indeed, PRMT1 biology was particularly responsive to myogenic cues. We also show that inhibition of PRMT1 in skeletal muscle cells results in morphological deficiencies, as well as decrements in mitochondrial biogenesis and respiratory function. Thus, complementing recent in vivo work (Blanc et al., 2017), this investigation supports a critical role for PRMT1 specifically in the optimal progression of muscle differentiation. This study enhances our understanding of PRMT biology during skeletal muscle plasticity elicited by myogenesis and identifies a mitochondrially-mediated mechanism that links PRMT1 inhibition to defects in skeletal muscle development.

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References

Batut, J., Duboé, C., & Vandel, L. (2011). The methyltransferases PRMT4/CARM1 and PRMT5 control differentially myogenesis in zebrafish. *PLoS ONE*, *6*(10). https://doi.org/10.1371/journal.pone.0025427

Bedford, M. T., & Clarke, S. G. (2009). Protein arginine methylation in mammals: who, what, and why. *Molecular Cell*, *33*(1), 1–13. https://doi.org/10.1016/j.molcel.2008.12.013

Bentzinger, C. F., Wang, Y. X., & Rudnicki, M. A. (2012). Building muscle: molecular regulation of myogenesis. *Cold Spring Harbor Perspectives in Biology*, *4*(2), a008342. https://doi.org/10.1101/cshperspect.a008342

Bischoff, R., & Heintz, C. (1994). Enhancement of skeletal muscle regeneration. *Developmental Dynamics*, *201*(1), 41–54. https://doi.org/10.1002/aja.1002010105

Bissinger, E.-M., Heinke, R., Spannhoff, A., Eberlin, A., Metzger, E., Cura, V., … Jung, M. (2011). Acyl derivatives of p-aminosulfonamides and dapsone as new inhibitors of the arginine methyltransferase hPRMT1. *Bioorganic & Medicinal Chemistry*, *19*(12), 3717–3731.https://doi.org/10.1016/j.bmc.2011.02.032

Blanc, R. S., & Richard, S. (2017). Regenerating muscle with arginine methylation. *Transcription*, e1291083. https://doi.org/10.1080/21541264.2017.1291083

Blanc, R. S., Vogel, G., Li, X., Yu, Z., Li, S., & Richard, S. (2016). Arginine methylation by PRMT1 regulates muscle stem cell fate. *Molecular and Cellular Biology*, *37*(3), MCB.00457-16. https://doi.org/10.1128/MCB.00457-16

Blanc, R. S., Vogel, G., Li, X., Yu, Z., Li, S., & Richard, S. (2017). Arginine Methylation by PRMT1 Regulates Muscle Stem Cell Fate. *Molecular and Cellular Biology*, *37*(3), e00457-16. https://doi.org/10.1128/MCB.00457-16

Braun, T., Bober, E., Buschhausen-Denker, G., Kohtz, S., Grzeschik, K. H., Arnold, H. H., & Kotz, S. (1989). Differential expression of myogenic determination genes in muscle cells: possible autoactivation by the Myf gene products. *The EMBO Journal*, *8*(12), 3617–25. http://www.ncbi.nlm.nih.gov/pubmed/2583111

Braun, T., Buschhausen-Denker, G., Bober, E., Tannich, E., & Arnold, H. H.

(1989). A novel human muscle factor related to but distinct from MyoD1 induces myogenic conversion in 10T1/2 fibroblasts. *The EMBO Journal*, *8*(3), 701–9. http://www.ncbi.nlm.nih.gov/pubmed/2721498

Buckingham, M., & Rigby, P. W. J. (2014). Gene Regulatory Networks and Transcriptional Mechanisms that Control Myogenesis. *Developmental Cell*, *28*(3), 225–238. https://doi.org/10.1016/j.devcel.2013.12.020

Calvo, J. A., Daniels, T. G., Wang, X., Paul, A., Lin, J., Spiegelman, B. M., … Rangwala, S. M. (2008). Muscle-specific expression of PPARgamma coactivator-1alpha improves exercise performance and increases peak oxygen uptake. *Journal of Applied Physiology (Bethesda, Md. : 1985)*, *104*(5), 1304–1312. https://doi.org/10.1152/japplphysiol.01231.2007

Cha, B., & Jho, E.-H. (2012). Protein arginine methyltransferases (PRMTs) as therapeutic targets. *Expert Opinion on Therapeutic Targets*, *16*(7), 651–664. https://doi.org/10.1517/14728222.2012.688030

Charge, S. B. P., & Rudnicki, M. A. (2004). Cellular and Molecular Regulation of Muscle Regeneration. *Physiological Reviews*, *84*(1). http://physrev.physiology.org/content/84/1/209.long

Chen, S. L., Dowhan, D. H., Hosking, B. M., & Muscat, G. E. (2000). The steroid receptor coactivator, GRIP-1, is necessary for MEF-2C-dependent gene expression and skeletal muscle differentiation. *Genes & Development*, *14*(10), 1209–28. https://doi.org/10.1101/gad.14.10.1209

Chen, S. L., Loffler, K. A., Chen, D., Stallcup, M. R., & Muscat, G. E. O. (2002). The coactivator-associated arginine methyltransferase is necessary for muscle differentiation: CARM1 coactivates myocyte enhancer factor-2. *Journal of Biological Chemistry*, *277*(6), 4324–4333. https://doi.org/10.1074/jbc.M109835200

Cibelli, J., Emborg, M. E., Prockop, D. J., Roberts, M., Schatten, G., Rao, M., … Mirochnitchenko, O. (2013). Strategies for improving animal models for regenerative medicine. *Cell Stem Cell*, *12*(3), 271–4. https://doi.org/10.1016/j.stem.2013.01.004

Collu-Marchese, M., Shuen, M., Pauly, M., Saleem, A., & Hood, D. A. (2015). The regulation of mitochondrial transcription factor A (Tfam) expression during skeletal muscle cell differentiation. *Bioscience Reports*, *35*(3). https://doi.org/10.1042/BSR20150073

Cornall, L., Hryciw, D., Mathai, M., & Mcainch, A. (2012). Generation and Use of Cultured Human Primary Myotubes. Retrieved from http://cdn.intechopen.com/pdfs/25595/InTech-Generation_and_use_of_cultured_human_primary_myotubes.pdf

Dacwag, C. S., Bedford, M. T., Sif, S., & Imbalzano, A. N. (2009). Distinct Protein Arginine Methyltransferases Promote ATP-Dependent Chromatin Remodeling Function at Different Stages of Skeletal Muscle Differentiation. *Molecular and Cellular Biology*, *29*(7), 1909–1921. https://doi.org/10.1128/MCB.00742-08

Dacwag, C. S., Ohkawa, Y., Pal, S., Sif, S., & Imbalzano, A. N. (2007). The protein arginine methyltransferase Prmt5 is required for myogenesis because it facilitates ATP-dependent chromatin remodeling. *Molecular and Cellular Biology*, *27*(1), 384–94. https://doi.org/10.1128/MCB.01528-06

Davis, R. L., Weintraub, H., & Lassar, A. B. (1987). Expression of a single transfected cDNA converts fibroblasts to myoblasts. *Cell*, *51*(6), 987–1000. https://doi.org/10.1016/0092-8674(87)90585-X

Dhar, S., Vemulapalli, V., Patananan, A. N., Huang, G. L., Lorenzo, A. Di, Richard, S., … Bedford, M. T. (2013). Loss of the major Type I arginine methyltransferase PRMT1 causes substrate scavenging by other PRMTs. https://doi.org/10.1038/srep01311

Di Lorenzo, A., & Bedford, M. T. (2011). Histone arginine methylation. *FEBS Letters*, *585*(13), 2024–31. https://doi.org/10.1016/j.febslet.2010.11.010

Dimauro, I., Pearson, T., Caporossi, D., & Jackson, M. J. (2012). A simple protocol for the subcellular fractionation of skeletal muscle cells and tissue. *BMC Res. Notes*, *5*, 513. https://doi.org/10.1186/1756-0500-5-513

