Characterization of the Epigenetic Signature Underlying Early Myogenic Differentiation

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Thesis submitted to the
Faculty of Graduate and Postdoctoral Studies in partial fulfillment of the
requirements for the Doctorate in Philosophy degree in Cellular and Molecular Medicine

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Authorization

Figures (6-13) listed in chapter 3 are from my published manuscripts in open access journals.

Figures (7-9) are published in *Epigenetics*.

Khilji, S*., **Hamed, M***., Chen, J., and Li, Q. (2018). Loci-specific histone acetylation profiles associated with transcriptional coactivator p300 during early myoblast differentiation. *Epigenetics*, *13*(6), 642–654. https://doi.org/10.1080/15592294.2018.1489659

Figures (10-13) are published in *Nucleic Acids Research*.

Hamed, M., Khilji, S., Dixon, K., Blais, A., Ioshikhes, I., Chen, J., and Li, Q. (2017). Insights into interplay between rexinoid signaling and myogenic regulatory factor-associated chromatin state in myogenic differentiation. *Nucleic Acids Research*, *45*(19), 11236–11248. https://doi.org/10.1093/nar/gkx800

*Co-first author

Figures (6) is published in *Scientific Reports*.

Chen, J., Wang, Y., **Hamed, M**., Lacroix, N., & Li, Q. (2015). Molecular Basis for the Regulation of Transcriptional Coactivator p300 in Myogenic Differentiation. *Scientific Reports*, 5(1):13727. https://doi.org/10.1038/srep13727

Abstract

Although skeletal myogenesis is largely controlled by myogenic regulatory factors, epigenetic modifications have recently emerged as an essential regulatory mechanism of gene expression. Molecular regulation of stem cell differentiation is exerted through both genetic and epigenetic factors over distal enhancer regions. Understanding the mechanistic action of active or poised enhancers is therefore, imperative for the control of stem cell differentiation. Based on the genome-wide co-occurrence of different epigenetic marks in proliferating myoblasts, we have generated a chromatin state model to profile differentiation- and rexinoid-responsive histone acetylation in early myoblast differentiation. Here, we delineate the functional mode of transcription regulators during early myogenic differentiation using genome-wide chromatin state association. We define a role of transcriptional coactivator p300, when recruited by muscle master regulator MyoD, in the establishment and regulation of myogenic loci at the onset of myoblast differentiation. In addition, we reveal an enrichment of loci-specific histone acetylation at p300 associated active or poised enhancers, mainly when enlisted by MyoD. We have previously established that bexarotene, a clinically approved agonist of retinoid X receptor (RXR), promotes the specification and differentiation of skeletal muscle lineage. Hence, we investigated the genome-wide impact of rexinoids on myogenic differentiation and uncovered a new mechanism of rexinoid action, which is mediated by the nuclear receptor and largely reconciled through direct regulation of MyoD gene expression. In addition, we determined rexinoid-responsive residue-specific histone acetylation at a distinct chromatin state associated with MyoD and myogenin.

Finally, through ChIP-seq and RNA-seq analyses, we have identified dystroglycan (Dag1) as a differentiation-dependent and a rexinoid-responsive model target, and we revealed a possible co-regulation of Dag1 by p300 and MyoD accompanied by enrichment of loci-specific histone acetylation. Taken together, we provide novel molecular insights into the regulation of myogenic enhancers by p300 in concert with MyoD. Furthermore, we provide novel mechanistic perceptions into the interplay between RXR signaling and chromatin states pertinent to myogenic programs in early myoblast differentiation. Our studies present a valuable insight for driving condition-specific chromatin state or enhancers pharmacologically to treat muscle-related diseases and for the identification of additional myogenic targets and molecular interactions for therapeutic development.

Acknowledgments

I would like to thank my supervisor, Dr. Qiao Li, for recruiting me to her lab as a Master's student without previous experience in molecular biology and then continuing to mentor me as a Ph.D. candidate. I would like to thank her for believing in me and for her guidance, patience and advice during the journey of my Ph.D. I am grateful for my time in her lab.

I would like to thank past and present members of the Li lab (Saadia, Matt, Katie and Hamood) for our long conversations, as well as their continuous help and support at all times. I would also like to thank the Blais' laboratory students for all their technical advice on qChIP and ChIP-seq experiments.

I would like to thank my thesis advisory committee members Dr. Alex Blais, Dr. Chris Kennedy and Dr. Wandong Zhang for their constructive criticism, suggestions, support and critical advice during my Ph.D. studies.

I would like to extend my acknowledgment to the source of my funding and scholarship: Umm Al-Qura University in Makkah, Saudi Arabia. I would like to thank them for allowing me to pursue my passion for research.

My sincerest thanks go to my father, Hamed, my amazing brother Abdulaziz and my best sisters, Sarah, Shahad and Raghad for their constant support, love and prayers. Special thanks go to my friends who helped me pass through the hard times and made my work much more enjoyable.

Special thanks to my family members: my husband Ghazi and my children: Mustafa, Ghena and Eyad for their endless help and patience during my master's and Ph.D. You were and will always be the source of my happiness and strength. Thank you for

supporting me throughout the good and bad days and for creating lots of good memories. My life without you is meaningless; I am so grateful for having you in my life.

Finally, I wish to dedicate this thesis to my mother Muna, who came to Canada in 2012 to take care of my children when I spent uncountable hours in the lab working and studying day and night. Without you, none of this would be possible.

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List of abbreviations

Angptl4	Angiopoietin Like 4
Asb2	Ankyrin Repeat and SOCS Box Containing 2
ATCC	American Type Culture Collection
Bex	Bexarotene
bHLH	Basic-helix-loop-helix
bp	Base pair
CBP	CREB-binding protein
cDNA	Complementary Deoxyribonucleic acid
CER	Core enhancer region
ChIP	Chromatin immunoprecipitation
ChIP-seq	Chromatin immunoprecipitation-high throughput sequencing
CO_2	Carbon dioxide
Dag1	Dystroglycan
DBD	DNA-binding domain
DGC	Dystrophin-associated glycoprotein complex
DM	Differentiation medium
DMD	Duchenne muscular dystrophy
dmd	Dystrophin
DMEM	Dulbecco's Modified Eagle's Medium with high glucose
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DRR	Distal regulatory region

DSHB	Developmental Studies Hybridoma Bank,
DTT	Dithiothreitol
E-box	Enhancer box motif
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
ENCODE	Encyclopedia of DNA Elements
ERK	Extracellular signal-regulated kinases
ERα	Estrogen receptor alpha
ES	Embryonic stem
ESCs	Embryonic stem cells
FBS	Fetal bovine serum
FAT	Factor acetyltransferase
FXR	Farnesoid X receptor
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
Gbr2	Growth factor receptor-bound protein 2
GCN5	General Control of Amino Acid synthesis 5-Like 2
GEO	Gene Expression Omnibus database
GM	Growth medium
GNAT	GCN5-related N-acetyltransferases
GO	Gene Ontology
Gpc6	Glypican-6 precursor
GTFs	*General transcription factors*
H1	Histone H1

H2A	Histone H2A
H2B	Histone H2B
H3	Histone H3
H3K18ac	Histone 3 acetylated on lysine 18
H3K27ac	Histone 3 acetylated on lysine 27
H3K27me3	Histone 3 trimethylated on lysine 27
H3K4me1	Histone 3 monomethylated on lysine 4
H3K4me2	Histone 3 dimethylated on lysine 4
H3K4me3	Histone 3 trimethylated on lysine 4
H3K63me3	Histone 3 trimethylated on lysine 63
H3K9ac	Histone 3 acetylated on lysine 9
H4	Histone H4
H4K8ac	Histone 4 acetylated on lysine 8
HAT	Histone acetyltransferase
HDACs	Histone deacetylases
HRE	Hormone response element
IF	Immunofluorescence
IgG	Immunoglobulin (Ig)
Igsf3	Immunoglobulin Superfamily Member 3
IGV	Integrative Genomics Viewer
Kb	Kilobase pair
Kd	Knockdown
kDa	Kilodalton

LBD	Ligand binding domain
LBP	Ligand binding pocket
LiCl	Lithium chloride
LXR	Liver X receptor
MACS	Model-based Analysis of ChIP-Seq
MEF2	Myocyte enhancer factor 2
Mef2c	Myocyte-specific enhancer factor 2C
Mef2α	Myocyte-specific enhancer factor 2A
ml	Milliliter
mM	Millimolar
Mm9	Mus musculus
MRF	Myogenic Regulatory Factor
Mrf4	Myogenic regulatory factor 4
mRNA	Messenger RNA
Myf5	Myogenic factor 5
MyHC	Myosin heavy chain
MyoD	Myogenic differentiation 1 (also known as Myod1)
Myog	Myogenin
NaCl	Sodium chloride
nM	Nanomolar
NP-40	Nonidet P40
NR	Nuclear receptor
P19 cells	Embryonic carcinoma cell lines

p300	Protein p300
Pax3	Paired box protein Pax-3
Pax7	Paired box protein Pax-7
PBS	Phosphate-buffered saline
PBS-T	PBS, 0.1% Triton X-100 (v/v)
PCAF	p300/CBP-associated factor
PCR	Polymerase chain reaction
PMSF	Phenylmethylsulfonyl fluoride
PPAR	Peroxisome proliferator-activated receptors
PPARγ	Peroxisome proliferator-activated receptors type gamma
PPARδ	Peroxisome proliferator-activated receptors type delta
PRR	Proximal regulatory region
PSCs	Pluripotent Stem Cells
PVDF	Polyvinylidene difluoride
PXR	Pregnane X receptor
qChIP	Quantitative chromatin immunoprecipitation
qPCR	Quantitative polymerase chain reaction
RA	Retinoic acid
RAR	Retinoic acid receptor
RNA	Ribonucleic acid
RNA pol II	RNA polymerase II
RNA-seq	Ribonucleic acid-sequencing
RNAi	RNA interfering system

RT	Room temperature
RT-qPCR	Reverse transcription-quantitative polymerase chain reaction
RXR	Retinoic X receptor
RXRγ	Retinoic X receptor-Gamma
RXRα	Retinoic X receptor-Alpha
RXRβ	Retinoic X receptor -Beta
SDS	Sodium dodecyl sulfate
shDag1	shRNA directed against dystroglycan
shp300	shRNA directed against p300
shRNA	Short hairpin RNA
shRXRα	shRNA directed against retinoid X receptor-alpha
Sntb1	Beta-1-syntrophin
Tbp	TATA-box-binding protein
TF	Transcription factors
THR	Thyroid hormone receptor
Tnnc1	Troponin C1
Tnnt1	Troponin T1
Tris-HCl	Hydroxymethyl hydrochloride
TSS	Transcriptional start site
VDR	Vitamin D receptor
α-Dag1	Alpha dystroglycan
β-Dag1	Beta dystroglycan
μM	Micromolar

Chapter 1. Introduction

Skeletal muscle development is a sophisticated series of cellular processes that involves collective intracellular and extracellular signaling of many evolutionary conserved factors (Bismuth and Relaix, 2010; Buckingham et al., 2003). The proper muscle formation and regeneration, both embryonically and postnatal, requires the correct expression of genes which are precisely coordinated through the sequential expression of myogenic regulatory factors (MRFs) including Myf5, MyoD, myogenin and Mrf4 (Figure1). MyoD contributes to myogenic differentiation, at least in part, through the recruitment of transcription co-factors such as p300 to the regulatory regions. As a transcriptional coactivator, p300 permits the accessibility of chromatin via acetylating histone tails, which either primes or activates the regulatory loci such as promoters or enhancers resulting in gene expression during myogenesis (Yang et al., 1996). Interestingly, nuclear receptor signaling, such as Retinoid X Receptor (RXR), has been shown to induce myogenic specification and stimulate early differentiation (Le May et al., 2011; AlSudais et al., 2016). Hence, a comprehensive understanding of the epigenetic regulation underlying the myogenic differentiation helps to decipher the roles of myogenic players leading to finding ways to cure muscle diseases or slow normal muscle aging to ensure a better lifestyle.

1.1. Skeletal muscle development

Muscle is the largest tissue by mass in the body and it is vital for body support and locomotion. Skeletal myogenesis is a multistep regulated process involving an enormous variety of gene expression that enables a muscle to form and function throughout sequential developmental phases from embryonic to adult. The development of muscle in vertebrates begins from a mesoderm-derived structure called somites. The somites are spherical epithelial structures that give rise to several cell lineages such as skeletal muscles, smooth muscles, brown fat and endothelial cells. The somites further differentiate into a dorsal portion, the so-called dermomyotome, along with the underlying compartment the myotome, which together comprise skeletal muscles (Brand-Saberi and Christ, 1999). Fetal myogenic precursor cells and postnatal satellite cells, the muscle stem cells that arise from the dermomyotome, are marked by the binding of paired/homeodomain family of transcription factors Pax3 and Pax7 (Gros et al., 2005; Kassar-Duchossoy et al., 2005; Relaix et al., 2005) (Figure 1). The dermomyotome is divided into the epaxial and hypaxial domains. The epaxial is made by cells migrating underneath from the dorsomedial lip of the dermomyotome, which forms the deep back muscles. Nevertheless, the hypaxial is generated by the ventrolateral lip of the dermomyotome located either ventrally to form the diaphragm and the body wall muscles, or laterally to form the limb muscles (Brand-Saberi and Christ, 1999; Brent and Tabin, 2002).

1.2. Formation of muscle fibers

During embryonic myogenesis, skeletal muscle is formed by the fusion of mononucleated myoblasts making multinucleated myotubes that eventually construct bundles of striated muscle fibers called mature myofibers. Myofibers are composed of bundles of myofibril, which is a complex organelle comprised an array of myofilaments, the sarcomeres (Sanes,

2003). Myofibers are surrounded by a basal lamina that harbors a specialized plasma membrane called the sarcolemma, which provides structural stability to the myofibers (Rahimov and Kunkel, 2013). Between the sarcolemma, of their residing myofibers, and the basal lamina lies the satellite cells, a regenerative cell population that proliferates and differentiates into myoblasts upon stimulation such as extensive physical exercise, injury or disease (Yin, Price, and Rudnicki, 2013; Mauro, 1961).

The sarcolemma allows for signal transduction partly through a group of proteins called dystrophin-associated glycoprotein complex (DGC). The DGC stabilizes the muscle plasma membrane by connecting extracellular components to the myofiber cytoskeleton (Rahimov and Kunkel, 2013). The DGC proteins involve dystrophin, dystroglycan, syntrophins, utrophin, sarcoglycan, sarcopan and dystrobrevin (Bhat et al., 2018). Loss of function mutation of the dystrophin gene, dmd, causes Duchenne Muscular Dystrophy (DMD), a disease characterized by continuous regeneration, elevated fibrosis, inflammation and loss of muscle mass (Serrano et al., 2011; Mercuri and Muntoni, 2013; Shieh, 2013). During vertebrate myogenesis, the fusion of myoblasts to each other or to myotubes is a highly regulated process; however, it is still poorly understood. Several processes, regulated by myogenic transcription factors and co-factors, are involved in myocyte and myotube formation, starting with recognition and adhesion of myoblasts that require membrane receptors and membrane repair proteins (Melanie et al., 2008). Besides, the elongation phase of the newly formed myofibers requires the fusion of myoblasts at their extremities (Gu et al., 2016; Williams and Goldspink, 1971).

1.3. Dystroglycan

Dystroglycan is the core component of the DGC (Ervasti et al., 1990), and it is highly expressed in skeletal muscle, epithelial and neuronal tissues (Durbeej et al., 1998; Durbeej and Campbell, 1999; Matsumuraa et al., 1993). It is represented in the DGC by two subunits, termed α (156 KDa) and β (43 KDa), which are produced from the posttranslational cleavage of a single mRNA species encoded by a single gene (Ibraghimov-Beskrovnaya et al., 1992). Dystroglycan α is an extracellular protein that functions as an extracellular matrix receptor with high affinity to laminin-2, perlecan and agrin, and interacting non-covalently with β-Dag1, which is the transmembrane portion of Dag1 protein (Sciandra et al., 2013), in turn, β-Dag1 acts as a scaffold for proteins such as Gbr2 and ERK, which are involved in signal transduction (Cavaldesi et al., 2001; Spence et al., 2004). Moreover, the cytosolic domain of β-Dag1 is anchored to actin via its interaction with dystrophin (Suzuki et al., 1994; Jung et al., 1995; Rosa et al., 1996).

1.4. Types of muscular dystrophies

In order for the muscle to function properly, it requires accurate signaling along the muscle fibers to contract and regenerate efficiently after muscle injury (Schmidt et al., 2011). Defective muscle proliferation and/or differentiation can lead to several muscle-related diseases. The DGC proteins, which receive much attention from researchers after the discovery of the dystrophin gene in humans, is a multimeric protein complex that ensures a mechanical reinforcement of the sarcolemma by providing a link between the extracellular matrix and the actin cytoskeleton (Petrof et al., 1993). The dystrophin mutation was first discovered in the 1980s in a DMD patient (O'Brien and Kunkel, 2001). Loss of function mutation of dystrophin causes loss of muscle membrane integrity and progressive cycles of regeneration and

degeneration that in turn, lead to prominent inflammation, fibrosis and eventual progressive weakness of muscle mass and function (Serrano et al., 2011; Guiraud et al., 2015). Dysfunction of the DGC is also implicated in distinct forms of muscular dystrophies. Abnormal glycosylation of Dag1, for example, is associated with several muscular dystrophies such as Fukuyama congenital muscular dystrophy, Walker–Warburg syndrome, muscle–eye–brain disease, congenital muscular dystrophy types 1C and 1D, and autosomal recessive limb-girdle muscular dystrophy type 2 (Kobayashi et al., 1998; Martin Brockington et al., 2001; Brockington et al., 2001; Longman et al., 2003; Toda et al., 2003; Barresi and Campbell, 2006; Hara et al., 2011; Gao and McNally, 2015). Furthermore, a mutation in sarcoglycan causes limb-girdle muscular dystrophy (Bonnemann et al., 1996). Therefore, understanding the mechanism of Dag1 regulation during differentiation can benefit in improving the pathology of muscle-related diseases.

1.5. Transcription factors in myogenesis

Both prenatal and postnatal myogenesis occurs through the coordinated function of a combinatorial regulatory network of extrinsic and intrinsic transcription factors and co-factors acting temporally and sequentially to ensure appropriate muscle formation. While much of our current accumulated knowledge of skeletal myogenesis was gained from loss-of-function and gain-of-function experiments performed on mouse, fruit fly, fish and avian models, most of these transcription factors and co-factors are well conserved from invertebrates to vertebrates. Among these transcription factors are the Pax family, which is expressed and function early in myogenesis.

Pax3 and Pax7 genes regulate the development of early striated muscle arising from somites in the trunk. It has been shown that genetic ablation of *Pax3* was embryonically lethal

and prevented the occurrence of Pax7-positive cells (Hutcheson et al., 2009), suggesting that Pax3 is a key factor in the regulation of myogenesis for skeletal muscle (Comai and Tajbakhsh, 2014). During muscle formation, myogenic commitment and differentiation are associated with the expression of a group of transcription factors termed the myogenic regulatory factors (MRFs), which includes Myf5, MyoD, myogenin and Mrf4 (Figure 1) (Buckingham and Rigby, 2014). Prenatal and postnatal myogenesis share some functional and molecular similarities including the chronological appearance of upstream transcription factors (e.g., Pax7 and Myf5) and the commitment and differentiation factors (e.g., MyoD, Myog), (Parker, Seale, and Rudnicki, 2003).

Myogenic regulatory factors belong to class II basic-helix-loop-helix (bHLH) family of transcription factors that dimerize with sequence-specific binding factors and histone-modifying enzymes to form transcriptional regulatory complexes in order to promote skeletal muscle formation (Blais et al., 2005; Braun et al., 1992; Braun and Arnold, 1995). The bHLH family members bind to the DNA consensus sequence known as the E-box (CANNTG), which is present on specific myogenic enhancers and promoters (Blackwell and Weintraub, 1990; Weintraub et al., 1990). These proteins cooperate with another family of transcription factors known to be essential in myogenesis termed Myocyte Enhancer Factor 2 (MEF2) (Davidson et al., 1996). Subsequently, the MRFs bind MEF2 family of transcription factors and cooperate to repress or activate the expression of an array of skeletal muscle gene (Blais et al., 2005; Molkentin et al., 1995; Naidu et al., 1995). For example, myogenin expression is regulated by the binding of both MyoD and MEF2 at the *myogenin* promoter in differentiating myocytes, both *in vivo* and *in vitro* (Edmondson et al., 1992; Cheng et al., 1993; Rampalli et al., 2007). Likewise, it has been shown that MRFs can regulate the transcription of each other and their own transcription, creating cross- and auto-regulatory loops, respectively (Thayer et al., 1989;

Braun et al., 1989).