Duguez, S., Féasson, L., Denis, C., & Freyssenet, D. (2002). Mitochondrial biogenesis during skeletal muscle regeneration. *American Journal of Physiology - Endocrinology and Metabolism*, *282*(4). http://ajpendo.physiology.org/content/282/4/E802.long

Edmondson, D. G., & Olson, E. N. (1989). A gene with homology to the myc imilarity region of MyoDl is expressed uring myogenesis and is sufficient to activate the muscle differentiation program. http://genesdev.cshlp.org/content/3/5/628.full.pdf

Fortini, P., Ferretti, C., Iorio, E., Cagnin, M., Garribba, L., Pietraforte, D., … Dogliotti, E. (2016). The fine tuning of metabolism, autophagy and differentiation during in vitro myogenesis. *Cell Death and Disease*, *7*(3), e2168. https://doi.org/10.1038/cddis.2016.50

Frankel, A., & Clarke, S. (2000). PRMT3 is a distinct member of the protein arginine N-methyltransferase family. Conferral of substrate specificity by a zinc-finger domain. *The Journal of Biological Chemistry*, *275*(42), 32974–82. https://doi.org/10.1074/jbc.M006445200

Goulet, I., Gauvin, G., Boisvenue, S., & Côté, J. (2007). Alternative splicing yields protein arginine methyltransferase 1 isoforms with distinct activity, substrate specificity, and subcellular localization. *The Journal of Biological Chemistry*, *282*(45), 33009–21. https://doi.org/10.1074/jbc.M704349200

Grounds, M. D., White, J. D., Rosenthal, N., & Bogoyevitch, M. A. (2002). The Role of Stem Cells in Skeletal and Cardiac Muscle Repair. *Journal of Histochemistry & Cytochemistry*, *50*(5), 589–610. https://doi.org/10.1177/002215540205000501

Handschin, C., Rhee, J., Lin, J., Tarr, P. T., & Spiegelman, B. M. (2003). An autoregulatory loop controls peroxisome proliferator-activated receptor gamma coactivator 1alpha expression in muscle. *Proceedings of the National Academy of Sciences of the United States of America*, *100*(12), 7111–6. https://doi.org/10.1073/pnas.1232352100

Hasty, P., Bradley, A., Morris, J. H., Edmondson, D. G., Venuti, J. M., Olson, E. N., & Klein, W. H. (1993). Muscle deficiency and neonatal death in mice with a targeted mutation in the myogenin gene. *Nature*, *364*(6437), 501–506. https://doi.org/10.1038/364501a0

Hawke, T. J., & Garry, D. J. (2001). Myogenic satellite cells: physiology to molecular biology. *Journal of Applied Physiology*, *91*(2). http://jap.physiology.org/content/91/2/534.long

Herzberg, N. H., Zwart, R., Wolterman, R. A., Ruiter, J. P. N., Wanders, R. J. A., Bolhuis, P. A., & van den Bogert, C. (1993). Differentiation and proliferation of respiration-deficient human myoblasts. *Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease*, *1181*(1), 63–67. https://doi.org/10.1016/0925-4439(93)90091-E

Hinterberger, T. J., Sassoon, D. A., Rhodes, S. J., & Konieczny, S. F. (1991). Expression of the muscle regulatory factor MRF4 during somite and skeletal myofiber development. *Developmental Biology*, *147*(1), 144–156. https://doi.org/10.1016/S0012-1606(05)80014-4

Hood, D. A., Tryon, L. D., Carter, H. N., Kim, Y., & Chen, C. C. W. (2016). Unravelling the mechanisms regulating muscle mitochondrial biogenesis. *Biochemical Journal*, *473*(15). http://www.biochemj.org/content/473/15/2295.long#sec-4

Iwasaki, H., Kovacic, J. C., Olive, M., Beers, J. K., Yoshimoto, T., Crook, M. F., … Nabel, E. G. (2010). Disruption of protein arginine N-methyltransferase 2 regulates leptin signaling and produces leanness in vivo through loss of STAT3 methylation. *Circulation Research*, *107*(8), 992–1001. https://doi.org/10.1161/CIRCRESAHA.110.225326

Iwasaki, H., & Yada, T. (2007). Protein arginine methylation regulates insulin signaling in L6 skeletal muscle cells. *Biochemical and Biophysical Research Communications*, *364*(4), 1015–1021. https://doi.org/10.1016/j.bbrc.2007.10.113

Jahnke, V. E., Sabido, O., & Freyssenet, D. (2009). Control of mitochondrial biogenesis, ROS level, and cytosolic Ca2+ concentration during the cell cycle and the onset of differentiation in L6E9 myoblasts. *AJP - Cell Physiology*, *296*(5), C1185--94. https://doi.org/10.1152/ajpcell.00377.2008

Jeong, H.-J., Lee, H.-J., Vuong, T. A., Choi, K.-S., Choi, D., Koo, S.-H., … Moraes, C. (2016). Prmt7 Deficiency Causes Reduced Skeletal Muscle Oxidative Metabolism and Age-Related Obesity. *Diabetes*, *65*(7), 1868–82. https://doi.org/10.2337/db15-1500

Jornayvaz, F. R., & Shulman, G. I. (2010). Regulation of mitochondrial biogenesis. *Essays in Biochemistry*, *47*, 69–84. https://doi.org/10.1042/bse0470069

Karalaki, M., Fili, S., Philippou, A., & Koutsilieris, M. (2009). Muscle regeneration: cellular and molecular events. *In Vivo (Athens, Greece)*, *23*(5), 779–96. https://doi.org/23/5/779 [pii]

Kassar-Duchossoy, L., Gayraud-Morel, B., Gomès, D., Rocancourt, D., Buckingham, M., Shinin, V., & Tajbakhsh, S. (2004). Mrf4 determines skeletal muscle identity in Myf5:Myod double-mutant mice. *Nature*,

431(7007), 466–471. https://doi.org/10.1038/nature02876

Kawabe, Y. I., Wang, Y. X., McKinnell, I. W., Bedford, M. T., & Rudnicki, M. A. (2012a). Carm1 regulates Pax7 transcriptional activity through MLL1/2 recruitment during asymmetric satellite stem cell divisions. *Cell Stem Cell*, *11*(3), 333–345. https://doi.org/10.1016/j.stem.2012.07.001

Kawabe, Y. I., Wang, Y. X., McKinnell, I. W., Bedford, M. T., & Rudnicki, M. A. (2012b). Carm1 regulates Pax7 transcriptional activity through MLL1/2 recruitment during asymmetric satellite stem cell divisions. *Cell Stem Cell*, *11*(3), 333–345. https://doi.org/10.1016/j.stem.2012.07.001

Kelly, D. P., & Scarpulla, R. C. (2004). Transcriptional regulatory circuits controlling mitochondrial biogenesis and function. *Genes and Development*. https://doi.org/10.1101/gad.1177604

Kim, S. J., Yoo, B. C., Uhm, C. S., & Lee, S. W. (2011). Posttranslational arginine methylation of lamin A/C during myoblast fusion. *Biochimica et Biophysica Acta - Proteins and Proteomics*, *1814*(2), 308–317. https://doi.org/10.1016/j.bbapap.2010.11.006

Lacroix, M., El Messaoudi, S., Rodier, G., Le Cam, A., Sardet, C., & Fabbrizio, E. (2008). The histone-binding protein COPR5 is required for nuclear functions of the protein arginine methyltransferase PRMT5. *EMBO Reports*, *9*(5), 452–8. https://doi.org/10.1038/embor.2008.45

Larsen, S. C., Sylvestersen, K. B., Mund, A., Lyon, D., Mullari, M., Madsen, M. V., … Nielsen, M. L. (2016). Proteome-wide analysis of arginine monomethylation reveals widespread occurrence in human cells. *Science Signaling*, *9*(443). http://stke.sciencemag.org/content/9/443/rs9.long

Larsson, N.-G., Wang, J., Wilhelmsson, H., Oldfors, A., Rustin, P., Lewandoski, M., … Clayton, D. A. (1998). Mitochondrial transcription factor A is necessary for mtDNA maintance and embryogenesis in mice. *Nature Genetics*, *18*(3), 231–236. https://doi.org/10.1038/ng0398-231

Le Grand, F., Grifone, R., Mourikis, P., Houbron, C., Gigaud, C., Pujol, J., … Maire, P. (2012). Six1 regulates stem cell repair potential and self-renewal during skeletal muscle regeneration. *The Journal of Cell Biology*, *198*(5). http://jcb.rupress.org/content/198/5/815?ijkey=197a484fa738a98000c477a3a5451d1e0288ee9f&keytype2=tf_ipsecsha

Ljubicic, V., Khogali, S., Renaud, J.-M., & Jasmin, B. J. (2012). Chronic AMPK stimulation attenuates adaptive signaling in dystrophic skeletal muscle. *AJP: Cell Physiology*, *302*(1), C110–C121. https://doi.org/10.1152/ajpcell.00183.2011

Mallappa, C., Hu, Y. J., Shamulailatpam, P., Tae, S., Sif, S., & Imbalzano, A. N. (2011). The expression of myogenic microRNAs indirectly requires protein arginine methyltransferase (Prmt)5 but directly requires Prmt4. *Nucleic Acids Research*, *39*(4), 1243–1255. https://doi.org/10.1093/nar/gkq896

Meyer, C., Dostou, J. M., Welle, S. L., & Gerich, J. E. (2002). Role of human liver, kidney, and skeletal muscle in postprandial glucose homeostasis. *American Journal of Physiology. Endocrinology and Metabolism*, *282*(2), E419–E427. https://doi.org/10.1152/ajpendo.00032.2001

Neuenkirchen, N., Chari, A., & Fischer, U. (2008). Deciphering the assembly pathway of Sm-class U snRNPs. *FEBS Letters*, *582*(14), 1997–2003. https://doi.org/10.1016/j.febslet.2008.03.009

Ott, M. O., Bober, E., Lyons, G., Arnold, H., & Buckingham, M. (1991). Early expression of the myogenic regulatory gene, myf-5, in precursor cells of

skeletal muscle in the mouse embryo. *Development*, *111*(4). Retrieved from http://dev.biologists.org/content/111/4/1097.long

Paul, C., Sardet, C., & Fabbrizio, E. (2012). The histone- and PRMT5-associated protein COPR5 is required for myogenic differentiation. *Cell Death and Differentiation*, *19*(5), 900–908. https://doi.org/10.1038/cdd.2011.193

Pawlak, M. R., Scherer, C. A., Chen, J., Roshon, M. J., & Ruley, H. E. (2000). Arginine N-methyltransferase 1 Is Required for Early Postimplantation Mouse Development, but Cells Deficient in the Enzyme Are Viable. *Molecular and Cellular Biology*, *20*(13), 4859–4869. https://doi.org/10.1128/MCB.20.13.4859-4869.2000

Remels, A. H. V., Langen, R. C. J., Schrauwen, P., Schaart, G., Schols, A. M. W. J., & Gosker, H. R. (2010). Regulation of mitochondrial biogenesis during myogenesis. *Molecular and Cellular Endocrinology*, *315*(1), 113–120. https://doi.org/10.1016/j.mce.2009.09.029

Reporter, M., & Corbin, J. L. (1971). NG, NG-Dimethylarginine in myosin during muscle development. *Biochemical and Biophysical Research Communications*, *43*(3), 644–650. https://doi.org/10.1016/0006-

291X(71)90663-2

Rhein, V. F., Carroll, J., Ding, S., Fearnley, I. M., & Walker, J. E. (2013). NDUFAF7 Methylates Arginine 85 in the NDUFS2 Subunit of Human Complex I *. https://doi.org/10.1074/jbc.M113.518803

Rochard, P., Rodier, A., Casas, F., Cassar-Malek, I., Marchal-Victorion, S., Daury, L., … Cabello, G. (2000). Mitochondrial activity is involved in the regulation of myoblast differentiation through myogenin expression and activity of myogenic factors. *Journal of Biological Chemistry*, *275*(4), 2733–2744. https://doi.org/10.1074/jbc.275.4.2733

Rutter, J., Winge, D. R., & Schiffman, J. D. (2010). Succinate dehydrogenase - Assembly, regulation and role in human disease. *Mitochondrion*, *10*(4), 393–401. https://doi.org/10.1016/j.mito.2010.03.001

Saleem, A., Carter, H. N., & Hood, D. A. (2014). p53 is necessary for the adaptive changes in the cellular milieu subsequent to an acute bout of endurance exercise. *American Journal of Physiology. Cell Physiology*, *306*(3), 241–249. https://doi.org/10.1152/ajpcell.00270.2013

Sassoon, D., Lyons, G., Wright, W. E., Lin, V., Lassar, A., Weintraub, H., & Buckingham, M. (1989). Expression of two myogenic regulatory factors myogenin and MyoDl during mouse embryogenesis. *Nature*, *341*(6240), 303–307. https://doi.org/10.1038/341303a0

Scarpulla, R. C. (2008). Transcriptional Paradigms in Mammalian Mitochondrial Biogenesis and Function. *Physiological Reviews*, *88*(2). Retrieved from http://physrev.physiology.org/content/88/2/611

Seyer, P., Grandemange, S., Busson, M., Carazo, A., Gamaléri, F., Pessemesse, L., … Wrutniak-Cabello, C. (2006). Mitochondrial activity regulates myoblast differentiation by control of c-Myc expression. *Journal of Cellular Physiology*, *207*(1), 75–86. https://doi.org/10.1002/jcp.20539

Sha, L., Daitoku, H., Araoi, S., Kaneko, Y., Takahashi, Y., Kako, K., & Fukamizu, A. (2017). Asymmetric Arginine Dimethylation Modulates Mitochondrial Energy Metabolism and Homeostasis in Caenorhabditis elegans 2 3. *Mol. Cell. Biol*. https://doi.org/10.1128/MCB.00504-16

Shiozu, H., Higashijima, M., & Koga, T. (2015). Association of sarcopenia with swallowing problems, related to nutrition and activities of daily living of elderly individuals. *Journal of Physical Therapy Science*, *27*(2), 393–6.

https://doi.org/10.1589/jpts.27.393

Sin, J., Andres, A. M., Taylo R, D. J. R., Weston, T., Hiraumi, Y., Stotland, A., … Gottlieb, R. A. (2016). Mitophagy is required for mitochondrial biogenesis and myogenic differentiation of C2C12 myoblasts. *Autophagy*, *12*(2), 369–380. https://doi.org/10.1080/15548627.2015.1115172

Swiercz, R., Cheng, D., Kim, D., & Bedford, M. T. (2007). Ribosomal protein rpS2 is hypomethylated in PRMT3-deficient mice. *The Journal of Biological Chemistry*, *282*(23), 16917–23. https://doi.org/10.1074/jbc.M609778200

Tang, J., Frankel, A., Cook, R. J., Kim, S., Paik, W. K., Williams, K. R., … Herschman, H. R. (2000). PRMT1 is the predominant type I protein arginine methyltransferase in mammalian cells. *The Journal of Biological Chemistry*, *275*(11), 7723–30. https://doi.org/10.1074/JBC.275.11.7723

Tee, W.-W., Pardo, M., Theunissen, T. W., Yu, L., Choudhary, J. S., Hajkova, P., & Surani, M. A. (2010). Prmt5 is essential for early mouse development and acts in the cytoplasm to maintain ES cell pluripotency. *Genes & Development*, *24*(24), 2772–7. https://doi.org/10.1101/gad.606110

Teyssier, C., Ma, H., Emter, R., Kralli, A., & Stallcup, M. R. (2005). Activation of nuclear receptor coactivator PGC-1alpha by arginine methylation. *Genes & Development*, *19*(12), 1466–73. https://doi.org/10.1101/gad.1295005

Vergara, M. N., & Canto-Soler, M. V. (2012). Rediscovering the chick embryo as a model to study retinal development. Retrieved from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3541172/pdf/1749-8104-7-22.pdf