Mutation studies involving the myogenic regulatory factors during embryogenesis revealed unique but also redundant roles and expression patterns in skeletal myogenesis (Comai and Tajbakhsh, 2014). Overall, these studies concluded two main areas of function for MRFs: a myogenic commitment by Myf5, Mrf4 and MyoD, and myogenic differentiation by MyoD, myogenin and Mrf4, (reviewed in Fong and Tapscott, 2013; Megeney and Rudnicki, 1995; Comai and Tajbakhsh, 2014). Myf5 is the first MRF to be expressed during embryogenesis, right before myotome formation (Kassar-Duchossoy et al., 2004). Myf5 null mice exhibit normal muscle development with mild defects in trunk muscle formation. Similarly, mice null for MyoD alleles displayed normal muscle development; however, these mice present delayed onset of branchial arches, limbs, tongue and diaphragm muscle formation (Kablar et al., 1998), implying a redundant function between Myf5 and MyoD. A later study showed that mice lacking both Myf5 and MyoD completely deficient of skeletal muscle formation (Rudnicki et al., 1993). However, Kassar-Duchossoy and colleagues showed that the methods used to target Myf5 by the previous group also disrupted Mrf4 transcription and that a partial rescue of Mrf4 resulted in a rescue of myogenesis (Kassar-Duchossoy et al., 2004). These results suggest that, during embryogenesis, Mrf4 can function as a muscle determination factor to compensate for the absence of Myf5 and MyoD. In fact, this result is in agreement with the expression pattern of Mrf4 as it is observed before the expression of MyoD in hypaxial and epaxial somites during muscle formation in embryogenesis and it is re-expressed later during the fetal stage (Kassar-Duchossoy et al., 2004).

Myogenin functions downstream of Myf5, MyoD and Mrf4, and has a unique non-overlapping function that cannot be compensated by other MRFs during embryogenesis. Unlike other MRFs, mice lacking myogenin display poorly developed skeletal muscles (complete absence of fetal myofibers), whereas the myoblast formation is unaffected (Nabeshima et al., 1993; Hasty et al., 1993), which implies a role for myogenin in terminal myogenic differentiation. On the other hand, the knockin of *myogenin* into the *Myf5* locus was not able to rescue skeletal muscle lineage in the absence of MyoD (Wang et al., 1997). Furthermore, expression of normal MyoD levels cannot compensate for the absence of myogenin in this context (Davie et al., 2007). These knockout studies imply that Myf5 and MyoD have distinctive intrinsic functions to myogenin, rather than merely having different sequential expression patterns. Similar to myogenin, the ablation of Mrf4-positive cells results in a severe decrease in the number of differentiated myofibers (Haldar et al., 2008). This result is further supported by the ability of *myogenin*-promoter-*Mrf4* transgene to partially rescue the muscle defect identified in myogenin mutant mice (Zhu et al., 1997).

MyoD was the first MRF identified to have the ability to enforce the myogenic program in non-muscle cells (Davis et al., 1987). The other three MRFs later also displayed similar potential in the myogenic conversion of non-muscle cells (Braun et al., 1989; 1990; Edmondson and Olson, 1989; Rhodes and Konieczny, 1989; Wright et al., 1989). In brief, Myf5, MyoD have roles in muscle lineage determination, and are partially redundant, since mice lacking either of them individually exhibit no apparent skeletal muscle defect (Braun and Arnold, 1995; Kassar-Duchossoy et al., 2004; Patapoutian et al., 1995; Rudnicki et al., 1992; 1993; Zhang et al., 1995). Nonetheless, myogenin and Mrf4 are more directly associated to myogenic differentiation and trigger the expression of myotube-specific genes (Hasty et al., 1993; Kassar-Duchossoy et al., 2004).

Figure 1

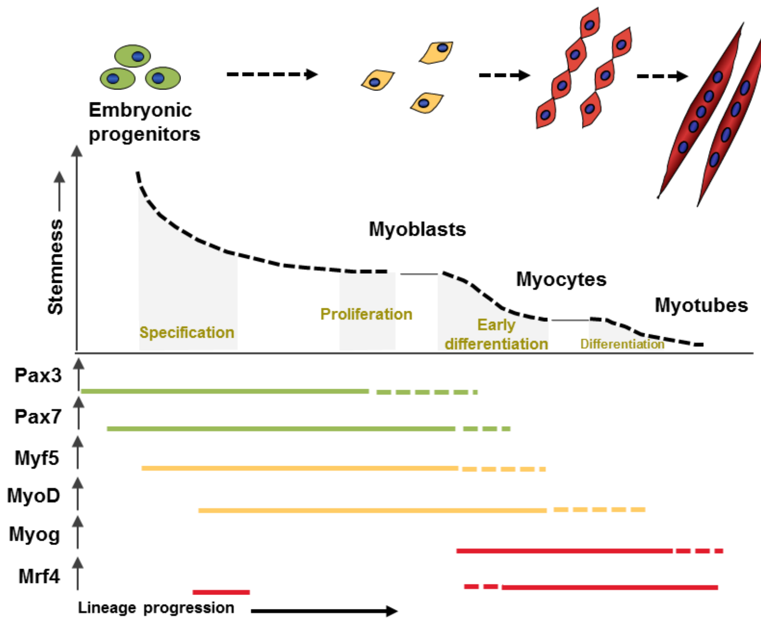

Figure 1. The regulation of skeletal myogenesis by myogenic regulatory factors.
During progenitor cell differentiation into myotubes, Pax3 and Pax7 play roles in the specification of progenitors to the myogenic lineage, while Myf5, MyoD, myogenin and Mrf4 contribute to the commitment and further differentiation of myogenic progenitors into myotubes, modified from (Bentzinger, Wang, and Rudnicki, 2012).

1.6. Adult muscle regeneration

Satellite cells are adult cells and are considered as muscle stem cells. They are characterized by Pax7 expression and are important for postnatal muscle growth and repair (Gros et al., 2005). The knockout of Pax7 in mice causes the progressive loss of satellite cells although Pax7 is not essential for the specification of satellite cells per se (Günther et al., 2013; Oustanina et al., 2004; Maltzahn et al., 2013). While satellite cells also express Myf5, they are distinct from proliferating myoblasts, which express both Myf5 and MyoD (Cornelison and Wold, 1997; Zammit et al., 2004). Thus, satellite cells are a heterogeneous mix of both activated proliferating myoblasts and quiescent stem cells, which are predisposed to activation upon stimulation (Chang and Rudnicki, 2014). The ablation of satellite cells results in impaired muscle tissue formation and infiltration of muscle tissue by inflammatory and adipogenic cells (Sambasivan et al., 2011).

Upon muscle injury, satellite cells can go through symmetric (Chang and Rudnicki, 2014) or asymmetric (Dumont et al., 2015) division, to generate myoblasts and/or to maintain the satellite cell pool for a possible future need (Yin, Price, and Rudnicki, 2013; Biressi and Rando, 2010). Muscles are repaired either by sarcolemmal resealing of membrane disruptions or cell-mediated repair mechanism through healing the torn membrane or recruiting the machinery governing myoblast fusion, respectively, to allow for the survival or formation of new muscle fibers (Abmayr and Pavlath, 2012; Kim et al., 2015). When triggered, satellite cells begin to proliferate, differentiate and fuse with one another or with existing myofibers to generate myoblasts as a response to MRFs and the factors released in the affected area (Robertson et al., 1990; 1993). How MRFs regulate gene expression during early myoblast differentiation is subsequently affected by their DNA binding partner and through their

association with the HATs, histone acetyltransferases (Puri et al., 1997; Sartorelli et al., 1997; Roth et al., 2003).

1.7. Chromatin modification during myogenic differentiation

During myogenesis, the commitment to and execution of differentiation involve multiple changes in the transcriptional program within the cell to enable the development of mature myogenic cells. Furthermore, the structural state of the chromatin, along with RNA-pol II, has a significant impact on the transcriptional status of the target gene, which is demonstrated by the presence of transcriptional co-factors. These changes in the transcriptional program and the structural state of the chromatin are regulated by lineage determining and auxiliary transcription factors (Ng and Gurdon, 2008). These facts have been explored during the late differentiation process represented by myotube formation; however, chromatin features during the differentiation of the myoblasts into myocytes, which represents early stages of differentiation, has yet to be determined.

Posttranslational modification of histones, particularly, histone acetylation, is a method of epigenetic control involving a direct modification of the amino acid residues on histone tails, where the relative charge of the chromatin is altered and thereby, modify the ability of chromatin to interact with the underlying DNA and/or proteins required to regulate gene expression (Bannister and Kouzarides, 2011). The N-terminal tail is subjected to diverse posttranslational modifications such as acetylation, methylation, phosphorylation, ubiquitination and/or sumoylation, which occur by modifiers of chromatin (Jenuwein and Allis, 2001). In response to external stimuli, modifiers of chromatin containing chromatin-remodeling complexes are recruited to enhancer and promoter regions by transcription factors at the regulatory regions (Sartorelli and Caretti, 2005). These posttranslational modifications

may cause chromatin to become accessible or condensed and affect directly on the propensity of DNA to be recognized by transcription factors (Zhang et al., 2014). Therefore, the eukaryotic genome structure is roughly present as two states, highly condensed chromatin that impedes DNA transcription, known as heterochromatin, and relatively less compacted chromatin that is more transcriptionally accessible, called euchromatin (Campos and Reinberg, 2009).

Histone acetylation occurs on lysine amino acid residues, whereas methylation occurs on both lysine and arginine residues. Histone residues can be mono-, di- or tri-methylated and each of which is associated with specific transcriptional status (Bernstein et al., 2006). Acetylation is generally associated with locus activation while methylation, depending on its position and state, is related with locus repression or activation, (reviewed in Berger, 2002; 2007; Bulger, 2005; Kuo and Allis, 1998; Li et al., 2007; Shahbazian and Grunstein, 2007). For example, H3K4me3 has been associated with active transcription via facilitating the recruitment of Pol-II complex to gene regulatory regions (Pekowska et al., 2011), whereas H3K27me3 has been linked to transcription repression which is mutually exclusive from H3K4me3 (Kim et al., 2013).

Acetylation is a transient process, where an acetyl group is transferred to a lysine residue, changing its basic side chain into a neutral residue, and eventually opens chromatin to activate transcription (Shahbazian and Grunstein, 2007). On the other hand, deacetylation reverses this process by removing the acetyl groups from the lysine residues of histones, allowing the histones to condense DNA and thereby repressing gene expression (Ruthenburg et al., 2007). Such deacetylation takes place by enzymes called histone deacetylases (HDACs) (Cress and Seto, 2000). Thus, distinct histone modifications define the states of chromatin,

which creates either a synergistic or antagonistic interaction for chromatin-associated proteins (Jenuwein and Allis, 2001).

Transcriptional gene regulation during muscle formation is a highly complex process that requires the contact of transcription factors themselves to their DNA targets. This process can be inhibited by the organization of nucleosomes, as DNA on nucleosomes binds tightly to histones, which eventually creates an inaccessible transcriptional structure (Kornberg, 1974; Tapscott, 2005). Nucleosomes are formed by the wrapping of DNA around octamers of core histone proteins comprising H3 and H4 tetramer flanked by H2A and H2B dimers forming an approximately 165 bp core. Nucleosomes are arranged linearly as arrays on the DNA and folded into higher-order structures with histone H1 as a linker (Luger et al., 1997).

The myogenic regulatory factors recruit a cascade of cooperating transcription factors that remove or incorporate histone variants, alter nucleosome structure and post-translationally modify histones (Blum et al., 2012). Particularly, MyoD has been shown to activate muscle genes of a variety of differentiated cell types from different species such as human, rat and chicken (Weintraub et al., 1989). During myogenesis, coordinated action between these factors prevents the expression of differentiation-specific genes in myoblasts and facilitates the activation of these genes during differentiation. This regulation is achieved by manipulating the chromatin structure of myogenic regulatory sequences, which occurs in both a temporal and spatial manner (Creyghton et al., 2010; Alvaro Rada-Iglesias et al., 2011).

1.8. p300 HAT and myogenic differentiation

Activation of gene transcription is the primary mechanism that regulates proliferation, differentiation and other cellular processes (Farnham, 2009; Sonenberg and Hinnebusch, 2009). It is a highly coordinated multistep process that involves the binding of sequence-specific transcription factors followed by the recruitment of transcriptional coactivators (Puri et al., 1997; Sartorelli et al., 1997). One way the coactivators activate transcription is by favoring the binding of the general transcription factors (GTFs) to the transcriptional machinery that permits the recruitment of RNA polymerase II (Pol-II) on the DNA (Lu et al., 2009). In addition, coactivators modulate the chromatin state by histone modifications to adjust chromatin accessibility (Arvey et al., 2012). Coactivators like p300, CREB binding protein (CBP) and p300/CBP-associated factor (PCAF) can form multimeric complexes during transcriptional activation (Niederreither et al., 2008; Ogryzko et al., 1996) (Figure 2).

Since it does not bind DNA directly, p300 is recruited to the genomic regulatory regions via interaction with sequence-specific DNA-binding transcription factors (Sartorelli et al., 1997; Kim et al., 2009). Interestingly, it has been demonstrated that MyoD directly binds p300 (Puri et al., 1997; Sartorelli et al., 1997), suggesting a role of MyoD in recruiting p300 to the regulatory regions of myogenic genes leading to histone acetylation and activation of transcription during myogenic differentiation. p300 is a modular protein that comprises multiple well-defined domains such as CH1, KIX, CH3 domains (Figure 3). These domains are connected by long stretches of intrinsically random residues to the catalytic core of p300, which includes the HAT domain, CH2 and bromodomain. The bromodomain recognizes acetylated substrates, whereas the HAT domain acetylates histone and non-histone proteins (Figure 3), (Zeng et al., 2008). Both the p300 bromodomain and CH2 region have been shown to be required for chromatin binding (Ragvin et al., 2004)

During myogenesis, p300 facilitates muscle-specific gene activation by modulating the chromatin structure to be more accessible as it possesses an intrinsic histone acetyltransferase (HAT) domain, which is essential for myogenesis (Figure 3), (Chen et al., 2001; Ogryzko et al., 1996; Roth et al., 2003). Knockout studies have demonstrated that p300 is essential for normal muscle development in mice (Yao et al., 1998), as well as for the expression of Myf5 and MyoD (Roth et al., 2003). In addition, p300, but not CBP, acetyltransferase activity is required for Myf5 and MyoD expression in differentiated embryonic stem (ES) cells (Roth et al., 2003). Furthermore, deletion of p300, but not PCAF, has been shown to dramatically decrease the acetylation levels of H3K18 and H3K27, which are hallmarks of active transcription in myogenesis (Jin et al., 2011). Additionally, we have illustrated that the HAT activity of p300 is required for H3K27 acetylation specifically at the MyoD locus, suggesting that the intact histone acetyltransferase activity of p300 is necessary for gene expression during myogenic commitment and differentiation. Thus, investigation of the regulatory mechanisms governing loci-specific histone acetylation by p300 recruitment is a significant area of interest for understanding the mechanisms of transcription and gene expression. The importance of p300 for an array of cellular processes including skeletal myogenesis and myotube formation is well established (Blum et al., 2012; Polesskaya et al., 2001; Sterner and Berger, 2000; Chan and Thangue, 2001). However, the functional mode of transcriptional regulation involving p300, especially at the early stage of myoblast differentiation, remains less clear, given that lineage-specific enhancers and loci-specific acetylation play an essential role in the control of genes pertinent to differentiation. In this study, we investigated the association of p300 and loci-specific histone acetylation with distinct chromatin states at the onset of myoblast differentiation to discern the concerted action of MyoD and p300 in regulating myogenic expression.

Figure 2

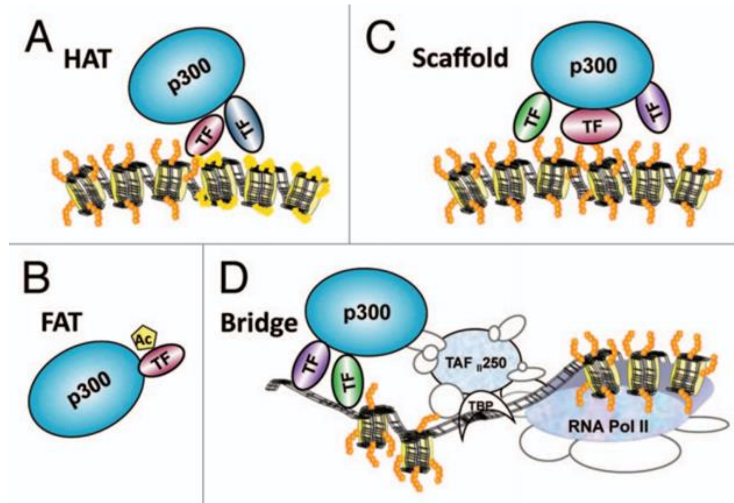

Figure 2. A schematic representation of transcriptional activation by p300.
The transcriptional coactivator p300 regulates gene transcription by acting as: (A) a histone acetyltransferase (B) factor acetyltransferase (C) a scaffold for multiple transcription factors on chromatin and (D) a bridge connecting the transcription factors to the basal transcriptional machinery (Chen and Li, 2011).

Figure 3

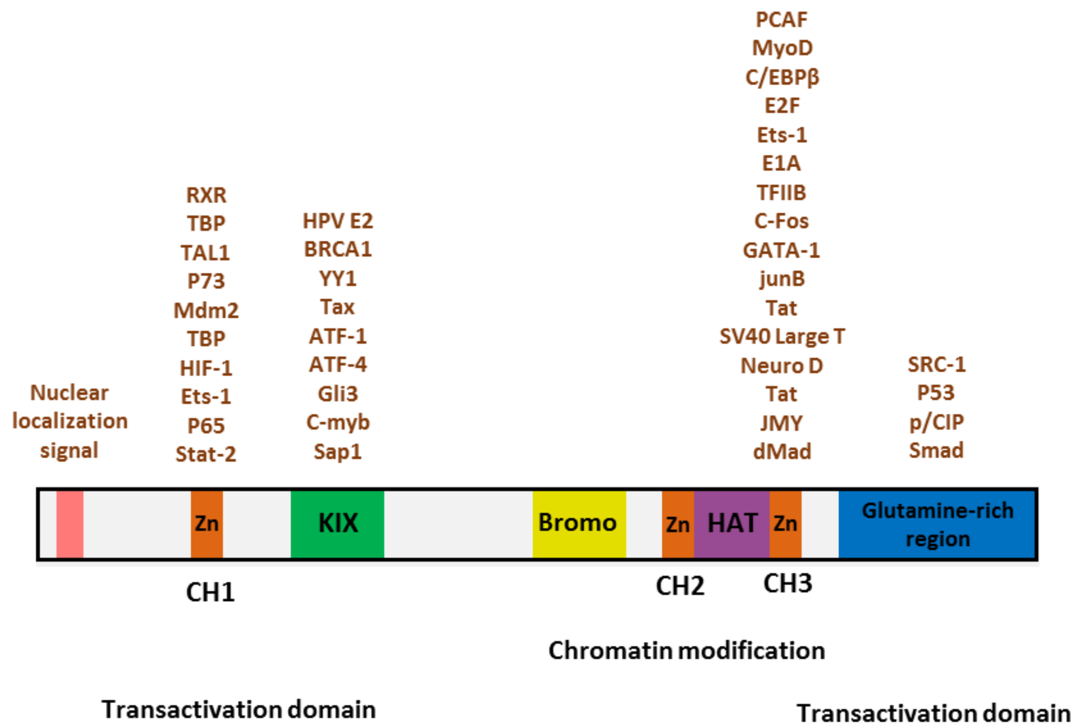

Figure 3. Domain organization of p300/CBP protein.

A schematic structure of p300/CBP protein showing the nuclear localization signal (pink), Transcriptional-adaptor zink-finger doamin1 (orange), Kinase inducible domain of CREB interacting domain (green), Bromodomain (yellow), Lysine acetyltransferase domain (purple) and C-terminal domain, Glutamine-rich region (blue). Modified from (Chan and Thangue, 2001).

1.9. Functional genomics and regulatory regions in myogenesis

Recently, a wealth of information has emerged from high-throughput genomics approaches and epigenetics, which enabled us to understand myogenic differentiation through mapping of protein-DNA interactions and epigenetic marks in a genome-wide manner at a base-pair resolution (Farnham, 2009). Chromatin immunoprecipitation followed by massive sequencing (ChIP-seq) has been extensively used in myogenesis in the past decade, contributing significantly to our understanding of transcriptional regulation during myogenic differentiation. The findings in recent years that certain DNA elements are associated with distinct histone modifications have offered a new pathway to identify networks of regulatory loci whose activities underpin the control of gene expression. As such, lineage-specific enhancers can be identified by promoter-distal enrichment in H3K4me1 and/or histone acetyltransferases recruitment (Hon et al., 2009; Krebs et al., 2011).