Wagatsuma, A., Kotake, N., & Yamada, S. (2011). Muscle regeneration occurs to coincide with mitochondrial biogenesis. *Molecular and Cellular Biochemistry*, *349*(1–2), 139–147. https://doi.org/10.1007/s11010-010-0668

Wagatsuma, A., & Sakuma, K. (2013). Mitochondria as a potential regulator of myogenesis. *The Scientific World Journal*. https://doi.org/10.1155/2013/593267

Wang, J., & Conboy, I. (2010). Embryonic vs. Adult Myogenesis: Challenging the "Regeneration Recapitulates Development" Paradigm. *Journal of Molecular Cell Biology*, *2*(1), 1–4. https://doi.org/10.1093/jmcb/mjp027

Wang, S.-C. M., & Muscat, G. E. O. (2013). Nuclear receptors and epigenetic signaling: novel regulators of glycogen metabolism in skeletal muscle. *IUBMB Life*, *65*(8), 657–664. https://doi.org/10.1002/iub.1181

Wang, S. M., Dowhan, D. H., Eriksson, N. A., & Muscat, G. E. O. (2012). CARM1/PRMT4 is necessary for the glycogen gene expression programme in skeletal muscle cells. *Biochemical Journal*, *444*(2), 323–331. https://doi.org/10.1042/BJ20112033

Wang, Y. P., Zhou, W., Wang, J., Huang, X., Zuo, Y., Wang, T. S., … Lei, Q. Y. (2016). Arginine Methylation of MDH1 by CARM1 Inhibits Glutamine Metabolism and Suppresses Pancreatic Cancer. *Molecular Cell*, *64*(4), 673–687. https://doi.org/10.1016/j.molcel.2016.09.028

Wei, H., Mundade, R., Lange, K. C., & Lu, T. (2014). Protein arginine methylation of non-histone proteins and its role in diseases. *Cell Cycle (Georgetown, Tex.)*, *13*(1), 32–41. https://doi.org/10.4161/cc.27353

Wu, Z., Puigserver, P., Andersson, U., Zhang, C., Adelmant, G., Mootha, V., … Spiegelman, B. M. (1999). Mechanisms Controlling Mitochondrial Biogenesis and Respiration through the Thermogenic Coactivator PGC-1.

Cell, *98*(1), 115–124. https://doi.org/10.1016/S0092-8674(00)80611-X

Yadav, N., Lee, J., Kim, J., Shen, J., Hu, M. C.-T., Aldaz, C. M., & Bedford, M. T. (2003). Specific protein methylation defects and gene expression perturbations in coactivator-associated arginine methyltransferase 1-deficient mice. *Proceedings of the National Academy of Sciences of the United States of America*, *100*(11), 6464–8. https://doi.org/10.1073/pnas.1232272100

Yang, Y., & Bedford, M. T. (2012). Protein arginine methyltransferases and cancer. *Nature Reviews Cancer*. https://doi.org/10.1038/nrc3409

Yang, Y., Hadjikyriacou, A., Xia, Z., Gayatri, S., Kim, D., Zurita-Lopez, C., … Bedford, M. T. (2015). ARTICLE PRMT9 is a Type II methyltransferase that methylates the splicing factor SAP145. https://doi.org/10.1038/ncomms7428

Yen, J., White, R. M., & Stemple, D. L. (2014). Zebrafish models of cancer: progress and future challenges. *Current Opinion in Genetics & Development*, *24*(100), 38–45. https://doi.org/10.1016/j.gde.2013.11.003

Yildirim, A. O., Bulau, P., Zakrzewicz, D., Kitowska, K. E., Weissmann, N.,

Grimminger, F., … Eickelberg, O. (2006). Increased protein arginine methylation in chronic hypoxia: Role of protein arginine methyltransferases. *American Journal of Respiratory Cell and Molecular Biology*, *35*(4), 436–443. https://doi.org/10.1165/rcmb.2006-0097OC

Yin, H., Price, F., & Rudnicki, M. a. (2013). Satellite cells and the muscle stem cell niche. *Physiological Reviews*, *93*(1), 23–67. https://doi.org/10.1152/physrev.00043.2011

Zhang, T., Günther, S., Looso, M., Künne, C., Krüger, M., Kim, J., … Braun, T. (2015). Prmt5 is a regulator of muscle stem cell expansion in adult mice. *Nature Communications*, *6*, 7140. https://doi.org/10.1038/ncomms8140

Figure Legends

Figure 1. Timecourse of skeletal muscle differentiation. A) Light microscope images (left column panels) of C_2C_{12} myoblasts (MB), and day 1 (D1), 3 (D3), 5 (D5), and 7 (D7) myotubes (MT). Immunofluorescence images (right column panels) of muscles cells throughout the differentiation timecourse stained with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI; blue) and embryonic myosin heavy chain (eMHC; red). Scale bar = 200 μm. B) Representative immunoblot (above) depicting myogenin levels throughout the experimental timecourse. Ponceau S stain (below) indicates equal loading between samples. C) Graphical summary of myogenin mRNA (gray line) and protein (black line) expression levels across the timecourse of myogenesis. Data are displayed as relative to MB levels. $n = 5$; *, $p < 0.05$ vs. MB mRNA content; *, $p < 0.05$ vs. MB protein content.

Figure 2. **Protein arginine methyltransferase gene expression during myogenesis.** A) Protein arginine methyltransferase 1 (PRMT1), PRMT4, and PRMT5 mRNA expression in MB, and D1, D3, D5, D7 myotubes displayed relative MB levels. B) Typical PRMT1, PRMT4, and PRMT5 immunoblots and amido black loading control image. C) Graphical summary of PRMT protein content throughout C_2C_{12} myogenesis. $n = 3$-4; *, $p < 0.05$ vs. PRMT1 levels in MB.

Figure 3. Global PRMT activity throughout myogenesis. Representative A) monomethylarginine (MMA), asymmetric dimethylarginine (ADMA), and symmetric dimethylarginine (SDMA) immunoblots at MB, and D1, D3, D5, and D7 stages of differentiation. A typical amido black stain is also shown to indicate consistent loading between samples. B) Graphical summary of MMA, ADMA, and SDMA species, expressed relative to MB levels. n = 5-7; *, $p < 0.05$ vs. MB ADMA content.

Figure 4. Specific PRMT methyltransferase activity during skeletal muscle differentiation. A) Representative PRMT1, PRMT4, PRMT5, histone 2B (H2B), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) immunoblots, as well as an amido black image of myonuclear lysates isolated from MB, D1, D3, D5, and D7 C_2C_{12} cells. B) Graphical depiction of myonuclear PRMT protein levels throughout myogenesis, expressed relative to the MB stage. *, $p < 0.05$ vs. MB PRMT1 content; *, $p < 0.05$ vs. MB PRMT4 content. C) Typical immunoblots of histone 4 arginine 3 (H4R3), H3R17, H3R8, total H4 and H3 protein content from MB, as well as D1, D3, D5, and D7 myotubes. A representative amido black stain is presented below. D) Graphical summary of histone arginine methylation status throughout myogenesis, depicted as the methylated form of the histone relative to the total histone amount, expressed relative to MB levels. *, $p < 0.05$ vs. MB H4R3 content; *, $p < 0.05$ vs. MB H3R17 content. n = 4-7.

Figure 5. PRMT1 inhibition during myogenesis. A) Representative H4R3 and H4 immunoblots, as well as amido black stain at C_2C_{12} differentiation days 1, 3, 5, and 7 in the vehicle (VEH) and TC-E 5003 (TC-E) treatment conditions. B) Graphical summary of H4R3 methylation status in the VEH and TC-E conditions expressed relative to levels in VEH D1. n = 5; *, $p < 0.05$ vs. VEH at the same timepoint; #, $p < 0.05$ main effect of time in the VEH.