Generally, enhancers can range from 200 bp to 1000 bp of DNA (Istrail and Davidson, 2005) and are found in long linear distances ranging from a few hundred bases to a few million bases from their target transcription start sites (TSS) (Bulger and Groudine, 2010; Heintzman et al., 2007; Kim et al., 2015). Enhancer regions are constitutively present but become active upon binding of specific transcription factors, which in turn recruit chromatin-modifying enzymes to the locus (Giacinti et al., 2006). The function of enhancers is governed by specific transcription factors that selectively bind distinct DNA sequences within the enhancer to control their activity, leading to activation or repression of target gene transcription (Lee et al., 1987). Gene expression programs associated with stem cell differentiation are largely regulated through distal regulatory elements characterized as enhancers, which can be further classified as ‘active’ or ‘poised’ based on the combinations of condition-specific histone

modification (Creyghton et al., 2010; A Rada-Iglesias et al., 2011). Studies in hematopoietic stem cells have revealed that poised enhancers are established in lineage progenitors before their activation and are involved in gene expression during lineage differentiation (Lara-Astiaso et al., 2014). In addition, poised enhancers marked bivalently by H3K4me1 and H3K27me3, are dynamically modified during terminal differentiation and myotube formation where H3K27me3 is replaced by H3K27ac (Creyghton et al., 2010; Asp et al., 2011). Therefore, enhancers marked by histone acetylation upon differentiation may reflect the activation of distinct gene programs regulated by lineage-specific transcription factors.

On the other hand, promoter regions are better characterized transcriptional regulatory sequences, given that their predictable location is approximately 50 to 300 bp upstream of the TSS (Butler and Kadonaga, 2002). Similar to enhancers, promoters are featured with enrichment of specific histone marks. Through analyzing the qChIP of lysine-acetylation marks at 124 E-box-containing promoter regions of a human B-cell line, Martinato and colleagues have observed changes in multiple histone marks upon Myc exogenous expression and found that the majority of targeted promoters display induction of H3K9ac, H4K8ac and H3K18ac (Martinato et al., 2008). Additionally, a later study reported that H3K9ac is further enriched at the *myogenin* promoter following 48-hours of C2C12 myoblast differentiation, suggesting that these histone marks are associated with active promoters (Chatterjee et al., 2016). Moreover, the increase in H3K9 acetylation has been shown to correlate with the activation of the nuclear receptor peroxisome proliferator-activated receptors (PPARγ) and has been shown to activate target gene expression in 3T3-L1 cells during adipogenesis (Jang et al., 2017). Interestingly, H4K8ac has also been linked to transcriptional activation (Wang et al., 2010) and was reported to be associated with p300 HAT activity (Wang et al., 2001). In

conclusion, the enrichment of histone acetylation is a highly tissue-specific event that occurs in various combinations at regulatory loci.

In human T-cells, the formerly mentioned histone marks are part of a "backbone" among other marks correlated with active promoters at a genome-wide level (Wang et al., 2008). One should keep in mind that certain chromatin modifications cannot co-exist. For instance, an acetyl group must be removed before a methyl group can be added by histone methyltransferases (Czermin et al., 2001; Zhang et al., 2002). In conclusion, specific and interlinked modification or histone acetylation may dictate distinct genomic states, which translates to distinct transcriptional outcomes.

1.10. Genomic mapping of transcription factors and histone modifications

Detailed mapping of transcription factor binding sites, coactivators and histone modifications is fundamental for deciphering the gene regulatory networks that underlie numerous biological processes. Since the invention of ChIP-seq (Johnson et al., 2007), various genomic studies have been widely performed to map the global binding sites of transcription factors and the deposition of histone marks during myogenic differentiation. One of the earliest ChIP-seq attempts mapped p300 genome-wide locations in embryonic stem cells (ES) cells to investigate the factors that could be involved in p300 recruitment to enhancer regions (Chen et al., 2008). *In vivo*, ChIP-seq was used to map p300 distribution in mouse embryonic forebrain, midbrain and limb tissue to accurately predict the p300-associated tissue-specific activity of enhancers. Most of the p300-associated loci were found further away from the TSS, indicating that p300 is mostly an enhancer-related coactivator (Visel et al., 2009); therefore, p300 occupancy is a critical chromatin signature of enhancers.

The human genome largely entails non-protein-coding DNA. Recently, progress has been made in annotating these non-coding loci via incorporating multiple chromatin modifications in order to generate a tissue-specific chromatin state model, to further decipher the molecular mechanism underlying a given cellular process. Therefore, the characterization of the chromatin states in myoblasts will help to reveal muscle-specific usage of regulatory DNA elements and the activity of muscle-specific enhancers. We previously generated a chromatin state model based on genome-wide co-occurrence of different epigenetic marks in committed proliferating myoblasts, using a hidden Markov model-based method. Through incorporating published ChIP-seq datasets for the promoter-associated mark H3K4me3 and RNA polymerase II (RNA Pol-II), enhancer-associated mark H3K4me1, transcription-associated mark H3K36me3 and the repressive mark H3K27me3 together with our own H3K9ac, H3K18ac, H3K27ac and H4K8ac ChIP-seq data in proliferating C2C12 myoblasts (GSE94558, Table 3), we established a model with 14-chromatin states, combined into five-chromatin states, reflecting diverse activities in gene expression prior to the onset of myoblast differentiation (Figure 4) (Hamed et al., 2017).

Remarkably, studies of histone acetylation in the context of terminal differentiation have observed a near-complete loss of H3K9ac in contrast to a high level of H3K18ac, on genes activated in myotubes, as compared to proliferating myoblasts (Asp et al., 2011; Blum et al., 2012). Furthermore, the association of MyoD to distinct E-box motifs generally corresponds to enhancer assembly, marked by H3K27ac, and muscle-specific gene expression during myotube formation (Blum et al., 2012; Fong et al., 2012). Therefore, MyoD binding is an index of myogenic enhancers (Creyghton et al., 2010), leading to the recruitment of histone acetyltransferases, which deposit the acetyl moiety required for enhancer activation (Blum et al., 2012). The presence or absence of specific transcription

factors and/or histone marks thus depicts global distinctive regulatory regions. For instance, the co-occurrence of p300, Pol-II, H3K4me1 and H3K27ac is associated with enhancers (Heintzman et al., 2009; Visel et al., 2009; Kim et al., 2015). Nevertheless, the temporal association of histone modifications and HATs at specific myogenic loci is less clear.

We have found earlier that H3K27ac is enriched at the MyoD core enhancer region (CER), a critical locus for MyoD expression, in a stepwise manner. In addition, the enrichment of H3K27ac at the CER coincides with the enriched occupancy of p300 in differentiating myoblasts (Hamed et al., 2013). MyoD expression is largely regulated by two enhancers within the *Myod1* locus, the CER located at -23 Kb and the distal regulatory region (DRR) located at -4 Kb(Asakura et al., 1995; Chen et al., 2004; Goldhamer et al., 1995; 1992; Tapscott et al., 1992). The CER has been shown to control normal spatiotemporal expression of MyoD to direct gene expression in muscle progenitor cells during embryogenesis (Goldhamer et al., 1995; Kablar et al., 1999), whereas the DRR, along with the proximal regulatory region (PRR at -275bp) are myoblast-specific response elements and thereby are important for the maintenance of MyoD gene expression (Tapscott et al., 1992; Asakura et al., 1995). Hence, enhancers marked with histone acetylation may reveal the activation of a cohort of gene programs regulated by distinct transcription factors during myogenic differentiation. Epigenetic modifications dictating the transition from myoblasts to late myotubes have been explored (Blum et al., 2012); however, we focus on the epigenetic changes in the conversion, specifically from myoblasts to myocytes.

Much of the work discovering the mechanism of muscle differentiation and fusion has been done *in vitro* using direct reprogramming, directed differentiation, or both. Direct reprogramming is achieved by converting of non-muscle cells including fibroblasts (Choi et al., 1990), into myogenic lineages via cellular manipulation such as the overexpression of

specific myogenic transcription factors like MyoD (Choi et al., 1990). On the other hand, directed differentiation is used to present specific signaling cues to proliferating cells *in vitro* to mimic the process *in vivo* during normal development, promoting them to differentiate. This is achieved by recapitulating the environment in the embryo during early differentiation to make muscle fibers with or without the addition of small molecule modulators, such as dimethyl sulfoxide (DMSO) and retinoic acid (RA), which enhance myogenic differentiation under specific environmental conditions (Francetic et al., 2012; Hamed et al., 2017). Adherent monolayer cultures of pluripotent stem cells (PSCs) have been used to represent more homogenous differentiation than embryoid bodies of mouse ES cells, which usually is heterogeneous and challenging to control lineage specification (Doetschman et al., 1985; Robbins et al., 1990).

A well-established cell line to study the molecular mechanism involved in myoblasts differentiation is C2C12, which is an immortalized myoblast cell line obtained through a continuous passaging of primary myoblasts derived from mouse limb muscles (Yaffe and Saxel, 1977). C2C12 cells have been extensively used in research, as they share a high correlation with transcriptional programs of isolated primary skeletal muscle cells (Szymanska et al., 1992). At a genome-wide scale, ChIP-seq analyses of MyoD (Cao et al., 2010) and gene expression profiling (Blais et al., 2005) from primary myoblasts is consistent with that of C2C12 during differentiation (Asp et al., 2011; Blais et al., 2005). Unlike primary skeletal myoblasts, C2C12 cells are less prone to spontaneous differentiation in culture, and they are a highly homogenous population of cells (Yaffe and Saxel, 1977), a characteristic that is required for genome-wide studies, making C2C12 cells a system of choice to investigate our hypotheses.

Figure 4

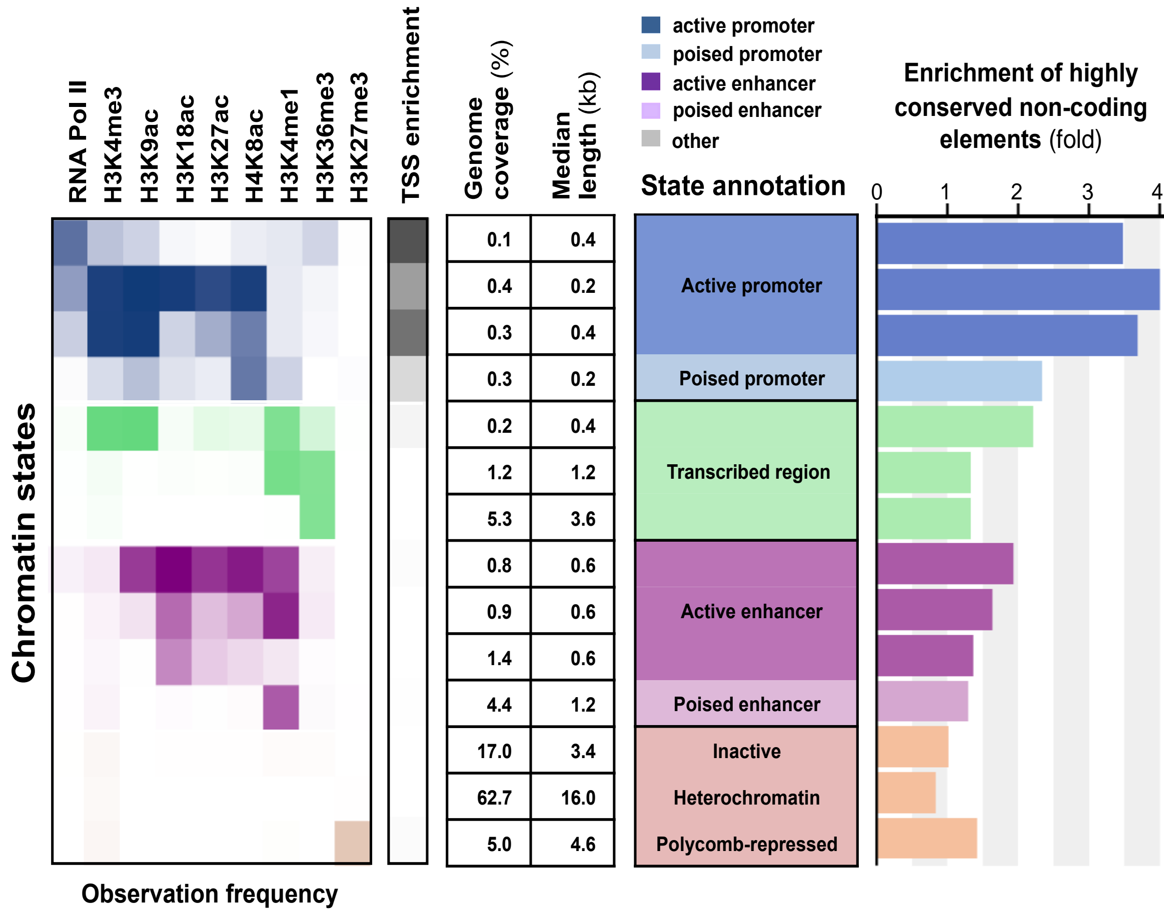

Figure 4. Characterization of the epigenome in proliferating myoblasts.

The 14-state chromatin state model was generated based on global ChIP-seq read enrichment for RNA Pol-II, H3K4me3, H3K9ac, H3K18ac, H3K27ac, H4K8ac, H3K4me1, H3K36me3 and H3K27me3 (GSE94558, Table 3). TSS enrichment was calculated as the ratio between the fraction of bases in the genome overlapping the feature and state and the joint probability that a base would overlap with the feature and state. Enrichment of highly conserved non-coding elements was calculated similarly (Hamed et al., 2017).

1.11. The retinoid X receptors

Nuclear receptors stimulate transcription upon activation by their agonists, and consequently, recruitment of coactivators at genomic regions takes place (Horwitz et al., 1996; McKenna et al., 1999). They are ligand-modulated transcription factors that respond to different hydrophobic molecules such as hormones, lipids, steroids and retinoids in order to influence gene expression. Upon ligand binding, the ligand binding domain folds into a α-helical sandwich, forming the ligand binding pocket, which is lined by hydrophobic specific residues. The agonist binding stabilizes the conformation of the ligand binding domain positioning a specific set of amino acids adequately for coactivator peptide interaction, and hence, the receptor-chromatin complex regulates their downstream transcription-related functions (Wurtz et al., 1996).

Nuclear receptors control transcription through recruiting specific co-regulators, transcription factors and components of the transcription initiation complex or RNA Pol-II to target regulatory regions (Acevedo and Kraus, 2004). In the absence of their agonists, nuclear receptors bind to co-repressors to keep target DNA in tightly coiled conformation, preventing accessibility to transacting factors. However, in the presence of agonists, nuclear receptors shift from binding co-repressors to coactivators. Subsequently, nuclear receptors bind to specific response elements within the DNA, termed hormone response element (HRE), located within the regulatory regions of the gene, often at the enhancers of their target genes, thereby, transcriptional activity is stimulated (Glass and Rosenfeld, 2000; McKenna and O'Malley, 2002; Yamada and Kakuta, 2014). Nuclear receptors have a high potential to be targeted pharmacologically as they have the ability to sense and respond to small-molecule ligands that can be modified to design drugs (Fan et al., 2013).

Nuclear receptors play essential roles in several physiological processes such as immunity, metabolism, lipid signaling, inflammation and myogenesis (Hollman et al., 2012; Lamers et al., 2012; Pascual-García and Valledor, 2012; Verhoeven et al., 2010). Notably, a previous study showed that adenoviral vector-mediated expression of the retinoic acid receptor (RAR) or RXR was able to induce *myosin heavy chain* (*MyHC*) reporter activity (Zhu et al., 2009), a gene that is continuously expressed during muscle development (Bi et al., 2017). Furthermore, studies conducted in our laboratory have reported that RXR-selective signaling contributes to the specification of skeletal muscle lineage (Le May et al., 2011). We have shown that rexinoid signaling, through RXR, also promotes myogenic differentiation (AlSudais et al., 2016). Therefore, we have focused on investigating the effect of RXR signaling on myogenic expression in early myoblast differentiation at a genome-wide scale.

Retinoid X receptors are ligand-inducible transcription factors that belong to the nuclear hormone receptor superfamily including non-steroid hormone receptors such as vitamin D receptor (VDR) and thyroid hormone receptor (THR), as well as steroid hormone receptors including estrogen receptor (ER) and androgen receptor (AR) (Szanto et al., 2004; Leid et al., 1992). Three distinctive isoforms of RXR have been identified: RXRα, RXRβ, and RXRγ, with RXRα being the predominant subtype expressed in adult skeletal muscle (Barbosa-Morais et al., 2012). RXR isoforms have different tissue distributions: RXRα is found in muscle, liver, lung, kidney, intestine and epidermis; RXRβ is ubiquitously expressed; and RXRγ is expressed in skeletal and cardiac muscles and brain (Dolle et al., 1994; Germain et al., 2006). Knockout studies showed that the function of RXR is crucial in early embryonic development. RXRα null mutants display myocardial and ocular abnormality and die in utero, whereas RXRβ and RXRγ null mice are viable and appear to be normal (Kastner et al., 1994;

Krezel et al., 1996). Just like other nuclear receptors, RXRs harbor multiple distinctive domains: N-terminal region (A/B), DNA-binding domain (C), hinge region (D), ligand binding domain (E) and C-terminal domain (F), (Figure 5). Within the LBD, there is a transactivation functional domain, a ligand binding pocket (LBP), a dimerization surface and a co-regulator-binding surface groove, (reviewed in Dawson and Xia, 2012; Mangelsdorf et al., 1995). RXRs are heterodimeric partners of about one-third of 48 other nuclear receptor superfamily members including VDR, THR, RAR, PPARs, liver X receptor (LXR) and farnesoid X receptor (FXR) (Mangelsdorf et al., 1995).

Given RXRs vast heterodimerization potential, they are involved in a broad array of cellular processes and signaling pathways. RXR isoforms can form homodimers, permissive heterodimers, or non-permissive heterodimers and can be either ligand-dependent or ligand-independent (Tanaka and Luca, 2009). When RXR forms permissive heterodimers, the complex can be indistinctly activated by ligands of either RXR or its partner due to the close proximity of the activation domain of the partner to RXR (Aranda and Pascual, 2001). However, when RXR forms non-permissive heterodimers, the ligand-induced transcriptional activity of RXR is inhibited (Forman et al., 1995). Therefore, RXR ligand binding only stimulates RXR homodimers or permissive heterodimers (Mangelsdorf et al., 1995), such as PPAR, LXR and pregnane X receptor (PXR). While RXRs, and their partner dimer, are constitutively bound to DNA, they require agonist binding to activate gene transcription (Leid et al., 1992; Umesono and Evans, 1989).

Rexinoids are compounds that bind to and modulate RXRs activities (Szanto et al., 2004; Altucci et al., 2007; Dawson and Xia, 2012; Kagechika, 2002; Nagpal and Chandraratna, 2000; Pérez et al., 2012). They are complexes that are chemically related to vitamin A, which bind to RXRs but not RARs, (reviewed in Yamada and Kakuta, 2014). Bexarotene (LGD1069)

is a synthetic rexinoid used in the treatment of mammary carcinogenesis (Orendas et al., 2012). Furthermore, it is a RXR-selective ligand and thereby is unable to activate RARs or transactivate the RXR-RAR heterodimer (Lehmann et al., 1992). We have shown that bexarotene acts through the function of RXRα, specifically to enhance myoblast differentiation, which coincides with the increase of MyoD and myogenin expressions (AlSudais et al., 2016).

The transcriptional function of nuclear receptors generally depends on the recruitment of HATs as part of the transcription complex at target promoters and enhancers in response to internal or external stimuli such as synthetic ligands (Shulman and Mangelsdorf, 2005). It has been shown that p300 acts as a transcription coactivator for nuclear receptor-mediated signaling (Kraus and Wong, 2002). p300/CBP has been associated to the function of glucocorticoid receptor, RARs and THR (Chakravarti et al., 1996; Kamei et al., 1996), In addition, p300 has been shown to act synergistically with ERα and RAR in order to enhance transcription initiation (Kraus and Kadonaga, 1998; Dilworth et al., 2000). Knockout studies have highlighted a distinct role of p300-mediated gene activation during induced myogenesis, in which p300 is required specifically for H3K18 and H3K27 acetylation (Jin et al., 2011). Moreover, previous studies have demonstrated that nuclear receptors are strongly associated with H3K18 acetylation (Jin et al., 2011; AlSudais et al., 2016). For example, it has been reported that, upon estrogen stimulation, the level of H3K18 acetylation increases at the ER-targeted promoters (Daujat et al., 2002). Moreover, H3K18ac decreased at targeted-gene enhancers after reducing p300 recruitment in the estradiol-stimulated MCF-7 breast cancer cell line, which significantly decreased p300-mediated H3K18 acetylation in the cells (Yi et al., 2017), indicating that p300, as a HAT, plays a vital role in nuclear receptor-targeted gene

transcription activation (Jin et al., 2011). Therefore, we focused our study on exploring the changes in histone acetylation in response to rexinoid, specifically during early myogenic differentiation.

Figure 5

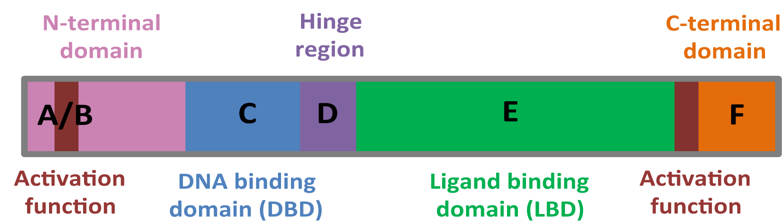

Figure 5. Nuclear receptor domain organization.
The structural basis of nuclear receptors shown here is the N-terminal domain (Pink), DNA-binding domain (blue), Hinge region (purple), ligand binding domain (green) C-terminal domain (orange) and the activation function domains (red) within the N- and C-terminal domains. Adapted from (Chandra, et al., 2017)

Rationale and hypotheses

The coactivator p300 is essential for the expression of muscle-specific genes. We have shown before that the histone acetyltransferase activity of p300 at the *MyoD* core enhancer region specifically is necessary for the expression of MyoD, implying the significant role of p300 in myogenic differentiation. Interestingly, RXR signaling has been shown to induce the specification of muscle lineage and to promote myogenic differentiation. Dystroglycan plays a role in maintaining muscle integrity along with other DGC complex proteins. However, it has been claimed lately that Dag1 expression sustains efficient regenerative ability of satellite cells.

Firstly, the compendium of p300-dependent distal regulatory elements that govern myogenic differentiation from myoblasts to myotubes has been explored. However, the features of these elements at an earlier stage of differentiation remain less clear. Considering that loci-specific acetylation at lineage-specific enhancers play a critical role in the control of genes required for differentiation, we hypothesize that p300 associates with distinct histone acetylation when occupying early active or poised myogenic enhancer loci.

In addition, we have shown earlier that rexinoid signaling, through RXRα, is required for ligand-promoted myogenic differentiation. However, the interplay of rexinoids with myoblast-specific chromatin state has not been identified yet. Therefore, we hypothesize that rexinoid signaling associates with specific histone acetylation at the active or poised enhancers of target genes in early myogenic differentiation.

Moreover, we identified a potential poised enhancer locus of *Dag1* that is occupied by p300. Thus, we hypothesize that dystroglycan is a p300-dependent target and is associated with specific acetylation upon myoblast differentiation.

To address these hypotheses, we have performed ChIP-seq to determine the DNA binding profiles of RXRα and p300, along with the deposition of histone acetylation marks, incorporated with RNA-seq during early differentiation and in response to rexinoid.

The specific aims of this study were:

(1) To investigate the molecular mechanisms of myogenic gene activation by p300 at the onset of myogenic differentiation

(2) To explore the mode of action of rexinoid signaling and its relation to myoblast-specific chromatin states in early myoblast differentiation

(3) To examine the molecular mechanism underlying the expression of dystroglycan in early myogenic differentiation.

Chapter 2. Materials and methods

2.1. Cell culture

C2C12 myoblasts acquired from the American Type Culture Collection were maintained in Dulbecco's Modified Eagle's Medium (D-MEM, Wisent) supplemented with 10% fetal bovine serum (HyClone) and 1% Penicillin/Streptomycin (P/S, Wisent) at 37°C with 5% CO_2. Similarly, D-MEM supplemented with 2% horse serum (Gibco) and 1% P/S was utilized as a differentiation medium. Cells were incubated at 37 °C with 5% CO_2. To induce the differentiation process, C2C12 cells were grown to a confluency of approximately 80% after which, they were switched to differentiation medium for a required amount of time. The addition of 50 nM bexarotene (LC Laboratories) was used as needed in the treatment condition.

2.2. shRNA knockdown

C2C12 myoblasts were grown in D-MEM supplemented with 10% fetal bovine serum to about 30% confluence and transduced at a MOI of 30 with lentiviral particles targeting p300 in the presence of Polybrene (5 µg/ml, Santa Cruz), according to the manufacturer's protocol (Santa Cruz Biotechnology). A nonsilencing shRNA was used as a negative control. Puromycin (2 µg/ml) was used to select pooled stable clones beginning two days after infection for a total duration of 5-8 days. Puromycin was purchased from Sigma.

2.3. Immunofluorescence microscopy

At the indicated time point, C2C12 cells were fixed with cold methanol for 20 minutes, rehydrated in 1X PBS for 5 minutes and incubated overnight with an antibody against myosin heavy chain (1:10 MF20, Developmental Studies Hybridoma Bank). Following rapid three 1X PBS washes, cells were incubated with 1:200 Alexa Flor®594 secondary antibody (Invitrogen) for 2 hours. The cells were also incubated with 0.1 μg/ml of Hoechst (Molecular Probes) to stain the nucleus. The coverslips were mounted on slides with 50% glycerol. Axiovert 200M microscope, AxioCam HRM camera and AxioVision Rel 4.8 software (Zeiss) were used to capture the images through a 10X objective. For each coverslip, five random images were analyzed. The myogenic index was determined as the percentage of myocyte nuclei in relation to the total number of nuclei. ImageJ software was used for cell counting. The student's *t*-test was used for statistical analysis. Each experiment was repeated at least three times.

2.4. Western blot analysis

C2C12 cells were lysed by incubation in whole-cell extract buffer (10% glycerol, 50 mM Tris-HCl pH 7.6, 400 mM NaCl, 5 mM EDTA, 1 mM DTT, 1 mM PMSF, 1% NP-40) on a rotator for 30 minutes at 4°C, and then the lysate was centrifuged at 14,000 × *g* at 4°C for 10 min. Protein concentration was determined by Bradford assay (Bio-Rad), using the Multiscan Spectrum Photospectrometer (Thermo Electron Corporation). The proteins were separated by 6%-12% SDS-PAGE and transferred to Immun-Blot PVDF membrane (BioRad). After transfer, membranes were blocked in 5% milk in PBS supplemented with 1% Tween-20 in PBS for 1 hour and then were sequentially probed with indicated primary and secondary antibodies. Immunoreactive bands were developed using Western Lightning

Chemiluminescence (Perkin Elmer) reagents and quantified using ImageLab software from BioRad. Antibodies specific for p300 was obtained from Santa Cruz Biotechnology (1:100, sc-584). Antibodies to myogenin (1:100 F5D), β-tubulin (1:100 E7), MyHC (1:100 MF20) and α-Dag1 (1:100 IIH6) were from Developmental Studies Hybridoma Bank. Antibodies for H4K8ac, H3K9ac, H3K18ac, H3K27ac and total H3 were obtained from Abcam (1:200 ab15823, 1:200 ab4441, 1:200 ab1191, 1:200 ab4729 and 1:200 H3 ab12079, respectively). Secondary antibodies used were: 1:2500 Anti-Mouse IgG (H+L) HRP Conjugate (Promega) and 1:2500 Anti-Rabbit IgG (H+L) HRP Conjugate (Promega). All experiments were repeated at least three times. Data were presented as the mean ± STDEV, where *p* values were determined using student's *t*-test.

2.5. Reverse transcription-quantitative PCR (RT-qPCR)

Total RNA was isolated from C2C12 myoblasts using Total RNA kit I (Omega) following the manufacturer's protocol. Reverse transcription to cDNA was performed using a High Capacity cDNA Reverse Transcription kit (Applied Biosystems). Total RNA was quantified by Nanodrop (ND-1000). Real-time PCR was conducted using a SYBR® Green and HotStarTaq DNA polymerase (Qiagen) on a CFX96 Touch Real-Time PCR Detection System (BioRad). Each sample was PCR amplified in triplicates. Results were analyzed by the threshold cycle (Ct) comparative method using TATA-Binding Protein (Tbp) or Ribosomal Protein S26 (Rps26) as an internal control. The experiments were repeated at least three times. Data were presented as the mean ± S.E.M, where *p* values were determined using student's *t*-test. MyoD, myogenin, Dag1, Angptl4, Tbp, Gpc6, Tnnt1, Igsf3, Asb2, Sntb1 and Tnnc1 gene specific primers are listed in Table1.

2.6. Chromatin immunoprecipitation (ChIP)

C2C12 cells were differentiated for 24 hours after, after which the cells were crosslinked with 1% formaldehyde for 20 min at room temperature (RT), lysed with lysis buffer (1% SDS, 50 mM Tris-HCl pH 8.0, 10 mM EDTA and 0.1% protease inhibitor cocktail (EMD Millipore Calbiochem™) and sonicated, followed by chromatin immunoprecipitation. Equal amounts of DNA were diluted with dilution buffer (20 mM Tris-HCl pH 8.0, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100 and 0.1% protease inhibitor cocktail) for immunoprecipitation with specific antibodies overnight at 4°C. Antibodies used for ChIP-seq were specific for H4K8ac, H3K9ac, H3K18ac and H3K27ac and were obtained from Abcam (ab15823, ab4441, ab1191, ab4729), whereas those for p300 and RXRα were from Santa Cruz (sc-584x and sc-553x). Other antibodies were used for ChIP experiments, p300 and MyoD were obtained from Santa Cruz (sc-584 and sc-32758), myogenin F5D was from the Developmental Studies Hybridoma Bank and H3K18ac was from Abcam (ab1191). For each batch of immunoprecipitation, corresponding normal IgG antiserum was used as a negative control. The immunoprecipitants were captured by incubation with dynabeads protein-A or G (Invitrogen) for 2 hours at 4°C. The immune complexes were washed sequentially for 20 min with washing buffers A (0.1% SDS, 20 mM Tris-HCl pH 8.0, 150 mM NaCl, 2 mM EDTA and 1% Triton X-100), buffer B (0.1% SDS, 20 mM Tris-HCl pH 8.0, 500 mM NaCl, 2 mM EDTA and 1% Triton X-100), buffer C (1% sodium deoxycholate, 20 mM Tris-HCl pH 8, 0.25 M LiCl, 1 mM EDTA and 1% NP-40) and twice for 10 min with TE buffer (10 mM Tris-HCl pH 8.0 and 1 mM EDTA). The immunocomplexes were then extracted with elution buffer $T_{50}E_{10}S_1$ (50mM Tris-HCl, 10mM EDTA and 1% SDS) for 30 min at room temperature. Reverse crosslinking was performed at 65°C overnight. DNA for ChIP was purified using the DNA purification kit (Qiagen), whereas the DNA for ChIP-seq was purified using the

MinElute Spin Columns Kit (Qiagen) and input chromatin DNA was used as control. The chromatin DNA was then purified and real-time PCR was performed using a SYBR® Green and HotStarTaq DNA polymerase (Qiagen) on a CFX96 or CFX384 Touch Real-Time PCR Detection System (BioRad). Each sample was amplified in triplicate PCR reactions. Purified input DNA was used to create a standard curve during the PCR amplification for each immunoprecipitant. Quantification was analyzed as the abundance of immunoprecipitated target DNA as a percentage of input chromatin DNA (enrichment as the percentage of input). Each ChIP was repeated at least three times.

2.7. ChIP-seq data processing

Purified DNA was sequenced by the McGill University Genome Quebec Innovation Centre with Illumina HiSeq 2000 as single-end 50 nucleotide reads, according to Illumina instructions with input chromatin DNA used as control. Sequencing reads were mapped to the mouse genome build mm9 using Bowtie, allowing for three mismatches and reporting the single best alignment per 50 bp read. Picard was used to filter out replicated reads (http://picard.sourceforge.net/), and BAM files were converted into BED files with the BEDTools suite (Quinlan and Hall 2010). For visualization of ChIP-seq signals in the Integrative Genomics Viewer, aligned reads were extended by 125 bp at their 3′ end and basewise signal intensity was computed. Peak detection was performed using the model-based analysis of ChIP-seq (MACS) software (Zhang et al., 2008) (*v*1.0.0) with a *P*-value threshold of 1×10^{-5}. Peak annotation followed by gene ontology (GO) using GREAT v3.0.0 (McLean et al., 2010) utilizing the whole genome as background and the single nearest gene association rule.

2.8. Chromatin State Model

ChIP-seq datasets were obtained from the NCBI Gene Expression Omnibus (GEO) for RNA Pol-II under the accession number GSM721286 and H3K4me1 under GSM721288 (Asp et al., 2011), while H3K4me3 under GSM918415 and for H3K36me3 under GSM918417 (Yue et al., 2014). The corresponding input for Pol-II and H3K4me1 were obtained under GSM721306 (Asp et al., 2011), and for H3K4me3 and H3K36me3 under GSM918421 (Yue et al., 2014). Genome-wide binding sites for MyoD and myogenin were obtained for MyoD_GM under accession number GSM915186, for MyoD_24h under GSM915183 and myogenin_24h under GSM915159 (Yue et al., 2014). The chromatin state model was generated using ChromHMM (Ernst and Kellis, 2012). The enrichment of transcription start sites (TSSs), Pol-II binding sites and highly conserved non-coding elements (HCNCEs) was calculated as a ratio between the fraction of nucleotides overlapping between the feature and state and the joint probability of observing the feature and state. HCNCEs for the mm9 build were identified using genomic evolutionary rate profiling (Davydov et al., 2010).

2.9. Analysis of histone enrichment and transcription factor binding sites

The enrichment of histone acetylation at the MRF binding sites was calculated with ngsplot (Shen et al., 2014), which calculates the coverage vectors for each query region based on specified alignment files. Following normalization and transformation on the coverage, an average profile is created as the number of reads in 20 bp bins within a 2 kb region, centered at the peaks and normalized to the total number of mapped reads (in millions) in the dataset. Two-sided Wilcoxon signed-rank test was used for statistical analysis. Homer (Heinz et al., 2010) was used to perform de novo motif analysis for p300 and RXRα ChIP-seq peaks, allowing for motif identification within 100 bp region from the peak center. Overlapping peaks

were calculated as being located within 100 bp of one another with the mergePeaks tool within Homer.

2.10. Genomic data deposition

The ChIP-seq and RNA-seq data generated and analyzed for this study have been deposited in the National Center for Biotechnology Information Gene Expression Omnibus (GEO) database and are accessible through GEO Series accession numbers GSE109636 (p300 ChIP-seq data), GSE94558 (RXR and histones ChIP-seq data) and GSE94560 (RNA-seq data).

Chapter 3. Results

3.1. p300 is required for early myogenic differentiation

To delineate the function of the p300, we used a shRNA knockdown approach in C2C12 myoblasts to reduce the endogenous levels of p300 protein. Western blotting analyses showed that the introduction of p300 shRNA into proliferating myoblasts effectively knocked down the level of p300 as well as significantly decreased the expression of myogenin, which is a lineage-specific marker of differentiation protein, by roughly 75% (Figure 6A and B). In addition, the expression of myogenin mRNA was significantly inhibited as shown by RT-qPCR analyses (Figure 6C). Immunofluorescence staining of myosin heavy chain (MyHC), a marker of terminal myogenic differentiation revealed a decrease in myotube formation in p300 knockdown cells as compared to control non-silencing shRNA (Figure 6D). Concurrently, there was a significant decrease in myoblast differentiation and fusion (Figure 6E). Therefore, these data reveal the importance of p300 in C2C12 myogenic differentiation, which agrees with previous findings that showed that there was a myogenic differentiation impairment in ES cells lacking p300 (Shikama et al., 2003).

Figure 6

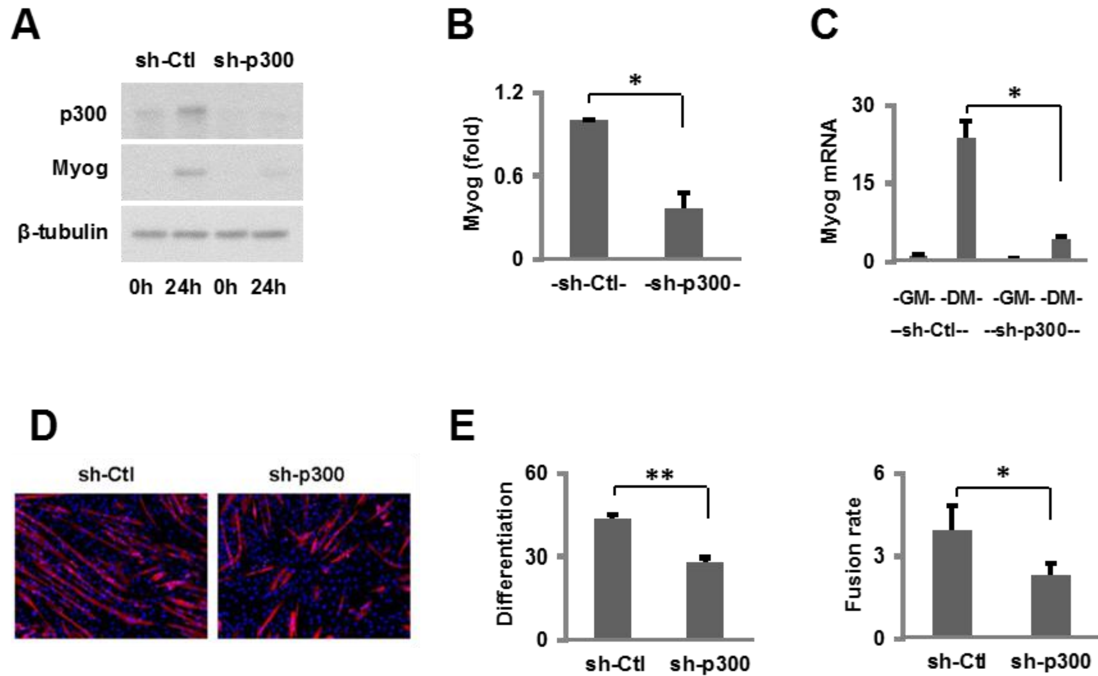

Figure 6. p300 is required during early myogenic differentiation.
C2C12 cells transfected with p300 shRNA (sh-p300) and non-silencing shRNA as controls (sh-Ctl) were differentiated for 24 and utilized for Western blotting. (A) The levels of p300 and myogenin proteins were analyzed on day 1 of differentiation. The blots were then stripped and re-probed for β-tubulin as loading controls. (B) Quantification of the myogenin blots is presented as the fold change relative to the non-silencing shRNA control normalized to β-tubulin (*$p < 0.05$, n = 3). (C) p300 knockdown cells were subjected to RT-qPCR analysis of myogenin mRNA on day 1 of differentiation. Quantification is presented as the fold change relative to proliferating myoblasts normalized to TBP as an internal control (*$p < 0.05$, n=3) and was calculated using the formula $2^{-\Delta\Delta CT}$. (D) Shown are the representative microscopic images of myosin heavy chain (red) and nuclei (blue) co-stained after 4 days of differentiation. (E) Differentiation was defined as the percentage of myocyte nuclei relative to the total number of nuclei, whereas the fusion rate was defined as the average number of nuclei per myocyte. Error bars are the standard deviations of four independent experiments (*$p < 0.05$, **$p < 0.01$).

3.2. Histone acetylation is enriched at p300-associated loci upon differentiation

Since p300 is a histone acetyltransferase and its HAT activity is necessary for myogenic differentiation (Polesskaya et al., 2001; Shikama et al., 2003), we examined the global levels of p300, myogenin and also global histone acetylation levels during early myogenic differentiation. The levels of myogenin protein increased more than 10-fold upon early differentiation, whereas p300 expression remained relatively steady (Figure 7A and B). Furthermore, Western blot analysis showed no significant changes in H3K9ac, H3K18ac and H3K27ac, whereas H4K8ac was augmented by roughly 2-fold (Figure 7A and B). Therefore, our analyses indicate that if there is a differentiation-dependent change in the levels of p300 and residue-specific histone acetylation, it may occur in a loci-specific manner rather than globally. In order to investigate the role of p300 during the early stages of myogenic differentiation, we set out to perform ChIP-seq of p300 in C2C12 cells. While maintaining a high correlation with transcriptional programs of primary myoblasts, C2C12 myoblasts are less prone to spontaneous differentiation (Asp et al., 2011; Blais et al., 2005) and thus they are a system of choice for genome-wide studies of histone modification and associated transcriptional regulators, including p300 in myotubes and proliferating myoblasts (Blum et al., 2012). ChIP-seq was performed in proliferating myoblasts and myoblasts that had been allowed to differentiate for 24 hours. Following model-based ChIP-seq analysis (MACS) of p300 read enrichment, 5,405 and 2,060 high confidence peaks were identified in proliferating and differentiating myoblasts, respectively (Figure 7C). The average PhastCons conservation scores of p300-associated sites were analyzed as a measurement of the technical validity of the p300 enriched sites identified by MACs (Figure 7D).

To further explore the characterization of p300-bound loci, we used a chromatin state model based on genome-wide co-presence of different epigenetic marks, in committed proliferating myoblasts (Hamed et al., 2017), by incorporating our own ChIP-seq of H3K9ac, H3K18ac, H3K27ac, H4K8ac and H3K27me3 (GSE94558) along with published ChIP-seq datasets (Asp et al., 2011; Yue et al., 2014; Davydov et al., 2010). Utilizing the chromatin state model, we next annotated different categories of p300 read signals through the progression of myoblast differentiation and found that p300 peaks are predominantly associated with active enhancers (Figure 7E). Additionally, the percentage of poised enhancers escalates from 5% in p300 peaks unique to proliferation to 15% in peaks unique to differentiation (Figure 7E), supporting a role for p300 in the activation of myogenic-specific loci during early differentiation.