Figure 6. Effect of PRMT1 inhibition on the progression of myogenic differentiation. A) Light microscopy images of C_2C_{12} muscle cells at D1, D3, D5, and D7 of differentiation in the VEH (left column) or TCE condition (right column). B) Immunofluorescence images stained with eMHC (red) and DAPI (blue) of VEH- (left column) and TCE-treated cells (right column). Scale bar = 200 μm. Graphical summaries of C) myotube fusion index, D) myotube length, E) width, and F) myotube surface area of VEH- and TCE-treated cells across the experimental timecourse. n = 3-5; #, $p < 0.05$ main effect of time in the VEH and TC-E conditions; ¶, $p < 0.05$ main effect of treatment; *, $p < 0.05$ vs. VEH at the same timepoint.

Figure 7. Effect of PRMT1 inhibition on mitochondrial biogenesis during muscle development. A) Representative immunoblots of mitochondrial complex I (CI), CII, CIII, CV, peroxisome proliferator-activated receptor γ coactivator 1-α (PGC-1α), as well as amido black stain, in VEH- and TC-E-treated cells across the differentiation timecourse. Graphical summaries of B) CI, C) CII, D) CIII, E) CV, and F) PGC-1α protein content expressed relative to the levels in VEH D1 cells. *n* = 4; #, $p < 0.05$ main effect of time in the VEH and TC-E conditions; ¶, $p < 0.05$ main effect of treatment; *, $p < 0.05$ vs. VEH at the same timepoint.

Figure 8. Effect of PRMT1 inhibition on mitochondrial respiration. Graphical summaries of rates of mitochondrial oxygen consumption in A) D1, B) D3, C) D5, and D) D7 cells with the substrates malate and glutamate (Mal + Glut), ADP, cytochrome c (Cyto c), pyruvate (Pyr), and succinate (Succ) in VEH and TC-E treatment conditions. E) CI and F) CI + CII oxygen consumption values across the differentiation timecourse in the two experimental conditions. *n* = 3-5; #, $p < 0.05$ main effect of time in the VEH and TC-E conditions; ¶, $p < 0.05$ main effect of treatment; *, $p < 0.05$ vs. VEH-treated cells.

Figure 1

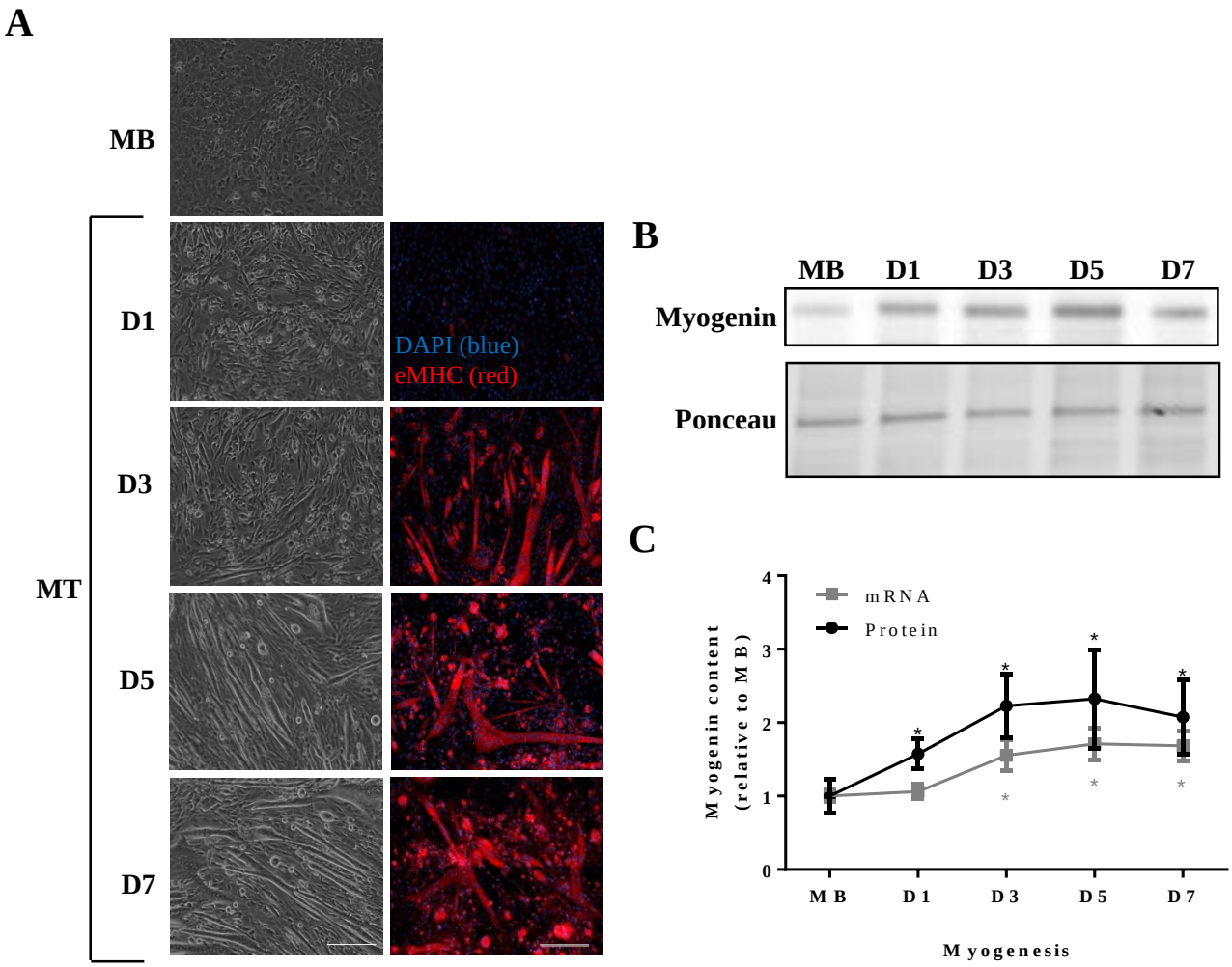

A
MB
MT
D1
D3
D5
D7
DAPI (blue)
eMHC (red)
B
MB
D1
D3
D5
D7
Myogenin
Ponceau
C
mRNA
Protein
Myogenin content (relative to MB)
0
1
2
3
4
MB
D1
D3
D5
D7
Myogenesis