Since histone acetylation is required for the modulation of regulatory regions, we also profiled loci-specific changes in histone acetylation during early differentiation (Figure 7E and F). We observed a differentiation-dependent enrichment observed not only for H3K18ac and H3K27ac, which are known acetylation targets of p300 (Jin et al., 2011) but also in H4K8ac and H3K9ac particularly at differentiation-unique p300 loci. Interestingly, H3K9ac showed the most prominent relative increase as compared to the other acetylation marks (Figure 7E). Therefore, the level of p300 occupancy is closely associated with loci-specific histone acetylation during early myoblast differentiation.

Figure 7

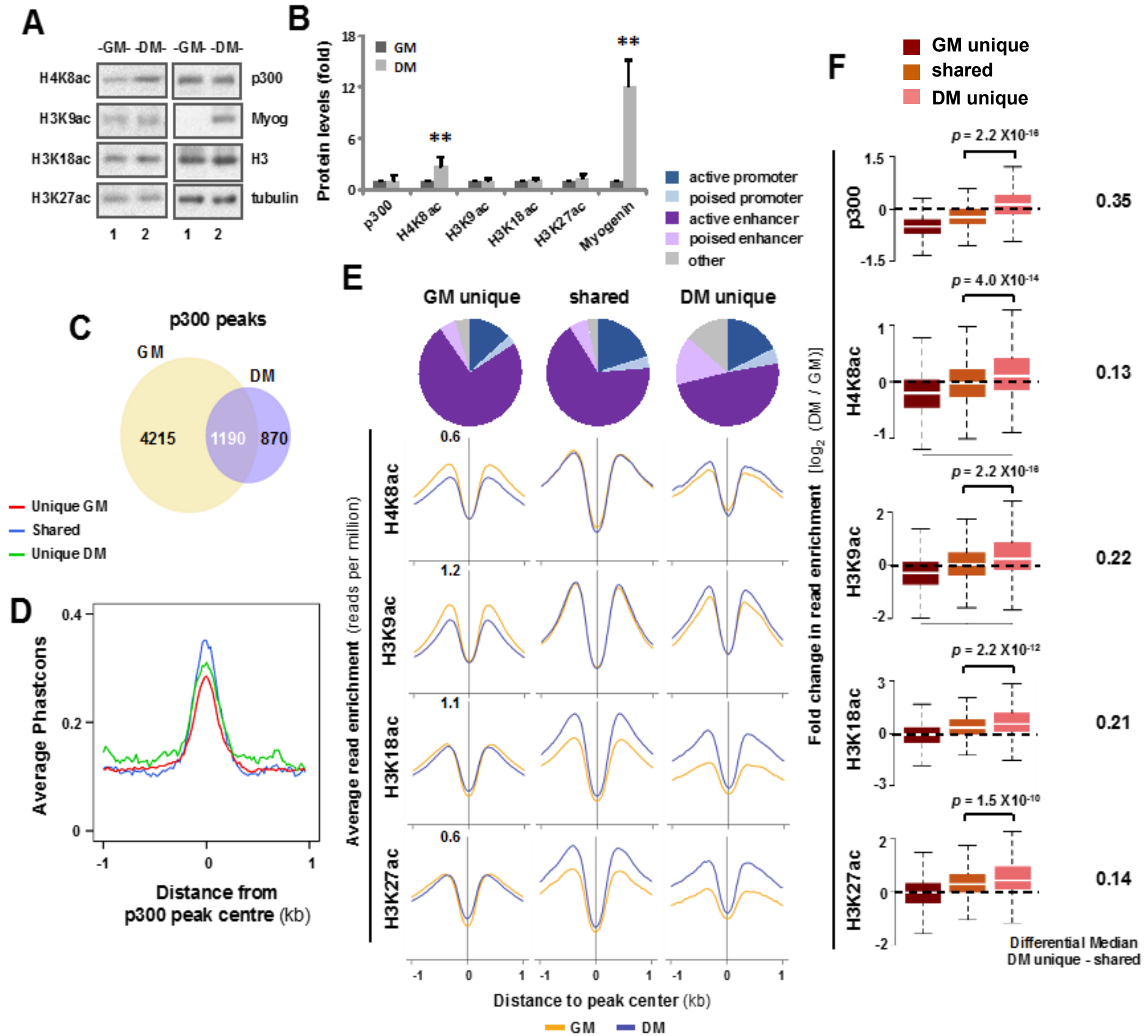

Figure 7. Histone acetylation is enriched at p300-associated loci upon differentiation.
(A) C2C12 cells were differentiated for 24 hours followed by Western blotting analysis for p300, myogenin, and histone marks (H4K8ac, H3K9ac, H3K18ac and H3K27ac) with β-tubulin as a loading control. (B) Quantification of the Western blots in panel A is presented as fold change relative to proliferating myoblasts (GM) (error bars: SD; n = 3; *$p < 0.05$). (C) Union analysis of p300 peaks in proliferating C2C12 (GM) and myoblasts differentiated for 24 hours (DM). (D) The average PhastCons conservation scores of p300 peaks in proliferating (GM) and differentiated myoblasts (DM). (E) Chromatin state distribution of p300 loci. The average read enrichment profiles of H4K8ac, H3K9ac, H3K18ac, and H3K27ac spanning 2 kb across the indicated p300 loci annotated above. (F) Boxplots present a log2-fold change in signal enrichment at the p300 loci. The differential median between enrichment at p300 loci unique to differentiation or shared are displayed to the right (Wilcoxon signed-rank test, *$p < 0.05$).

3.3. MyoD and p300 mainly co-localized at enhancers during early differentiation

As a transcriptional coactivator, p300 does not directly bind to DNA but is recruited by DNA-binding transcription factors. Thus, we searched for enriched motifs associated with p300-dependent loci. We first divided the p300-associated loci into groups that were unique to proliferation, shared or unique to differentiation as in Figure 7E. *De novo* motif analysis revealed that MyoD and myogenin motifs were found primarily at p300-associated shared or unique to differentiation loci but not unique to proliferation (Figure 8A). Collectively, our data indicate that p300 might be recruited predominantly by MRFs in order to regulate myogenic targets in early differentiation.

MyoD and myogenin binding sites were found to associate to p300 loci; however, since MyoD is involved in the initial step of myoblast differentiation, while myogenin is required for terminal differentiation and that MyoD functions upstream of myogenin (Moncaut et al., 2013), we analyzed the genome-wide co-localization of p300 and MyoD. For this purpose, we used the Caltech published genome-wide MyoD ChIP-seq data in proliferating and myoblasts differentiated for 24 hours under the ENCODE project consortium (http://encodeproject.org/ENCODE). Intriguingly, our chromatin state analysis showed that there is an increase in the occupancy of enhancers by p300 when co-localized with MyoD, particularly poised enhancers in differentiating myoblasts from about 8% to 12% and no such an increase was apparent in proliferating myoblasts (Figure 8B). Moreover, when the distance of p300 peaks to the nearest TSS was analyzed, we found that the majority of p300 peaks located in the promoter regions (0 to −1 kb) lacked MyoD overlap (Figure 8C). Nevertheless, more p300 peaks co-localized with MyoD were found distal to the promoters (−1 to −50 kb), in regions that are more likely to be enhancers, active or poised (Figure 8C).

Thus, an increase in p300 recruitment to MyoD-bound poised enhancers may be important for the initiation of myoblast differentiation. (Figure 8B and C).

To further explore the relationship of p300 and MyoD, we examined the histone profiles at p300-associated loci categorized with and without a MyoD overlap. As shown in Figure 8B, p300-associated regions displayed an increase in H3K18ac and H3K27ac acetylation regardless of the presence of MyoD. In contrast, H4K8 and H3K9 acetylation are distinct in that enrichment is only seen when p300 co-localizes with MyoD (Figure 8B). More interestingly, quantification of condition matching RNA-seq data (Hamed et al., 2017) revealed a significant increase in the expression of genes associated with p300 in differentiating myoblasts, but only when co-localized with MyoD (Figure 8D). Therefore, our data shed new light into the functional mode of p300 when recruited by MyoD at the early stage of myoblast differentiation, in that the changes in loci-specific histone acetylation correlate with differentiation-specific myogenic expression.

Figure 8

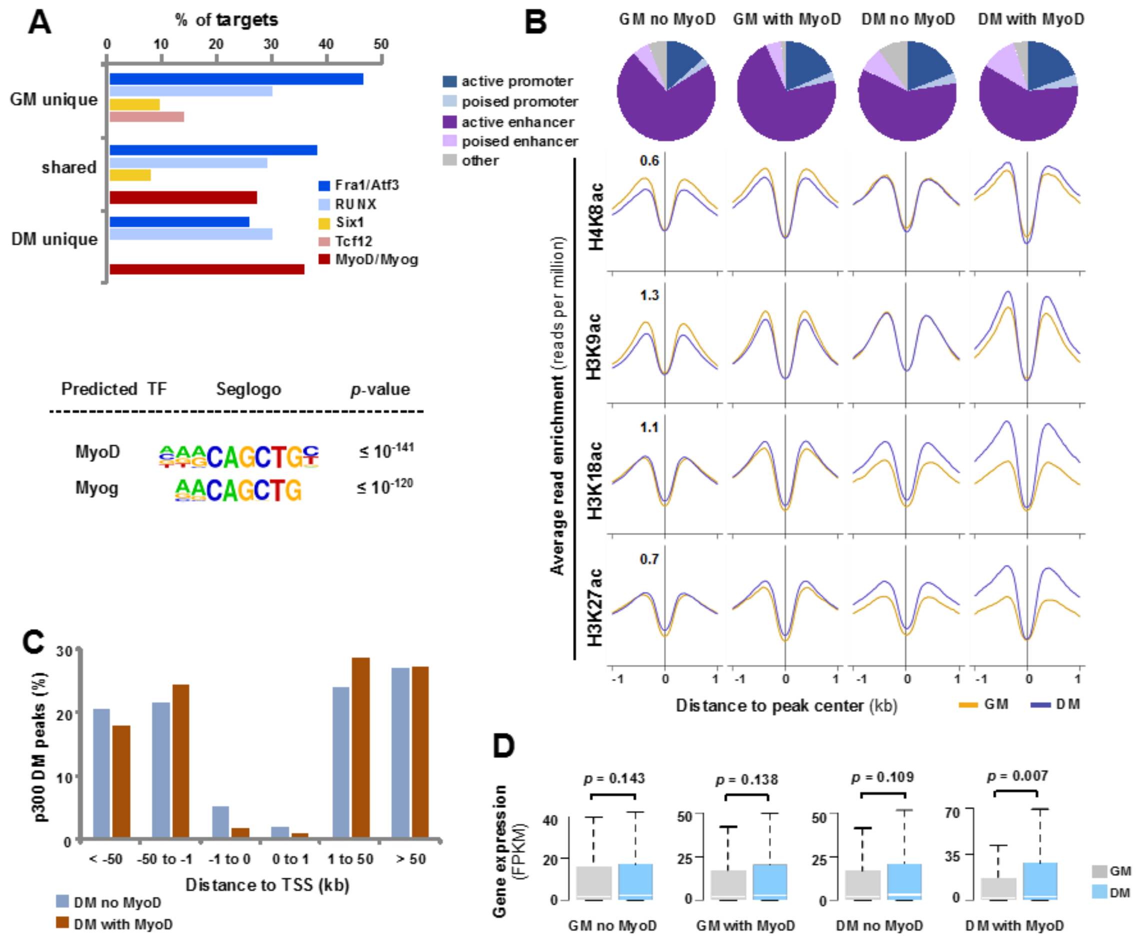

Figure 8. MyoD and p300 co-localization correlates with H4K8ac and H3K9ac enrichment. (A) De novo motif analysis was performed for the p300 loci as categorized in Figure 7C. The percentage of p300 loci bound by the top-ranked motifs are presented as a bar graph, with *p*-values for the motifs being equal to or lower than the one stated in each category presented at the bottom. (B) Chromatin state distribution of p300 loci in proliferating (GM) and differentiating (DM) conditions, with or without a MyoD overlap. The average read density of indicated histone acetylation spanning 2 kb across corresponding p300 loci is presented below. (C) The distance of p300 peaks with and without a MyoD overlap in relation to the closest TSS is quantified as a bar graph (D) The expression levels of ENSEMBL genes associated to the indicated categories of p300 peaks of panel B are plotted as FPKM measured by RNA-seq analysis (Wilcoxon signed-rank test, $*p < 0.05$).

3.4. Co-occupancy of p300 and MyoD at the enhancers of differentiation-dependent genes

To identify genes bound by and potentially regulated by p300 during myogenic differentiation, we examined selected target loci based on the ChIP-seq read coverage (Figure 9A) to evaluate the transcriptional dependency of these genes on p300. The designated loci for Tnnt1, Igsf3, Asb2 and Gpc6 genes displayed an increase in p300 and MyoD binding upon differentiation, as represented by read density (Figure 9A). We next performed ChIP for p300 and MyoD with normal IgG antiserum and a random locus as negative controls. The ChIP-qPCR analysis shows that the occupancy of p300 to the putative regulatory loci of Tnnt1, Igsf3, Asb2, and Gpc6 was significantly enriched by 24 hours of differentiation, corresponding to a concurrent increase in MyoD binding (Figure 9B). As poised/inactive lineage-specific enhancers play a determinant role in stem cell differentiation (Xu et al., 2009) and MyoD interacts with p300 (Puri, et al., 1997; Sartorelli et al., 1997), the recruitment of p300 by MyoD to target loci may thus be important for the expression of muscle-related genes. Therefore, examining the chromatin state associated with differentiation-specific regulators can be applied to delineate the molecular mechanisms by which gene expression is facilitated in a condition-specific manner. To assess the requirement of p300 for the expression of these target genes, we employed p300 shRNA knockdown myoblasts. As shown in Figure 9C, the mRNA expression of the indicated genes is significantly augmented within the first 24 hours of differentiation, however, their expression was reduced following the p300 knockdown. Together, these data suggest that p300 is indeed required for the expression of differentiation-dependent genes.

Figure 9

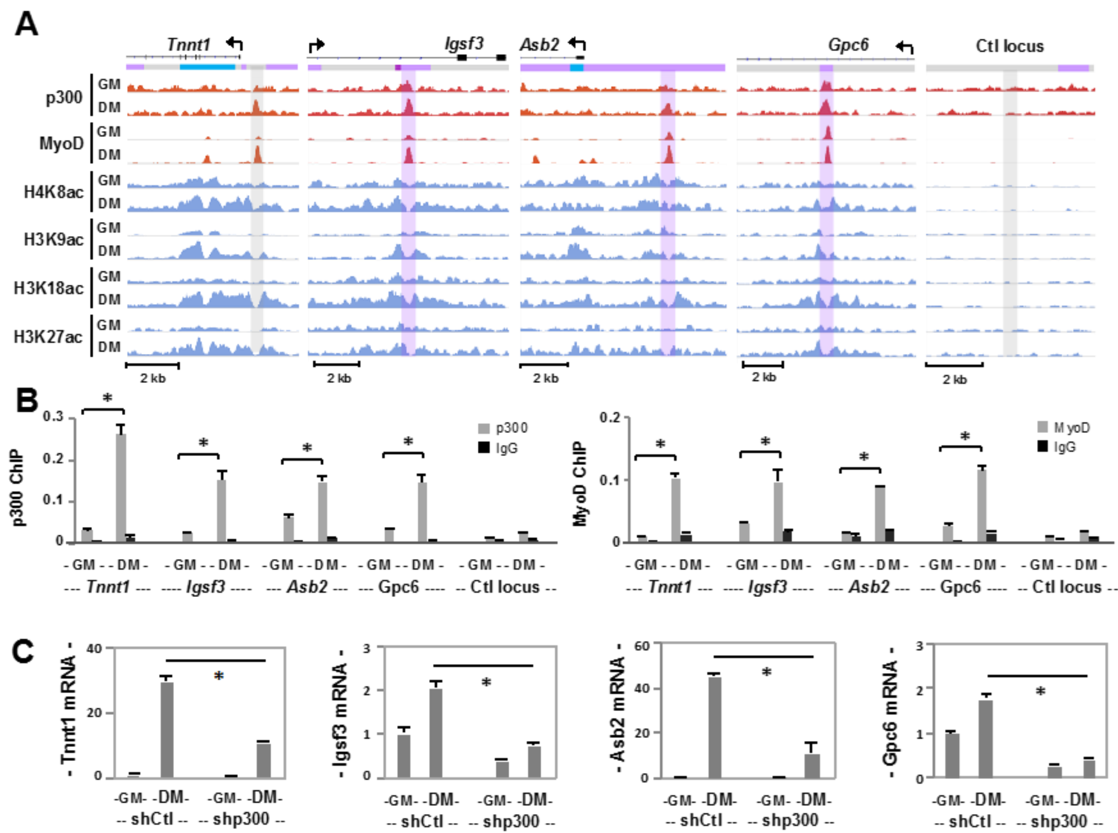

Figure 9. p300 is required for myogenic gene expression.
(A) Genome browser view of p300, MyoD and histone acetylation including H4K8ac, H3K9ac, H3K18ac and H3K27ac read density at *Tnnt1, Igsf3*, *Asb2*, and *Gpc6* loci (genes fall within the categories in panel B of figure 8). Black bars show Refseq gene position and the colors of ChromHMM track below correspond to that designated to each chromatin state as illustrated in Figure 8B. (B) For validation, ChIP-qPCR analysis was performed for identified target loci, using antibodies against p300 and MyoD. Normal IgG antiserum and a random locus (Ctl) were used as negative controls. Quantification is presented as the percentage of enrichment in relation to input chromatin DNA (error bars: SEM; n=3; *$p < 0.05$). C2C12 cells were differentiated for 24h following the introduction of p300 shRNA and non-silencing shRNA as controls. (C) RT-qPCR analysis of gene expression levels for p300-dependent and differentiation responsive targets (from panel B) is presented as fold change relative to proliferating myoblasts (GM), normalized to Tbp and calculated using the formula $2^{-\Delta\Delta CT}$ (error bars: SEM; n=3; *$p < 0.05$).

3.5. The effect of bexarotene is mediated through RXRα

We have previously shown that bexarotene enhances myogenic differentiation and fusion through the function of RXRα (AlSudais et al., 2016), indicating that RXRα ligand activation plays an essential role in augmenting muscle-related gene expression. Following RNA-seq analysis, we identified 187 bexarotene-responsive genes, which were upregulated following 24 hours of differentiation and treatment (Figure 10A), and many of these genes were associated with muscle-related functions (Figure 10B). Therefore, to identify direct genetic targets of RXR signaling during early myogenic differentiation, we performed ChIP-seq of RXRα in proliferating and differentiating C2C12 myoblasts in the presence or absence of bexarotene (GSE94558). In addition, to better understand the effects of rexinoid on epigenetic modifications, we also performed ChIP-seq for H4K8ac, H3K9ac, H3K18ac and H3K27ac in matching conditions (GSE94558).

Through MACS analysis of the RXRα read enrichment signals, 627 and 1207 RXRα high confidence peaks were identified in the treated and untreated condition, respectively. The average PhastCons conservation scores of RXRα binding sites represent one measure of the technical validity of the RXRα enriched sites identified by MACs (Figure 10C). Following a subsequent *de novo* motif search of RXRα-bound sequences, we identified a consensus nuclear receptor-binding motif as the top-ranking motif identified for both conditions (Figure 10D). This is in agreement with the functional mechanism of nuclear receptors such as RXRs, which are constitutively bound to their DNA sites, despite the absence of ligand (Pazin and Kadonaga, 1997; Rosenfeld et al., 2006; Torchia et al., 1998). In relation to this, we also identified a RXRα peak on *Angptl4*, a classical target of RXR signaling, locus in differentiating myoblasts regardless of bexarotene treatment (Figure 10E). In order to validate the effect of

bexarotene on the expression of *Angptl4*, we performed RT-qPCR and found that the mRNA levels of Angptl4 were indeed enhanced following bexarotene treatment of early differentiating myoblasts, however, the positive effects of bexarotene were obstructed by the introduction of RXRα shRNA knockdown (Figure 10F). Thus, these sets of data indicate that bexarotene-enhanced gene expression is indeed mediated by RXRα function as a transcription factor.