Figure 2

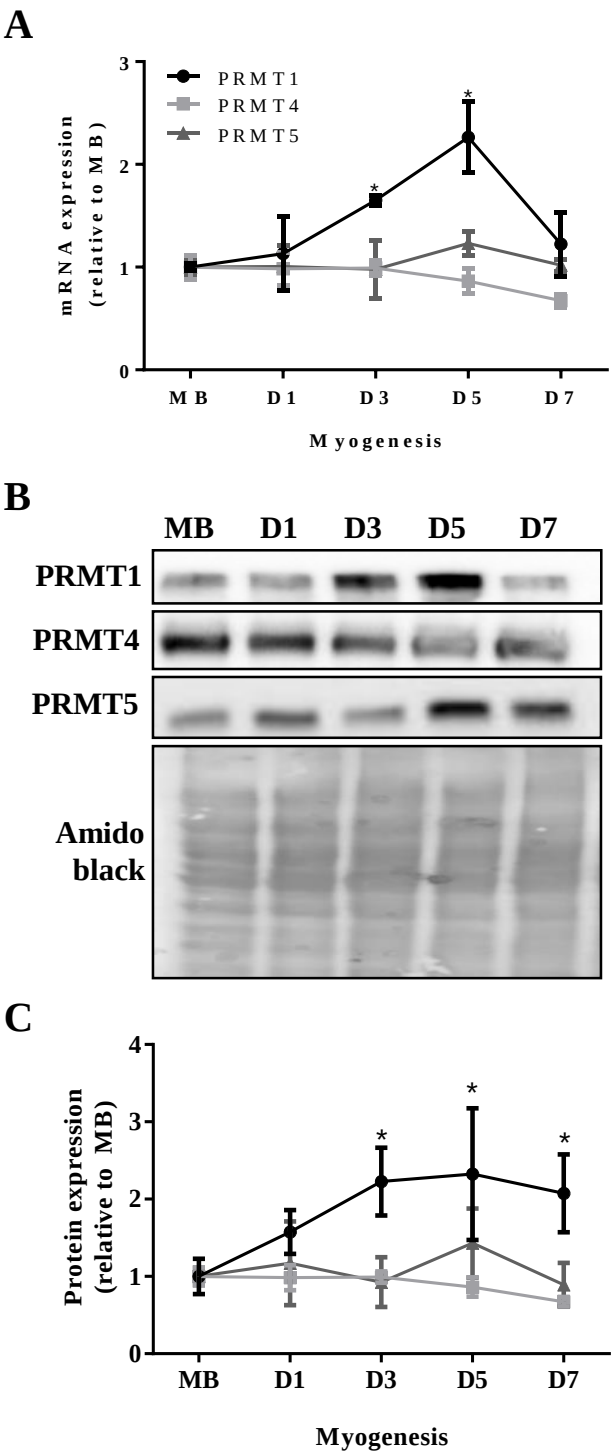

A
PRMT1
PRMT4
PRMT5
mRNA expression (relative to MB)
3
2
1
0
MB
D1
D3
D5
D7
Myogenesis
B
MB
D1
D3
D5
D7
PRMT1
PRMT4
PRMT5
Amido black
C
Protein expression (relative to MB)
4
3
2
1
0
MB
D1
D3
D5
D7
Myogenesis

Figure 3

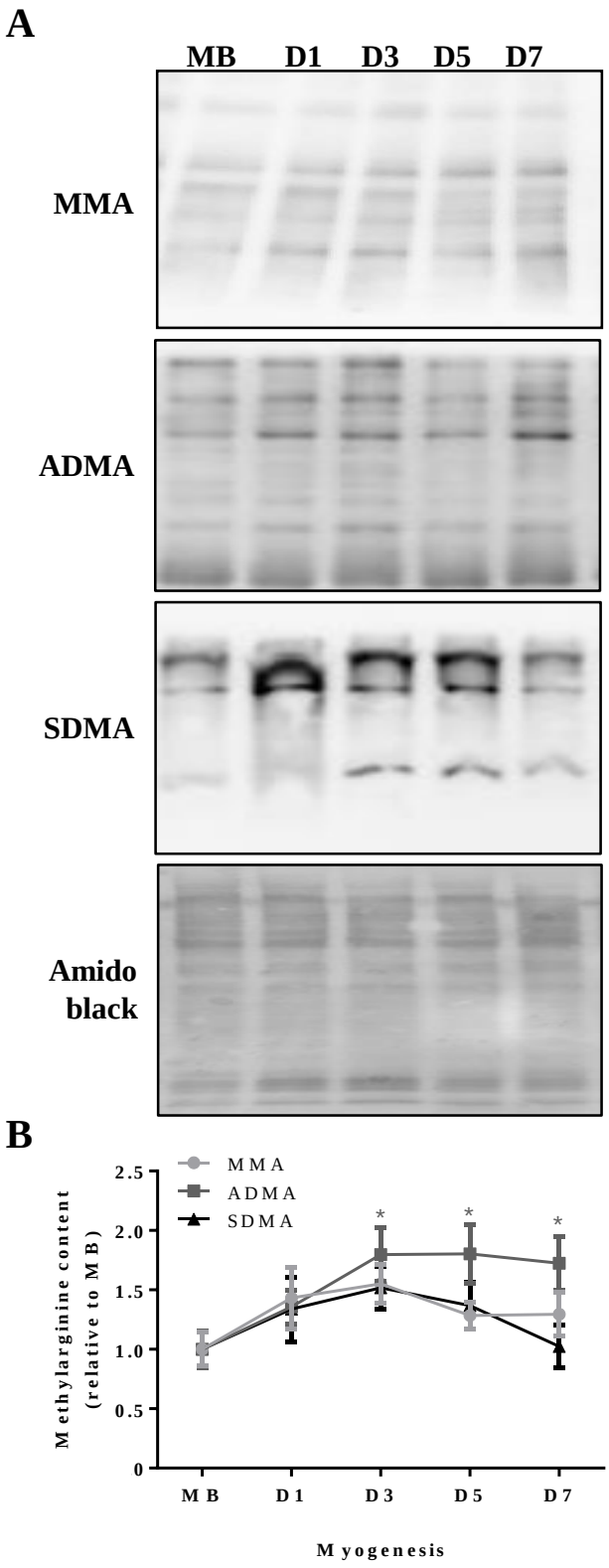

A
MB
D1
D3
D5
D7
MMA
ADMA
SDMA
Amido black
B
MMA
ADMA
SDMA
Methylarginine content (relative to MB)
2.5
2.0
1.5
1.0
0.5
0
*
*
*
MB
D1
D3
D5
D7
Myogenesis

Figure 4

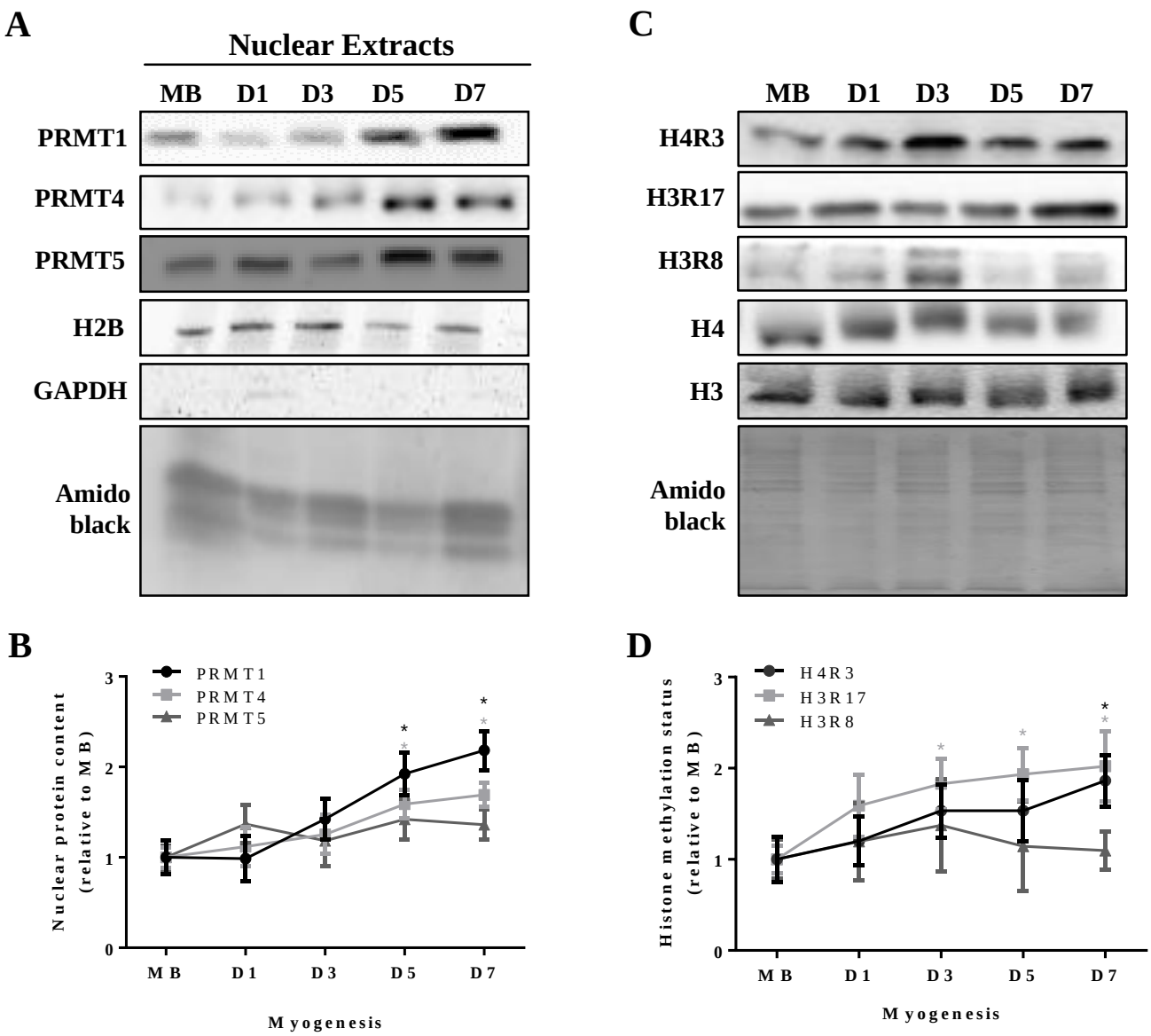

A
Nuclear Extracts
MB D1 D3 D5 D7
PRMT1
PRMT4
PRMT5
H2B
GAPDH
Amido black
C
MB D1 D3 D5 D7
H4R3
H3R17
H3R8
H4
H3
Amido black
B
PRMT1
PRMT4
PRMT5
Nuclear protein content (relative to MB)
Myogenesis
D
H4R3
H3R17
H3R8
Histone methylation status (relative to MB)
Myogenesis

Figure 5

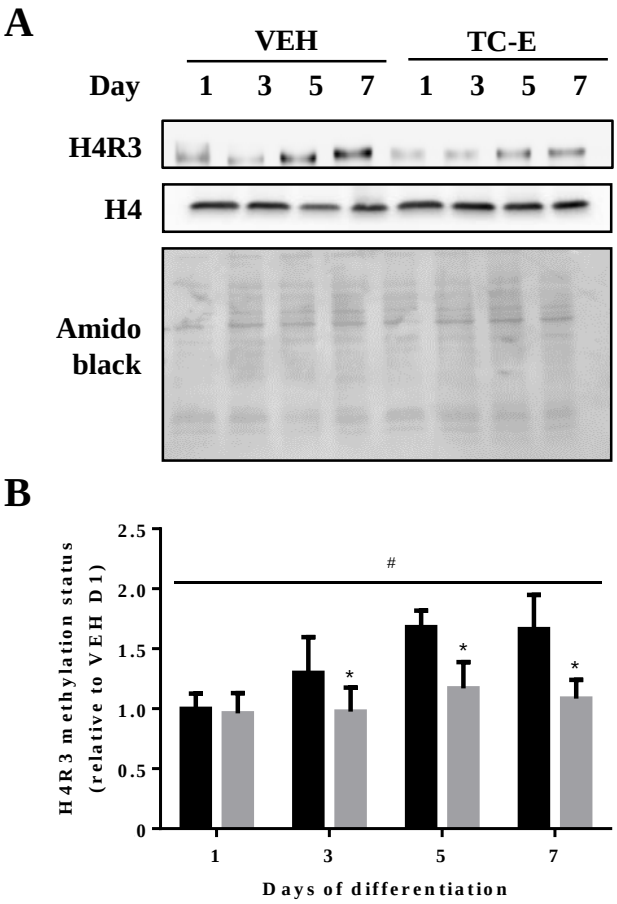

A
VEH
TC-E
Day
1 3 5 7 1 3 5 7
H4R3
H4
Amido black
B
H4R3 methylation status (relative to VEH D1)
2.5
2.0
1.5
1.0
0.5
0
#
*
*
*
1
3
5
7
Days of differentiation

Figure 6

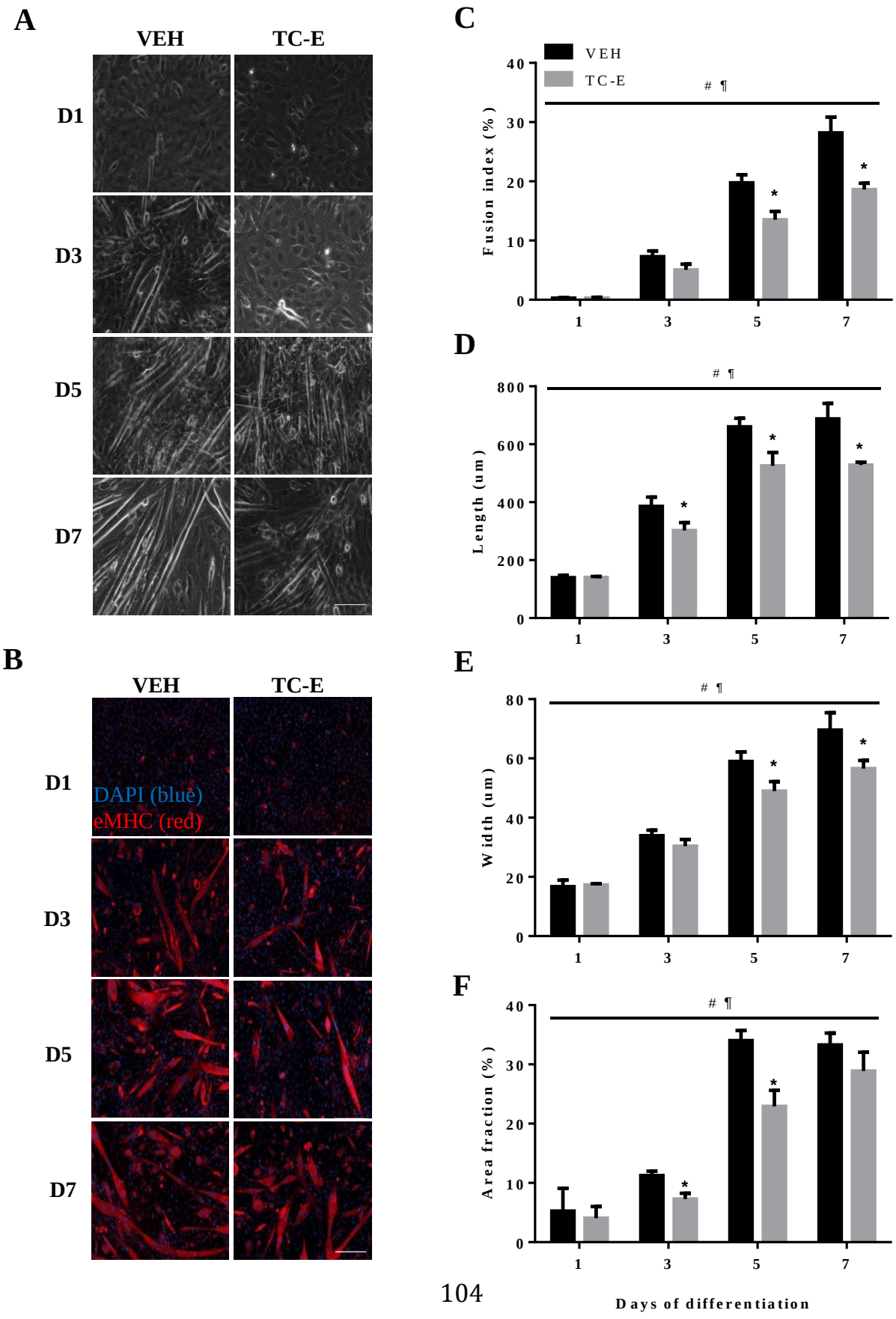

A
VEH
TC-E
D1
D3
D5
D7
B
VEH
TC-E
D1
DAPI (blue)
eMHC (red)
D3
D5
D7
C
VEH
TC-E
¶
Fusion index (%)
0
10
20
30
40
1
3
5
7
*
D
¶
Length (um)
0
200
400
600
800
1
3
5
7
*
E
¶
Width (um)
0
20
40
60
80
1
3
5
7
*
F
¶
Area fraction (%)
0
10
20
30
40
1
3
5
7
*
Days of differentiation