Figure 10

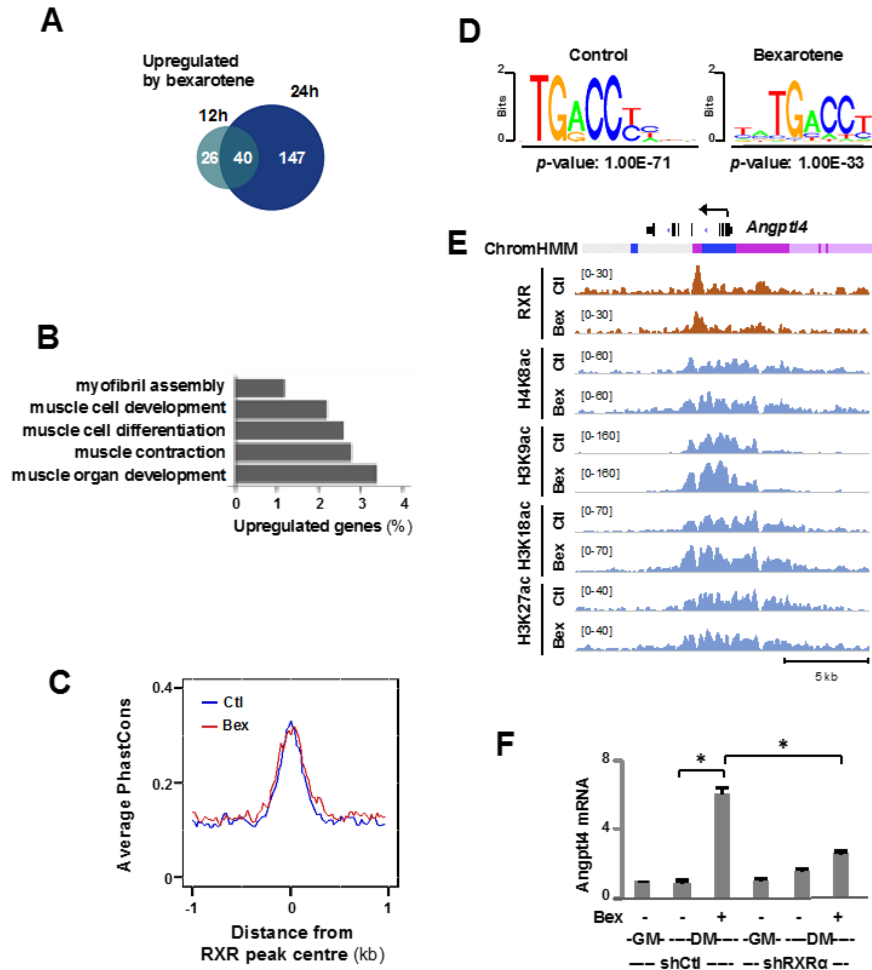

Figure 10. RXRα ChIP-seq identifies nuclear receptor-regulated loci.
(A) Overlap analysis of genes categorized as responsive to bexarotene after 12 or 24 hours of C2C12 differentiation, as identified by RNA-seq. (B) Gene ontology (GO) terms associated with genes that were responsive to bexarotene. (C) The average PhastCons conservation scores of RXRα peaks in myoblasts differentiated for 24 hours in the presence (Bex) or absence (Ctl) of bexarotene. (D) Consensus binding sequences of nuclear receptors were discovered through *de novo* motif analysis of RXRα peaks in myoblasts differentiated for 24h with or without bexarotene. (E) Genome browser view of RXRα and indicated histone acetylation signals at the *Angptl4* locus. Black bars show Ref-seq gene position and ChromHMM track colors below correspond to color designated to each chromatin state. RXR-knockdown (shRXR) myoblasts were differentiated in the presence or absence of bexarotene for 24 hours along with proliferating myoblasts (GM). A non-silencing shRNA (shCtl) was used in parallel as a control. (F) C2C12 myoblasts were differentiated for 24 h and subjected for RT-qPCR analysis. The mRNA levels of Angptl4 were assessed and plotted as fold change in relation to proliferating myoblasts after normalization to Tbp. Data calculated using the formula $2^{-\Delta\Delta CT}$ (error bars: SEM; n=3; *$p < 0.05$).

3.6. RXR directly binds to the MyoD core enhancer region

Since MyoD is a major myogenic regulatory factor and an early bexarotene-responsive gene, as shown by RT-qPCR time-course analysis (Figure 11A), we further explored the role of RXR signaling in relation to MyoD. Intriguingly, we observed occupancy of RXRα at the core enhancer region of *MyoD* (Goldhamer et al., 1992; 1995), ~20 kb upstream of the TSS, as well three additional regions (about 26, 34 and 38 kb upstream of the TSS), (Figure 11B). To complement the observation of RXRα binding at the *MyoD* locus, we assessed the mRNA levels of MyoD in differentiating myoblasts in the presence or absence of bexarotene treatment using a shRXRα cell line. Similar to Angptl4, the levels of MyoD mRNA were significantly augmented upon bexarotene treatment and this positive effect was diminished following shRXRα knockdown (Figure 11C). It has been demonstrated previously that H3K18ac is often associated with nuclear receptor signaling and rexinoid action (AlSudais et al., 2016; Jin et al., 2011). By performing qChIP of H3K18ac at *Angptl4* and *Myod1* regulatory loci, we found a significant increase of H3K18ac enrichment following rexinoid activation (Figure 11D). Taken together, our data suggest that a common mode of molecular regulation may mediate differential gene expression observed in rexinoid-enhanced myoblast differentiation, in that it may be reconciled largely through the regulation of MyoD gene expression.

Figure 11

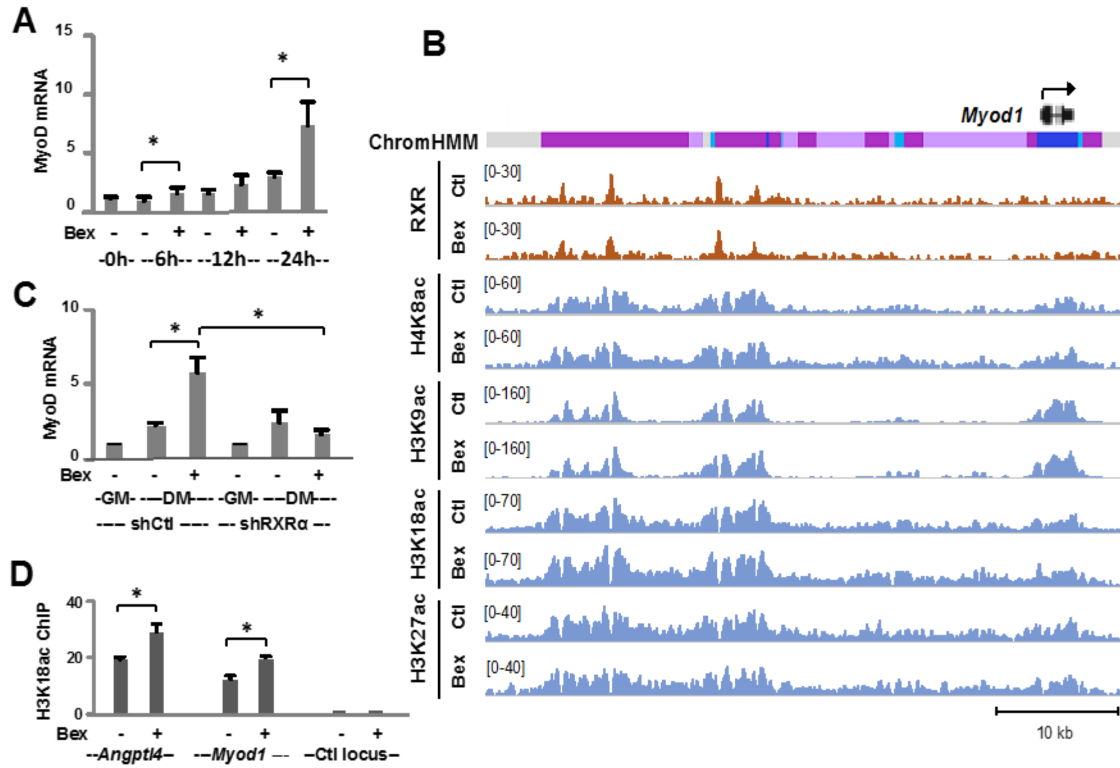

Figure 11. RXR regulates MyoD via its occupancy at the MyoD core enhancer region. C2C12 myoblasts were differentiated 0, 6, 12 and 24 hours and subjected for RT-qPCR analysis (A) Levels of MyoD transcripts were presented as fold change relative to proliferating myoblasts (0 h), normalized to internal control. Data calculated using the formula $2^{-\Delta\Delta CT}$ (error bars: SEM; n=3; *$p < 0.05$). (B) Genome browser view of RXR and indicated histone acetylation signals at the *MyoD* locus. Black bars show Ref-seq gene position and ChromHMM track colors below correspond to color designated to each chromatin state. RXR-knockdown (shRXR) myoblasts were differentiated in the presence or absence of bexarotene for 24 hours along with proliferating myoblasts (GM). A non-silencing shRNA (shCtl) was used in parallel as a control. (C) The mRNA levels of MyoD were assessed and plotted as fold change in relation to proliferating myoblasts after normalization to TBP. Data calculated using the formula $2^{-\Delta\Delta CT}$ (error bars: SEM; n=3; *$p < 0.05$). (D) H3K18ac enrichments at the *Angptl4* and *Myod1* loci were examined by qChIP analysis with an intergenic region as a control, using C2C12 cells differentiated for 24 hours. Enrichment was quantified as percentage to input chromatin DNA (error bars: SEM; n=3; *$p < 0.05$).

3.7. Loci-specific histone enrichment upon bexarotene treatment

Taking into consideration that rexinoids appear to act through MyoD expression and that MyoD and myogenin play sequential roles in the control of myogenic differentiation, we analyzed the changes in rexinoid-dependent residue-specific histone acetylation that are specifically associated with the two myogenic regulatory factors. For this purpose, we used the Caltech publically available MyoD and myogenin datasets under the ENCODE project performed in proliferating and myoblasts differentiated for 24 hours. A classification of MyoD and myogenin binding sites into distinct chromatin states displayed greater than 60% association of MyoD high confidence peaks to enhancer regions, whereas only 36% of myogenin binding sites were found to do the same (Figure 12A). Next, by examining the active and poised enhancer-associated histone changes of both MyoD and myogenin binding sites, we observed a differentiation-dependent enrichment in H3K18ac and H3K27ac at active enhancers (Figure 12B). However, there was an additional increase of H4K8ac and H3K9ac at MyoD- and myogenin-associated poised enhancers upon differentiation (Figure 12B). Intriguingly, we observed a further increase in all indicated histones at MyoD- and myogenin-associated poised enhancers following bexarotene treatment, whereas no such increase was detected at the active enhancer regions (Figure 12B). Our analyses hence suggest a possible role for MyoD and myogenin in the regulation of poised enhancers, particularly in the context of rexinoid signaling.

In differentiating myoblasts, the activation of myogenin expression is coupled with the binding of MyoD to the promoter and upstream enhancer regions of the myogenin locus (Faralli and Dilworth, 2012), which was characterized as poised or inactive regions in proliferating myoblasts based on our chromatin state model (Figure 12C). While myogenin

has been identified previously as a bexarotene responsive gene (AlSudais et al., 2016), RXR occupancy was not detected at the myogenin locus (GSM2478304, GSM2478305) (Hamed et al., 2017). However, MyoD associated H4K8ac, H3K9ac and H3K18ac signals at the poised enhancers were not only enriched by 24 hours of differentiation but also further increased following bexarotene treatment (Figure 12C). Altogether, as a direct RXRα genetic target (Figure 12), our data underline the importance of MyoD and affiliated histone acetylation in the activation of myogenic genes, including myogenin at the commencement of myogenic differentiation as well as in mediating the effect of rexinoids signaling in differentiating myoblasts.

Figure 12

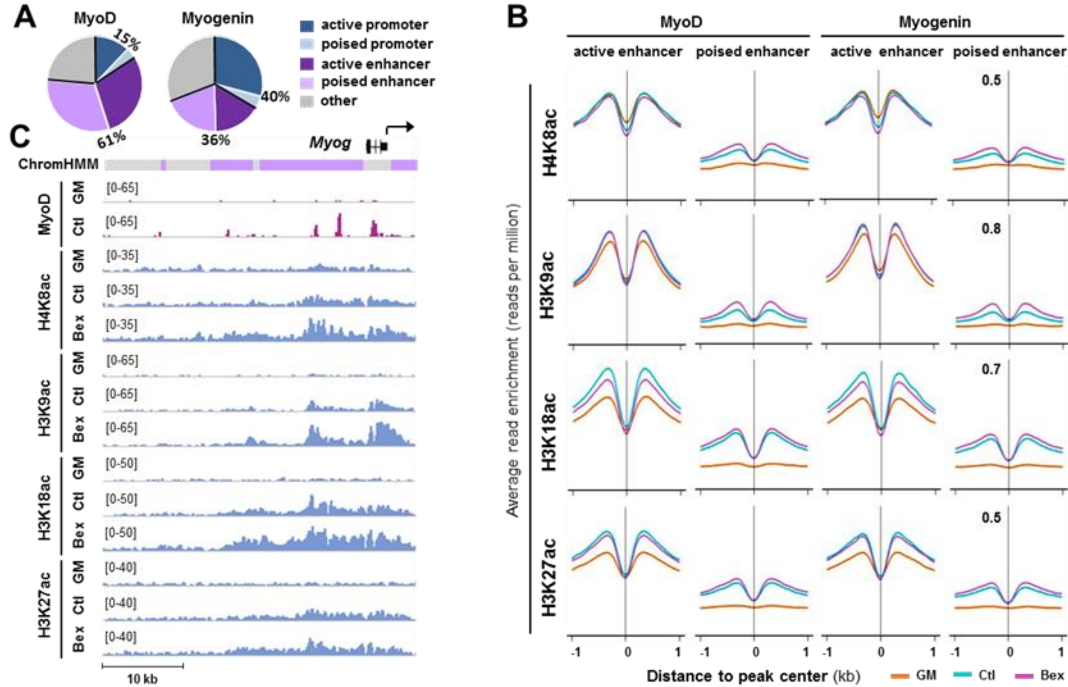

Figure 12. Loci-specific histone enrichment associated with MyoD and myogenin binding. (A) Genome-wide MyoD and myogenin binding sites in myoblasts differentiated for 24 hours were associated with distinct chromatin states. (B) The average read enrichment of H4K8ac, H3K9ac, H3K18ac and K3K27ac in proliferating myoblasts (GM), and myoblasts differentiated for 24 hours in the presence or absence of bexarotene is plotted at regions of ±1 kb of the MyoD and myogenin peaks (following 24 hours of differentiation) associated to active and poised enhancers. (C) Genome browser view of MyoD and histone acetylation signals at the *myogenin* locus. Black bars show Ref-seq gene position and ChromHMM track colors below correspond to color designated to each chromatin state.

3.8. MyoD and p300 colocalize to the regulatory regions of bexarotene-responsive genes

To delineate the potential mechanisms of MyoD-mediated rexinoid responsive gene expression, we first selected multiple poised enhancers as indicated by our chromatin state model, including a previously identified myogenin enhancer (Asp et al., 2011) along with Sntb1 and Tnnc1 that display localization of p300 and histone acetylation enrichment in the control differentiation condition as well as following bexarotene treatment (Figure 13A). Then, we conducted ChIP-qPCR analysis to examine the association of MyoD and p300 to the indicated poised enhancers (Figure 13B). The ChIP-qPCR results accurately validated the ChIP-seq enrichment profiles of p300 at these sites. As shown in Figure 13B, the association of p300 and MyoD was detected at the indicated loci by 24 hours of differentiation. Moreover, their enrichment was further significantly increased upon bexarotene treatment. Intriguingly, the association of myogenin was also significantly enriched at these poised enhancers to a similar degree following differentiation as well as with the addition of bexarotene (Figure 13B). Normal IgG antiserum and a random locus were used as negative controls in the analysis. To complement these findings, we also conducted RT-PCR analyses to assess the mRNA expression of these MyoD mediated rexinoid responsive genes upon bexarotene treatment at 24 hours of differentiation (Figure 13C). Our data show that the activation of the indicated genes correlated with the association of p300, MyoD and myogenin to their loci (Figure 13B and C). These sets of data suggest that bexarotene-responsive gene expression is mediated through the activation of poised enhancers by MyoD and myogenin as transcription factors and p300 as a histone acetyltransferase.

Figure 13

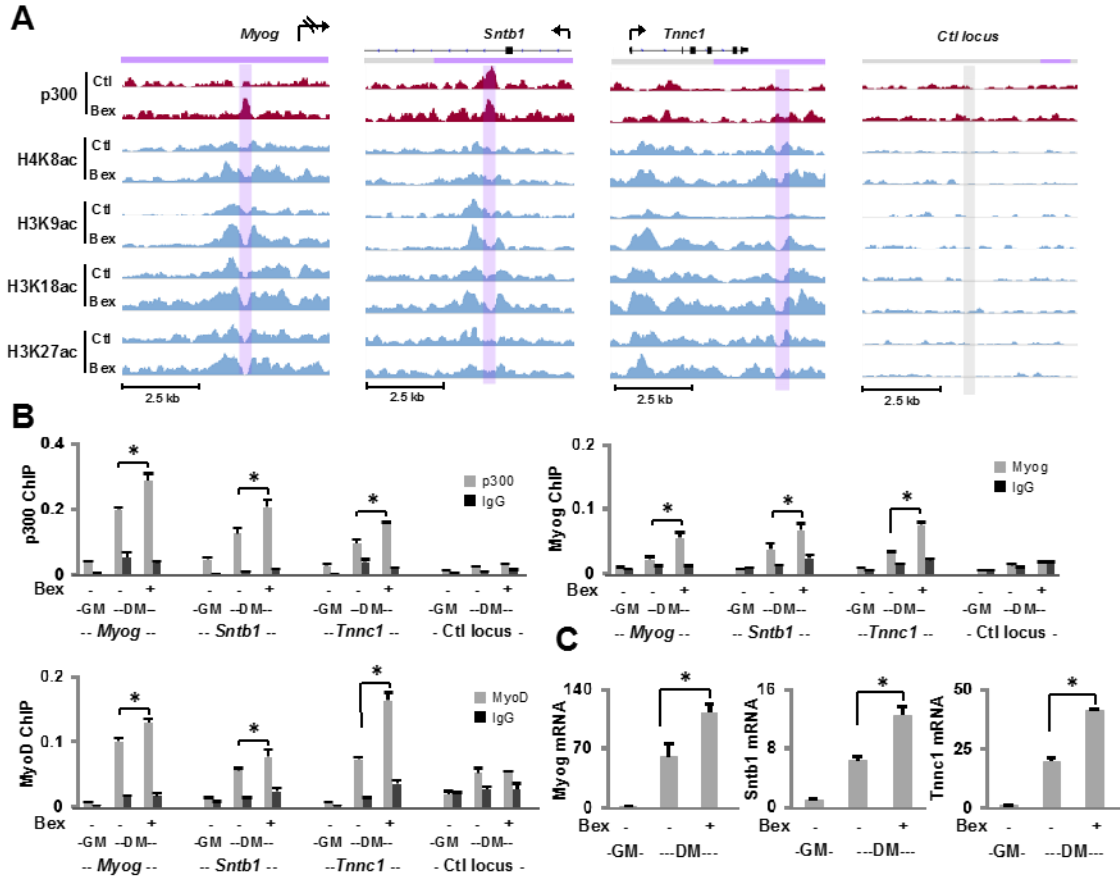

Figure 13. MyoD and p300 contribute to bexarotene-responsive gene regulation.
(A) Genome browser view of p300 and histone acetylation read density at *Myog, Sntb1*, *Tnnc1* and a *negative* locus. Black bars show Refseq gene position and the colors of ChromHMM track below correspond to distinct chromatin states. (B) ChIP-qPCR was performed for poised enhancers identified for Myog, Sntb1 and Tnnc1 using antibodies against p300, MyoD or myogenin. Normal IgG and a random locus (Ctl) were used as controls. Quantification is presented as the percentage of enrichment in relation to the input chromatin DNA (error bars: SEM; n=3; *$p < 0.05$). (C) RT-qPCR analysis of gene expression levels for MyoD mediated rexinoid responsive targets was calculated using the formula $2^{-\Delta\Delta CT}$ (from panel A) and is presented as fold change relative to proliferating myoblasts, after normalization to internal control (error bars: SEM; n = 3; *$p < 0.05$).

3.9. Bexarotene promotes dystroglycan expression in myoblast differentiation

Given that Dag1 is a bexarotene-responsive target as shown by RNA-seq analysis, we assessed the requirement of RXRα for Dag1 expression. First, we utilized primary myoblasts, physiologically relevant cells, differentiated for 12 and 24 hours in the presence or absence of bexarotene and observed that Dag1 mRNA levels were significantly augmented upon bexarotene treatment during primary myoblast differentiation (Figure 14A). As the effect of bexarotene is mediated via RXRα specifically (AlSudais et al., 2016), we sought to confirm whether bexarotene-enhanced Dag1 expression is also mediated through RXRα activation. We used C2C12 myoblasts co-treated with a RXR antagonist (UVI3003) with or without bexarotene treatment on day 1 of differentiation (AlSudais et al., 2016) and assessed the Dag1 mRNA expression levels. As shown in Figure 14B, the expression of Dag1 mRNA was enhanced within 24 hours of differentiation and further increased upon bexarotene treatment. Interestingly, co-treatment with UVI3003 mitigated the enhanced effect of bexarotene on Dag1 mRNA expression determined by RT-qPCR, while treatment with UVI3003 alone exhibited no effect. This suggests that bexarotene enhances Dag1 expression through RXR selective signaling. To further validate the role of RXRα in Dag1 regulation, we used RXRα knockdown C2C12 cells and found a significant reduction in Dag1 mRNA levels upon the introduction of RXRα shRNA knockdown. The knockdown also impaired the enhanced effect of bexarotene on Dag1 mRNA expression (Figure 14C). These sets of data suggest that RXRα activation is required for Dag1 regulation during bexarotene-enhanced myogenic differentiation.