Figure 7

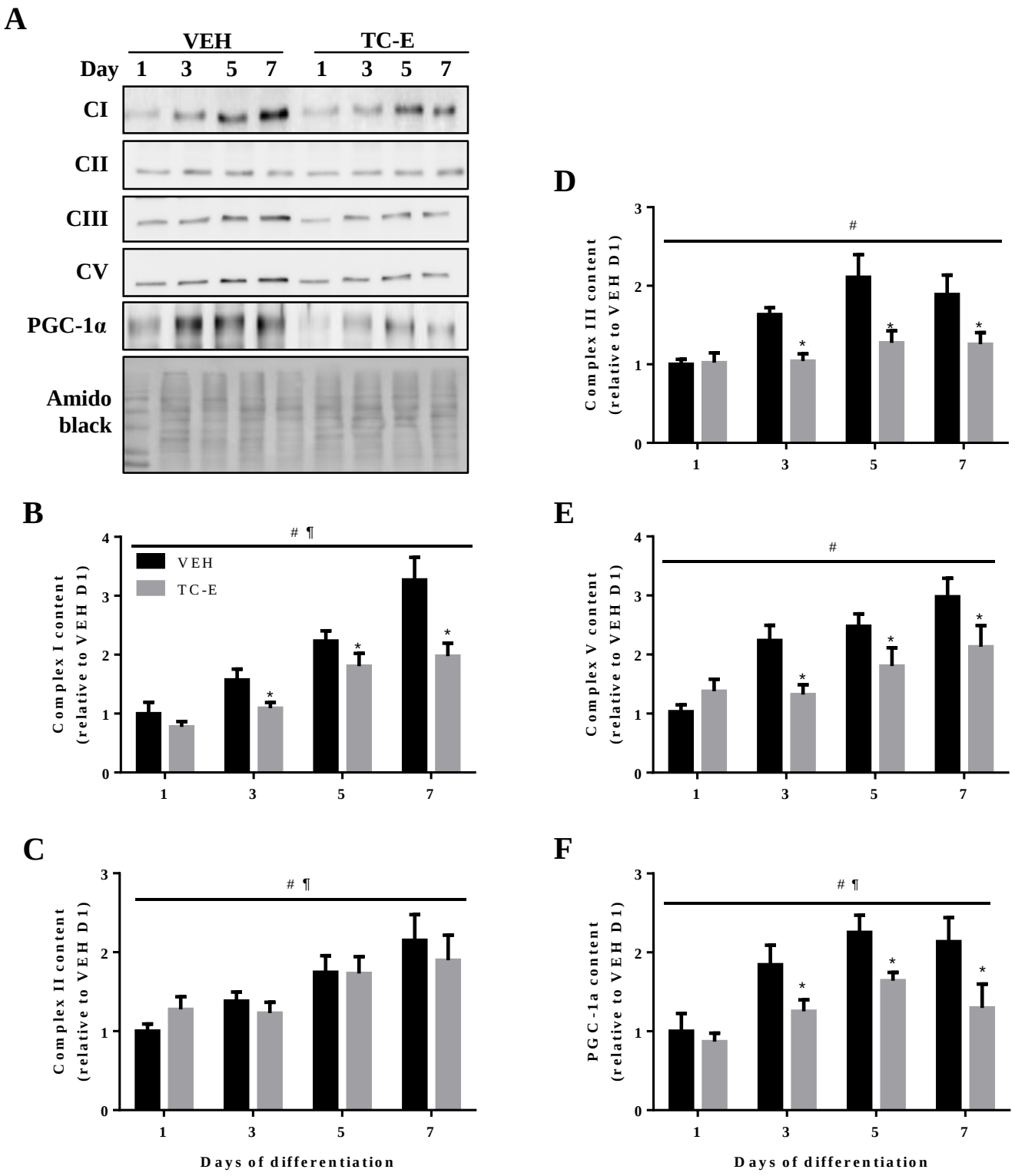
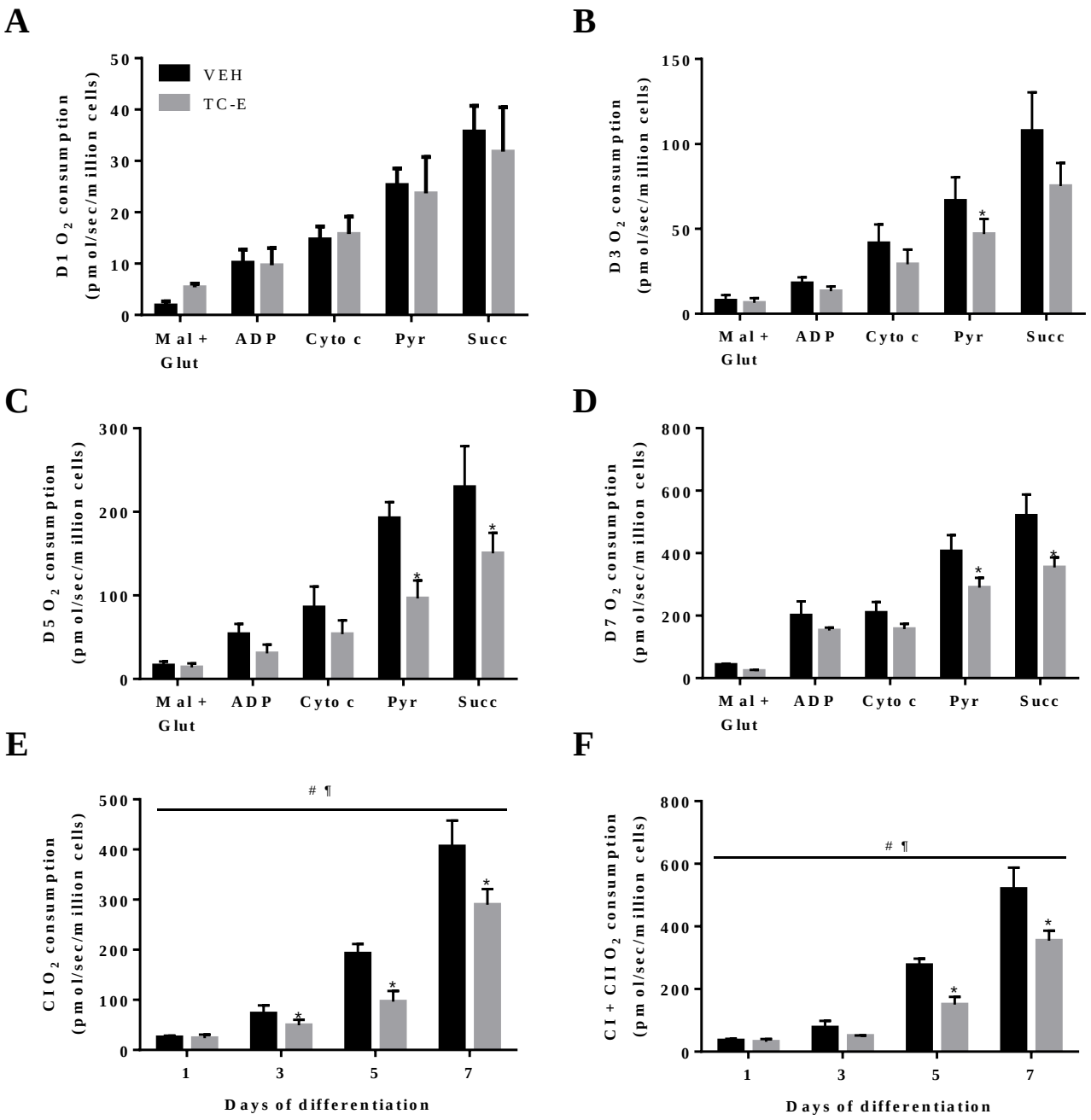

A
VEH
TC-E
Day 1 3 5 7 1 3 5 7
CI
CII
CIII
CV
PGC-1α
Amido black
B
Complex I content (relative to VEH D1)
VEH
TC-E
C
Complex II content (relative to VEH D1)
Days of differentiation
D
Complex III content (relative to VEH D1)
E
Complex V content (relative to VEH D1)
F
PGC-1a content (relative to VEH D1)
Days of differentiation

Figure 8


A
B
C
D
E
F
VEH
TC-E
D1 O2 consumption (pmol/sec/million cells)
D3 O2 consumption (pmol/sec/million cells)
D5 O2 consumption (pmol/sec/million cells)
D7 O2 consumption (pmol/sec/million cells)
CI O2 consumption (pmol/sec/million cells)
CI + CII O2 consumption (pmol/sec/million cells)
Mal + Glut
ADP
Cyto c
Pyr
Succ
Days of differentiation
¶
*