Figure 14

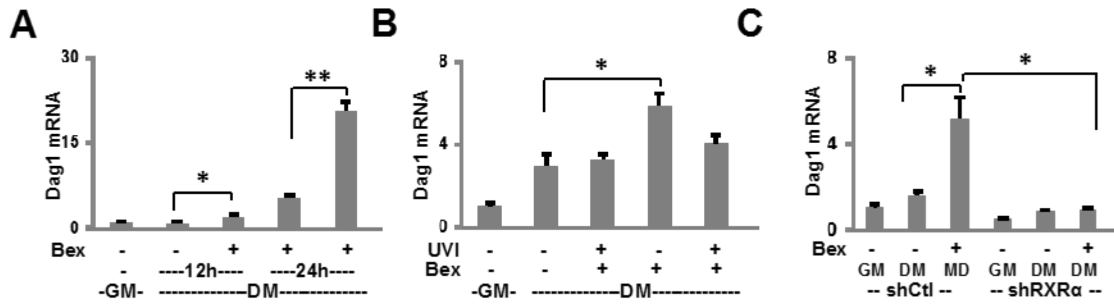

Figure 14. RXR signaling promotes Dag1 expression.
(A) Primary myoblasts were differentiated for 12 hours and 24 hours in the presence or absence of bexarotene (Bex). The levels of Dag1 mRNA were determined by RT-qPCR and presented as fold changes in relation to proliferating primary myoblasts (GM) normalized to Rps26 (*$p < 0.05$; **$p < 0.01$; n=3). (B) C2C12 cells were differentiated with and without bexarotene (Bex, 50nM) in combination with RXR antagonist UVI3003 (UVI, 5uM) for 24 hours. The levels of Dag1 mRNA were determined by RT-qPCR and presented as fold changes relative to proliferating myoblasts (GM), normalized to Rps26 (*$p < 0.05$; n=3). (C) RXRα knockdown cells were differentiated for 24 hours and subjected to RT-qPCR analysis. The levels of Dag1 mRNA were presented as fold changes relative to proliferating myoblasts (GM), normalized to TBP (*$p < 0.05$; n=3).

3.10. p300 is involved in Dag1 regulation during myoblast differentiation

Having identified Dag1 as a RXRα target, we utilized comprehensive ChIP-seq data analysis including p300 and indicated histone acetylation marks to study a potential p300-dependent regulatory region upstream of *Dag1* classified as a poised enhancer based on our chromatin state (Figure 15A), which is likely to be putative regulatory region as preliminary analyses show it to be highly conserved across vertebrate species including humans (Figure 15A). p300 associated H4K8ac, H3K9ac and H3K18ac signals at the poised enhancer were not only enriched by 24 hours of differentiation but also further increased following bexarotene treatment (Figure 15A). In order to validate the occupancy of p300 at the *Dag1* promoter and *Dag1* poised enhancer, we employed qChIP analysis. Primers spanning the summits of the p300 peaks were designed at -10 Kb of the *Dag1* gene TSS. Figure 15B and C show that the association of p300 to *Dag1* promoter and poised enhancer locus, respectively, was significantly enriched by 24 hours of differentiation. Moreover, the occupancy of p300 at *Dag1* loci was further increased upon bexarotene treatment (Figure 15B and C). Since MyoD is important for transmitting rexinoid signaling and the MyoD seems to cooperate with p300 to regulate gene expression during myogenic differentiation, we also examined the binding of MyoD to *Dag1* potential regulatory regions through qChIP analysis. Similar to p300, MyoD also increased upon differentiation and was further augmented upon bexarotene treatment (Figure 15D and E), indicating that p300 and MyoD, indeed, play principal roles in *Dag1* regulation during early enhanced-myogenic differentiation.

Next, we investigated the requirement of p300 in the regulation of Dag1 further. We used p300 shRNA knockdown C2C12 myoblasts to assess the protein and mRNA levels of Dag1 during myogenic differentiation. Figure 15F and G show that p300 depletion in C2C12

attenuated the levels of Dag1 protein expression by approximately 3- and 4-fold in bexarotene treated and untreated cells, respectively, as compared to respective controls (sh-Ctl). As an additional reference, myosin heavy chain, a muscle-specific gene, was significantly inhibited upon p300 knockdown (Figure 15F and H). Likewise, the bexarotene-enhanced Dag1 mRNA expression was attenuated by a knockdown of p300 (Figure 15I). In addition, the p300 knockdown hindered bexarotene-enhanced myogenin mRNA expression as determined by RT-qPCR analysis (Figure 15J). These data suggest that p300 is necessary for the regulation of *Dag1* likely via the acetylation of histones at *Dag1* putative regulatory regions during early myogenic differentiation. Taken together, these data suggest that Dag1 has an important role in early myogenic differentiation, as being regulated by p300 and MyoD, which are critical players in myogenesis.

Figure 15

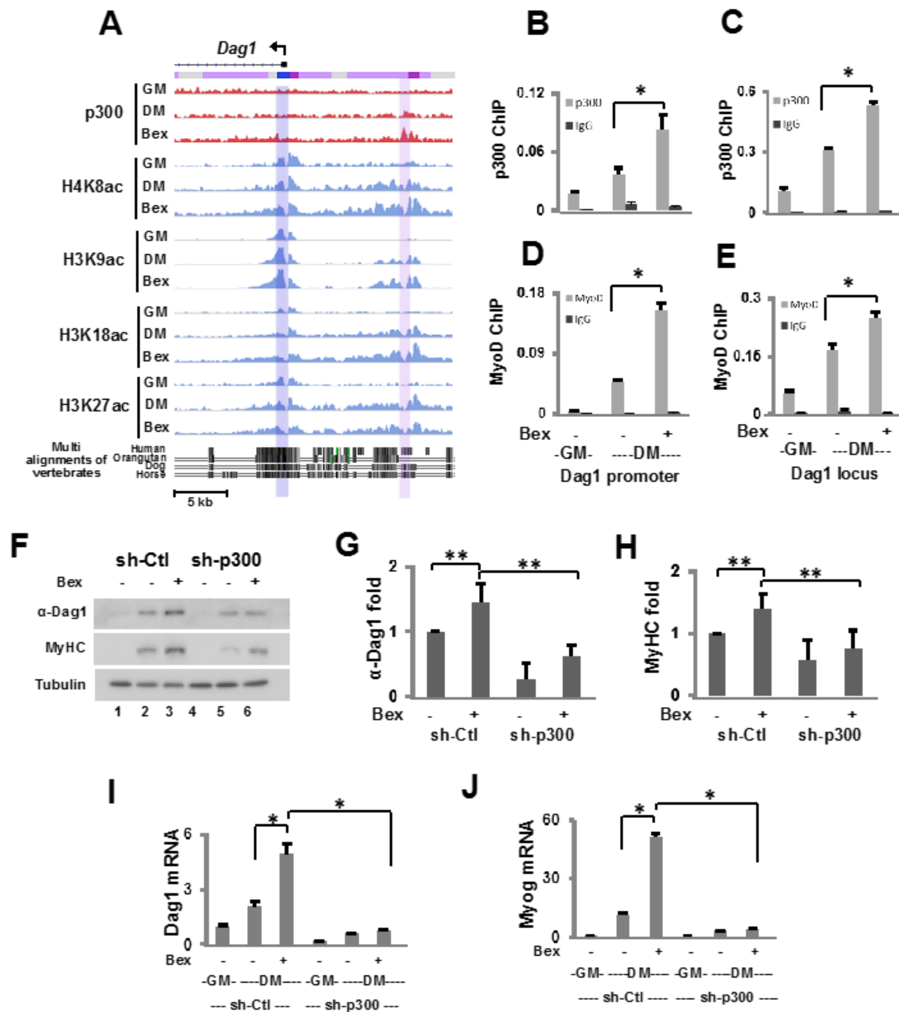

Figure 15. The regulation of *Dag1* during myogenic differentiation.

(A) Genome browser view of p300 and indicated histone acetylation signals at the *Dag1* locus. Black bars show Ref-seq gene position and the ChromHMM track colors below correspond to color designated to each chromatin state. The panel below displays the conservation of the Dag1 locus shown from the alignment of multi vertebrates by the UCSC genome browser. (B-D) C2C12 cells were differentiated for 24 hours (DM) in the presence or absence of bexarotene (Bex) with proliferating myoblasts as controls (GM). ChIP-qPCR analysis was performed at the (B&D) *Dag1* promoter and (C&E) *Dag1* locus, using antibodies against p300 or MyoD. Normal IgG antiserum and a random locus (Ctl) were used as negative controls. Quantification is presented as the percentage of enrichment in relation to input chromatin DNA (error bars: SEM; n = 3; *$p < 0.05$). (F) The levels of α-Dag1, MyHC, and β-Tubulin were analyzed using Western blotting following the introduction of p300 shRNA (sh-p300) into the parental C2C12 cells. (G) Quantification of α-Dag1 and (H) MyHC protein levels from panel A is presented as fold change in relation to day-1 untreated differentiating myoblasts (**$p < 0.01$; n=3). C2C12 cells for shp300 and shCtl were differentiated and subjected for qPCR analysis of myogenin (I) Dag1 and (J) myogenin mRNA. Quantification is presented as fold changes relative to undifferentiated myoblasts (GM), normalized to TBP (n=3).

3.11. Dystroglycan depletion attenuates myogenic differentiation

Through qRT-PCR of C2C12 myoblasts treated with bexarotene, Dag1 has been identified as a bexarotene-responsive gene (Figure 14A-C). Besides, dystroglycan is part of the DGC and it has been shown that Dag1 expression sustains efficient regenerative ability of satellite cells (Cohn et al., 2002), and thus, we were interested in assessing the functional requirement of Dag1 in the differentiation process. For this purpose, we performed an *in vitro* knockdown assay of Dag1, in which we used shRNA system to reduce the expression of Dag1 in C2C12 cells. As shown in Figure 16A and B, Dag1 protein expression continued to increase on day 1 and day 2 of differentiation and was further augmented upon bexarotene treatment in the control non-silencing shRNA. Additionally, the shDag1 condition shows a significant reduction of Dag1 levels. Likewise, a more than 50% decline in myosin heavy chain expression was observed by the knockdown of Dag1 expression, particularly on day 1 of differentiation. Intriguingly, there was no significant bexarotene-enhanced Dag1 or MyHC expression in shDag1 cell line (Figure 16A and B). We then assessed the impact of Dag1 reduction on C2C12 differentiation and fusion indices and found that there was nearly 50% inhibition in both indices. Similarly, bexarotene-enhanced differentiation and fusion were impeded in shDag1 C2C12 cells, demonstrating that Dag1 protein expression is required for early myoblast differentiation and for the enhanced effect of bexarotene on myogenic expression (Figure 16C and D). Taken together, these data suggest that Dag1 may be necessary for the rexinoid-enhanced early myoblast differentiation and fusion.

Figure 16

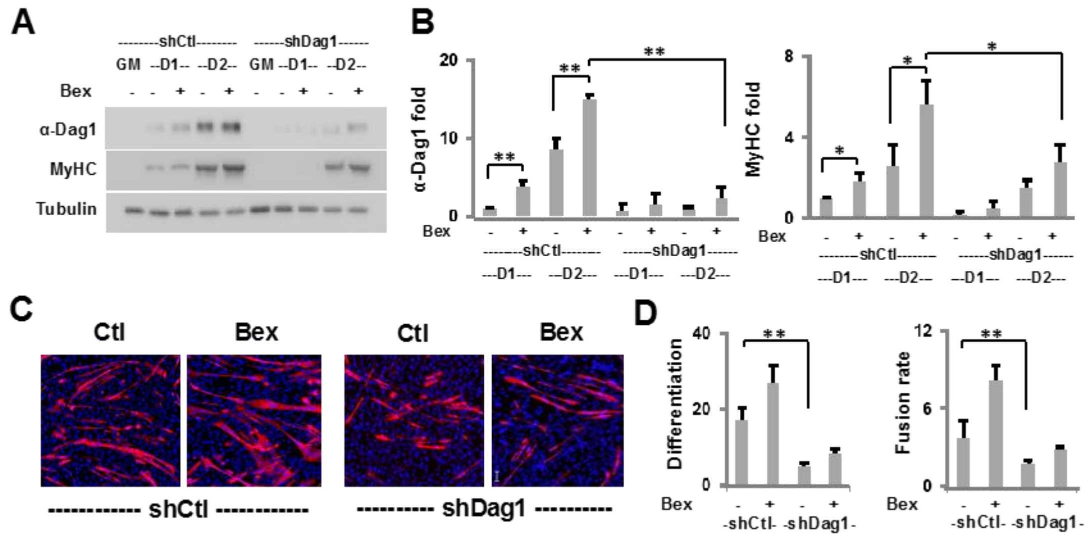

Figure 16. The effects of Dag1 inhibition on myoblast differentiation and fusion.
(A) C2C12 myoblasts were transfected with a small hairpin RNA targeting Dag1 and a non-silencing shRNA as control (sh-Ctl) to generate stable shDag1 cells. Myoblasts were then differentiated for 24 and 48 hours with and without bexarotene (Bex) treatment and subjected for Western blotting. MyHC is used as a marker to represent the differentiation process with β-Tubulin as a loading control. (B) Quantification of Dag1 and MyHC protein levels is presented as fold change in relation to day-1 untreated differentiating myoblasts (*$p < 0.05$; **$p < 0.01$; n=3). (C) Representative images stained for myosin heavy chain (red) and myoblast nuclei (blue) for Dag1 knockdown and related control in the presence or absence of bexarotene after 4 days of differentiation. (D) Differentiation was defined as the percentage of myogenic nuclei relative to the total number of nuclei. The fusion rate was defined as the average number of nuclei per myocyte (**$p < 0.01$; n=3).

Chapter 4. Discussion

Differentiation signaling pathways are affected by both genetic and epigenetic determinants. Given the role of enhancers in driving cell-fate specific gene programs, it is imperative to understand the mechanistic action of enhancers that contribute to cellular differentiation. Here, we utilize genome-wide chromatin state association to delineate the functional mode of transcription regulators in early myogenic differentiation. We define a role for transcriptional coactivator p300, when recruited by MyoD, in the establishment and regulation of myogenic loci during early myoblast differentiation. Furthermore, we reveal a significant enrichment of loci-specific histone acetylation, particularly of H4K8 and H3K9, at p300-associated loci, but only when enlisted by MyoD. Our studies provide novel mechanistic insights into the action of p300 as a coactivator in early differentiation and its functional mode in the regulation of myogenic enhancers.

In addition, we have examined the molecular pathways associated with rexinoid-enhanced myogenic differentiation using integral RNA-seq and ChIP-seq analyses. Intriguingly, bexarotene-responsive gene expression is mediated through RXR as a transcription factor and reconciled largely through direct regulation of MyoD gene expression. While histone acetylation at MyoD binding sites associated with different chromatin states increases upon differentiation, bexarotene augments the enrichments of H3K9ac and H4K8ac, particularly at poised enhancers. Our studies thus provide novel molecular insights into the interplay between rexinoid signaling and MRFs-associated chromatin states during early myogenic differentiation.

Many aspects of adult myogenesis resemble the embryonic muscle development episodes and related transcriptional and signaling mechanisms, inferring a tight control of the

genetic networks that govern cell fate during these processes. The genesis of skeletal muscle involves the specification and differentiation of stem and progenitor cells (Gros et al., 2005). These processes are regulated by gene transcription, which is regulated by sequence-specific DNA-binding transcription factors (Rudnicki et al., 2008), including MyoD which has been shown to be essential for myoblast differentiation (Weintraub et al., 1989). Furthermore, *in vitro* studies investigating the role of MRFs during myogenic differentiation demonstrated that ectopic expression of MyoD in non-muscle cells, such as fibroblasts, chondrocytes and neurons, is sufficient to convert them into myoblasts (Choi et al., 1990; Weintraub et al., 1989), through activation of muscle-specific genes. Moreover, mice lacking *MyoD* display muscle regeneration impairments (Asakura et al., 2007), suggesting that MyoD is essential for gene expression during myogenesis. Transcription factors, such as the myogenic regulatory factors, bind promoter and enhancer regions to activate target gene expression, a process which includes the recruitment of transcriptional activators, such as p300 to regulatory loci (Puri et al., 1997; Sartorelli et al., 1997). Transcriptional activators have several functions, including, but not limited to, recruiting basal transcriptional machinery to the target enhancer or promoter (Thomas and Chiang, 2006; Ptashne and Gann, 1997), and/or chromatin remodeling or modification (Näär et al., 2001) such as histone acetylation (Kuo and Allis, 1998; Legube and Trouche, 2003) (Figure 2). Moreover, nuclear receptor controlled gene expression also relies on the recruitment of histone acetyltransferase, as part of multi-protein and transcription-initiating apparatuses that regulate gene expression (Perissi and Rosenfeld, 2005).

4.1. p300 associates with enhancers in early myoblast differentiation

Genetic and epigenetic modifications have a major influence on the differentiation signaling pathways, which provides an additional evolutionary tool for demarcating the dynamic changes in transcription. Histone acetylation and deacetylation are catalyzed by HATs and HDACs, respectively, and play crucial roles in regulating gene programs (Bayle and Crabtree, 1997). In addition, transient acetylation and deacetylation occurring on loci marked with specific histone modifications suppress, activate or create a poised state in the loci primed for future activation when demanded (Wang et al., 2009). Previous studies have examined the role of p300 in myotube formation by comparing global p300 occupancy and histone modification of mature myotubes generated from C2C12 myoblasts (Blum et al., 2012). Although an *in vitro* system, it parallels the molecular processes in primary myoblasts; however, C2C12 myoblasts are more synchronized to differentiate and fuse into post-mitotic myotubes (Asp et al., 2011; Blais et al., 2005). Comprehensive gene expression analyses have also shown that myogenic differentiation ensues in a stepwise fashion, where sequential activation of a specific set of genes restructures the cells one step at a time toward the differentiated phenotype (Delgado et al., 2003). Nonetheless, at the early stage of differentiation, thousands of genes are differentially expressed (Hamed et al., 2017; Trapnell et al., 2010; Doynova et al., 2017), mostly within 24 hours of differentiation. As a result, epigenetic changes reflecting the alteration of gene programs should occur.

The HAT function of p300 is specifically essential during myogenic differentiation, where it has been shown to be required for the function of the MRFs including Myf5 and MyoD during skeletal muscle formation (Roth et al., 2003; Jin et al., 2011). Interestingly, the absence of HAT activity of CBP, a closely related transcriptional coactivating protein, did not

result in a prominent defect in muscle formation, and ES cells lacking CBP are still capable of forming myotubes (Roth et al., 2003). We have assessed the requirement of p300 in C2C12 myoblasts using a shRNA approach and shown that p300 is required for the differentiation and fusion of myoblasts as supported by the reduced expression of myogenin (Figure 6). In addition to p300, the involvement of PCAF in myogenic differentiation has been examined (Yang et al., 1996; Puri et al., 1997). PCAF is a member of the GNAT family of HATs and participates in locus-specific histone acetylation (Nagy and Tora, 2007). PCAF histone acetyltransferase activity is required for MyoD-DNA interaction and terminal cell cycle arrest during terminal differentiation (Puri et al., 1997; Sartorelli et al., 1999). Intriguingly, Puri and colleagues have shown that inhibition of either p300 or PCAF eliminates the myogenic program and they cannot compensate for each other, indicating a differential functional role of p300 and PCAF during muscle differentiation (Puri et al., 1997). We have investigated the temporal events of histones acetylation and histone acetyltransferase associations at *MyoD* loci specifically and found that p300 is sequentially enriched at *MyoD* regulatory regions, which is associated with augmented histone acetylation during myoblast differentiation (Hamed et al., 2013). Therefore, the focus of our study is not only to examine the transient chromatin dynamics that initiate and drive early myoblast differentiation, but also to explore the functional mode of p300 in regulating gene expression coupled to specific chromatin states.

4.2. p300 is associated with a distinct histone acetylation profile when occupying MyoD-dependent enhancers in early differentiation

By mapping global p300-associated loci, we show that a majority of p300 peaks are localized to enhancer regions (Figure 7), in agreement with previous reports (Wang et al., 2008; Blum et al., 2012). However, a substantial fraction of p300 peaks distributes specifically to the poised enhancer, accompanied predominantly by loci-specific histone acetylation in early myoblast differentiation (Figure 7). Binding of MyoD to myogenic enhancers in proliferating myoblasts leads to concomitant recruitment of p300 and deposition of H3K27ac (Blum et al., 2012). The recruitment of p300 principally by MyoD in early differentiation (Figure 8) suggests that enhancers identified through chromatin state association reflect the development of the skeletal lineage, which provides the rationale for us to explore the genome-wide co-presence of MyoD along with p300 in proliferating and early differentiating myoblasts.

Through co-localization analysis, we show that there is an apparent increase of p300 association to poised enhancers concurring with a MyoD presence in early differentiating myoblasts. However, no such enhancement was found in the undifferentiated state, suggesting a collaboration function between p300 and MyoD in activating poised enhancers, at the onset of differentiation (Figure 8B). Interestingly, this correlation is characterized by an increase in H4K8ac and H3K9ac, primarily in the presence of MyoD, in addition to H3K18ac and H3K27ac, which are augmented at p300-dependent loci regardless of the presence of MyoD (Figure 8B). Taken together, our data suggest a role for MyoD in p300 recruitment at lineage-specific enhancers to regulate muscle-related genes, as exemplified by the muscle-specific target *Tnnt1* (Figure 9).

In this study, we have identified a key lineage-specific regulatory signature and present a blueprint to explore novel regulators of myogenic differentiation. We provide evidence for the enrichment of not only H3K27ac, but also H4K8ac and, specifically, H3K9ac at p300- and MyoD-associated enhancers specific to myoblast differentiation (Figure 8B). While some parallels between our data and previous genome-wide studies performed in myotubes (Asp et al., 2011; Blum et al., 2012) exist, our studies present critical new findings. An example relates to the state of global histone acetylation of H3K9 and H3K18. The previous study comparing myotubes to proliferating myoblasts (Asp et al., 2011) presents a decrease in global histone acetylation levels following 72 hours of differentiation, whereas our studies show no significant change in global histone acetylation in myoblasts differentiated for 24 hours. We provide evidence that the differentiation process is mediated by transformations in residue-specific histone acetylation at lineage-specific loci, rather than global histone acetylation events. Our data compilation thus reflects the transformations required to drive the proliferating myoblasts into differentiation and provide greater insights into epigenetic changes that govern myogenic differentiation.

Thus far, we offer a novel molecular insight into the modification of myogenic enhancers by loci-specific histone acetylation in concert with p300 and muscle master regulator MyoD. Our data suggest the intriguing possibility that the cellular stage may be regulated through alteration of distinct chromatin states. It thus presents a valuable potential of driving chromatin state pharmacologically for the treatment of tissue-specific diseases.

4.3. MyoD is a direct genetic target of RXR

In knockout studies, RXRα has been shown to be required for development and its inactivation exhibits lethality *in vivo*, proving the important function of RXRα for early development (Sapin et al., 1997; Sucov et al., 1994; Kastner et al., 1994). *In vitro*, liganded RXRα plays a principal role during myogenic determination and differentiation (Le May et al., 2011; AlSudais et al., 2016). We have previously shown that bexarotene, a synthetic RXR agonist, enhances the commitment to skeletal muscle lineage (Le May et al., 2011). We have reported additionally that bexarotene enhances myogenesis at the differentiation and fusion levels, and this effect is blocked in cells lacking RXRα (AlSudais et al., 2016). Thus, we aimed to investigate the epigenetic changes that lead to the enhanced differentiation and fusion following RXRα activation. Being a dimeric partner of many nuclear receptors, RXRα has high potential to be involved in a wide range of cellular processes and signaling pathways; however, the molecular pathways associated with rexinoid enhanced myogenic differentiation are still unexplored.

The concept that the nuclear receptor superfamily can physically interact with the MyoD family (Froeschlé et al., 1998) establishes a mechanism for myogenic gene activation by nuclear receptor ligands. Froeschlé and colleagues reported localization of RXRα at E-box MyoD DNA-binding sites (Froeschlé et al., 1998), which led us to examine the rexinoid-responsive transcriptional program during myoblast differentiation. Here, we investigated how rexinoids affect global myogenic transcriptional mechanisms via ChIP-seq analyses of activated RXR utilizing C2C12 myoblasts.

We found that MyoD is an early rexinoid responsive gene, the active enhancer regions of MyoD are occupied by RXRα, and knockdown of RXRα hinders MyoD gene expression (Figure 11), suggesting that MyoD is a direct genetic target of RXR in early myogenic differentiation. At the onset of differentiation, MyoD activates the expression of both muscle-specific genes and cell cycle inhibitors, allowing irreversible exit from the cell cycle and progression into differentiation (Halevy et al., 1995; Weintraub et al., 1989). Given that the expression of MyoD is upregulated by bexarotene and RXRα is important for the positive effect of bexarotene on MyoD expression (Figure 11) and myoblast differentiation (AlSudais et al., 2016), a common mode of molecular regulation may mediate differential gene expression observed in bexarotene-enhanced myoblast differentiation.

Characterization of the chromatin states in proliferating C2C12 myoblasts reveals a muscle-specific usage of regulatory DNA elements, and the activity of muscle-specific enhancers is marked by local changes in residue-specific histone acetylation at the early stage of myoblast differentiation (Figure 12 and 13). While H3K9ac is generally considered as a global mark of promoter activity, we identify enrichment of H3K9ac at poised enhancers coupled with MyoD at the early stage of differentiation (Figure 12). Thus, H3K9ac may reflect the control of a discrete set of genes through the function of MyoD with the cooperation of myogenin in early myogenic expression and that MyoD plays important role in the activation of poised enhancers particularly in the milieu of rexinoid action. Besides H3K9ac, the enrichments of H4K8ac are also strongly coupled with MyoD and myogenin at the poised enhancers (Figure 12). Unlike H3K18ac and H3K27ac, which did not show a significant change at enhancer regions, H4K8ac was enriched with MyoD and myogenin binding at poised enhancers, as it has been known as a marker of active or transcriptionally-competent chromatin (Wang et al., 2008) (Figure 12B). This observation implies that MyoD plays key roles, in

cooperation with myogenin, in residue-specific histone acetylation in early myogenic differentiation. A previous study showed that Myf5 and MyoD share a broad set of binding sites; yet, they have different potentials to activate gene transcription (Conerly et al., 2016). Here, while we observed cooperation between MyoD and myogenin in regard to the activation of gene transcription in the context of rexinoid, we found that MyoD is preferentially associated with enhancers, while myogenin binds preferentially to promoters (Figure 12A). Furthermore, besides MyoD, we detected a co-localization of p300, particularly at poised enhancers of indicated bexarotene-responsive genes (Figure 13). Collectively, our findings demonstrate a role for MyoD as a transcription factor and p300 as a HAT in the activation of poised enhancers of bexarotene-responsive genes in early myoblast differentiation as well as in the interplay with rexinoid signaling to further endorse myogenic differentiation.

We here demonstrate that specific targeting of RXR signaling promotes the normal regulation of gene expression occurring during myoblast differentiation, and describe a novel molecular regulation of MyoD gene expression through the activation of RXR. A potential direction will be therefore to determine if rexinoids are able to similarly regulate myogenic transcription in a specific physiological context or disease models. Moreover, the model of rexinoid-enhanced myogenesis also offers an excellent system to identify additional genetic targets and molecular interactions for therapeutic development towards muscle wasting diseases by directly targeting epigenetic regulating mechanisms to promote efficient muscle regeneration in patients affected by muscle-wasting diseases.

4.4. RXR signaling promotes dystroglycan expression in myoblast differentiation

We have shown for the first time through RNA-seq analysis that Dag1 is a bexarotene-responsive gene targeted in rexinoid signaling in the early differentiation process. Although ChIP-seq analysis showed no enrichment of RXRα at the Dag1 locus, we postulated that RXRα indirectly targets Dag1 possibly through the function of MyoD. To validate this hypothesis, we examined the expression of Dag1 in C2C12 cells lacking RXRα expression and found that the bexarotene-enhanced expression of Dag1 was significantly diminished (Figure 14C). Hence, we aimed to assess the MyoD dependency of the previously identified *Dag1* promoter extending from -318 to -207 from the TSS (Rettino et al., 2009), in addition to a potential regulatory locus based on the deposition of histone acetylation and p300 occupancy, which is a mark of enhancer regions (Visel et al., 2009).

Enhancers play central roles in regulating tissue-specific gene expression and can be found at large distances from the genes they regulate. Based on genomic sequence studies, enhancers are primarily associated with RNA polymerase II recruitment and the histone acetyltransferase p300 as well as enrichment of distinct histone modifications such as H3K27ac and H3K4me1 (Karmodiya et al., 2012). In particular, p300 genome-wide studies in combination with cloning p300-associated regions for *in vivo* reporter assays yielded an overall 87% success rate in predicting tissue-specific enhancer activity (Visel et al., 2009). Thus, we utilized p300 enrichment as well as enhancer-related histone acetylation as predictors of enhancer activity. Our preliminary identification of regulatory regions was based on chromatin modifications in mouse myoblasts. However, we observed that the coding sequence of *Dag1* is highly conserved across mammalian species, including humans (Figure 15A). Moreover, a previous study demonstrated a strong correlation between the conservation of histone

modifications of mice and human samples, suggesting the presence of mechanisms involved in maintaining histone modifications against genetic changes (Woo and Li, 2012). Intriguingly, this study showed that the modifications in promoter regions or intergenic enhancer regions to be the most conserved (Woo and Li, 2012). These results support our rationale for investigating the indicated *Dag1* loci (Figure 15) to investigate its regulation in myogenic differentiation. Taking into consideration all these criteria, we were able to identify an intergenic putative p300-dependent enhancer, about 10 Kb upstream of *Dag1* TSS. We observed an enrichment of p300 histone acetylation targets, H3K18ac and H3K27ac, in addition to H4K8ac and H3K9ac following 24 hours of differentiation as well as upon bexarotene treatment (Figure 15A). Interestingly, the binding profile of MyoD and p300 in ChIP-qPCR analysis was strongly correlated, suggesting a role for MyoD and p300 in Dag1 regulation during myogenic differentiation.

The data collected from dystroglycanopathies patients, as well as the mutation studies of Dag1, reinforce the notion that correct expression and regulation of Dag1 is critical for muscle integrity during cycles of contraction and relaxation (reviewed in Whitmore and Morgan, 2014). Although an emerging number of studies have considered the function and modifications of Dag1 in muscle integrity and maintenance, the regulation of Dag1 is less understood. Therefore, we investigated the upstream regulation of Dag1 in myogenic differentiation.

4.5. p300 is required for dystroglycan expression during myogenic differentiation

Through genome-wide association and qPCR analysis, we have shown that p300 occupies the putative regulatory loci of *Dag1*. Thereby, we sought to assess the expression of Dag1 in cells lacking p300. We verified the use of shRNA previously to knockdown p300 expression in C2C12 cells and found that the lack of p300 in myoblasts leads to significant inhibition of myoblast differentiation and fusion (Jihong et al., 2015). This result is in agreement with the *in vivo* studies that demonstrated that mice lacking both p300 alleles exhibited apparent defects in cell differentiation (Yao et al., 1998). We therefore used shp300 C2C12 cells to assess the p300-dependent expression of Dag1. We have shown that Dag1 expression was significantly reduced in p300 knockdown cells (Figure 15G and I). Interestingly, bexarotene was not able to promote Dag1 expression in cells lacking p300. One could argue that the inhibition of Dag1 protein expression is due to a defect in the posttranslational modification effect, therefore; we also assessed the mRNA expression at the same time points and conditions. Similarly, p300 knockdown resulted in reduced Dag1 mRNA levels after 24 hours of C2C12 differentiation (Figure 15I). In addition, p300 knockdown mitigated the positive effect of bexarotene on Dag1 mRNA expression (Figure 15I). These data suggest that Dag1 is, at least in part, under the control of p300, when recruited by MyoD in early myogenic differentiation. Being an acetyltransferase, p300 is anticipated to be involved in the observed enrichment of H3K18ac and H3K27ac at putative *Dag1* loci as they are p300 targets (Jin et al., 2011). Moreover, p300 might be responsible for the increase in H4K8ac and H3K9ac at *Dag1* enhancer loci, particularly in the context of rexinoid. Therefore, the fact that MyoD and p300 co-occupy the indicated putative regulatory *Dag1* enhancer (Figure 15) infers that p300 regulates myogenic genes, when recruited by MyoD, through acetylating specific histone residues at the regulatory

loci. Given that Dag1 expression in satellite cells sustains continued efficient skeletal muscle regeneration *in vivo* (Cohn et al., 2002), it is suggested that lack of Dag1 negatively influences myogenic differentiation. Interestingly, we showed that the lack of Dag1 in the C2C12 myoblasts led to a significant decrease in the differentiation and fusion indices. Moreover, bexarotene treatment was not able to overcome the inhibition of cell differentiation (Figure 16). These results suggest that Dag1 expression is indeed essential for the early stages of differentiation.

Concluding remarks

We here provide novel molecular insight into the regulation of myogenic regions by p300, particularly when enlisted by MyoD during early myogenic differentiation. We reveal that the occupancy of p300 is accompanied by enrichment of loci-specific histone acetylation. Through our study of the molecular regulation of rexinoid responsive gene expression, we found that rexinoid signaling, which is mediated through RXR, is largely reconciled via direct regulation of MyoD gene expression in early myogenic differentiation. Lastly, although the regulation of Dag1 has been explored in myofibers, our work has established a novel mode of regulation of Dag1 by p300 as a histone acetyltransferase and MyoD as a transcription factor in early myogenic differentiation. Moreover, my Ph.D. work improved the understanding of the mode of action of histone acetyltransferases in mediating rexinoid signaling, providing mechanistic insight into ligand-activated gene transcription during myogenesis. Utilizing the model of rexinoid-enhanced myogenesis, we have uncovered a mechanistic regulation of the active and poised enhancers and propose an excellent system to discover molecular interactions for therapeutic development toward muscle-related diseases (Figure 17).

Figure 17

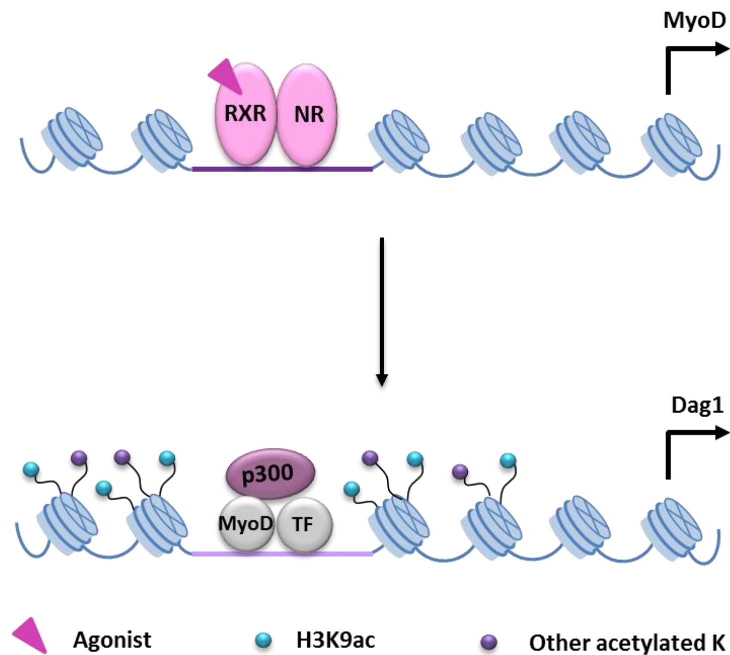

Figure 17. Summary and proposed models for my Ph.D. findings.
Upon agonist binding, RXRα binds the core enhancer region of MyoD, which mediates the function of rexinoids during the enhanced-myogenic differentiation. MyoD, as a master myogenic transcription factor, binds the regulatory regions of its genetic targets to facilitate histone acetylation through transcriptional coactivators recruitment. Recruitment of the transcriptional coactivator p300 is associated with differential lysine (K) acetylation, leading to enhanced gene expression.

Significance and future directions

Muscle wasting can occur as a result of a wide range of diseases such as inflammatory myopathies, cerebral palsy, cancer and muscular dystrophies (Meryon, 1852; O'Dwyer, Neilson, and Nash, 2008; Henry, Cohen, and Campbell, 2001). Particularly in Duchenne muscular dystrophy patients, the type II skeletal muscle fibers are more prone to damage (Webster et al., 1988). Interestingly, nuclear receptors, such as PPARδ which regulate transcription by heterodimerizing with RXR, play a key role in the regulation of mitochondrial respiration (Luquet et al., 2003), skeletal muscle lipid oxidation as well as the determination of skeletal muscle fiber types, where an activated form of PPAR was shown to increase the proportion of type I fibers (Wang et al., 2004), suggesting that RXR agonists may be able to promote muscle regeneration.

Our data suggest the intriguing possibility that the cellular stage may be regulated through alteration of distinct chromatin states. It thus presents a valuable potential of driving chromatin state pharmacologically for the treatment of tissue-specific diseases. Future prospects will therefore be to determine the functional capacity of the myogenic enhancers identified in a specific physiological context. The model of p300-associated cellular stage-specific enhancers provides an excellent system to initiate the identification of additional myogenic targets and molecular interactions for therapeutic development towards muscle-related diseases. Furthermore, the application of particular nuclear receptor ligands (agonists) is a potential avenue of research in muscle-related diseases to promote improved muscle fiber formation, metabolism and function (Gaudel et al., 2008).

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Table 1. Primer sets used for RT-qPCR analysis.

Gene	Forward Primer	Reverse Primer
Tnnt1	5’-TCCTGCAGCAAGCCTACC-3′	5′-ATCGCACCAATATCCTCACC-3′
Igsf3	5′-AAGTACAGATCGTTAGCACGGT-3′	5′-GGTGTGACATTCATACTCGCC-3′
Asb2	5′-TCCTGCAGCAAGCCTACC-3′	5′-ATCGCACCAATATCCTCACC-3′
Gpc6	5′-TTTTCGGCCCTACAACCCTG-3′	5′-TCTGTGACACTGTGCTGCAT-3′
Sntb1	5′-GAAGGAGAGATTCAACTGGACC-3′	5′-CCAAGCCCAGTCGTGTTATC-3′
Tnnc1	5′-GGAGCTGCAGGAGATGATTG-3′	5′-CCTCAGACTTCCCTTTGCTG-3′
MyoD	5′-TGCCTTCTACGCACCTGGA-3′	5′-ATCATGCCATCAGAGCAGTTGG-3′
Myog	5′-ATCCAGTACATTGAGCGCCTAC-3′	5′-AGCAAATGATCTCCTGGGTTGG-3′
Dag1	5′-GGTTGGCATTCCAGACGGTA-3′	5′-CCTGCTGCAGACACCTTGAT-3′
Angptl4	5′-GTTTGCAGACTCAGCTCAAGG-3′	5′-CCAAGAGGTCTATCTGGCTCTG-3′

Table 2. Primer sets used for ChIP-qPCR assay.

Gene	Forward Primer	Reverse Primer
Tnnt1	5′-GTGGTGACAACAGGCAGCTA-3′	5′-GTTAGAAAGAGGAGGGGCTGG-3′
Igsf3	5′-GCTCATCTAGGCCACTCAACA-3′	5′-GGACCCCGTCTGATGCAAT-3′
Asb2	5′-ACCCATTTGGCCCTCTTCAG-3′	5′-GGATGCTGTATCTCTGGCCC-3′
Gpc6	5′-CCAGCTGCTCTGTCAGTCAC-3′	5′-TGGCATGGAAACAACCAGGA-3′
Sntb1	5′-AGTCAGTTTTCTCCCTGCCC-3′	5′-TACCACATGTGTGGGACAGC-3′
Tnnc1	5′-AGTTGGGGGCTTTCAAGGAA-3′	5′-AATACAGTCACAGGGCGGAG-3′
MyoD	5′-TGCTTCTTTCGGCCAAGTAT-3′	5′-CCAACTGGCTGTGTTGTGAG-3′
Myog	5′-GCATTCTGGGAAGGGGTTAC-3′	5′-CCTGGGTGGAGATGTCTTTT-3′
Dag1-promoter	5′-CCTTTCCCAACTTCTCCCGAA-3′	5′-AGATACCGCTTGCGTTTTGC-3′
Dag1-locus	5′-GCTGGATCTTTGTGGGGCAT-3′	5′-TGAAAGGAGGAGCCATTGGT-3′
Angptl4	5′-CCAGCCAGGGAAAGTAGGAGA-3′	5′-CAGAAAGTGCCTGCATGCC-3′
Control	5′-ACAGACAACGCAGAGTACCG-3′	5′-GCCACACTCCAGACAAGATAGT-3′

Table 3. RNA-seq and ChIP-seq dataset access.

Organism	Cell line	Condition	Antibody	Data Type	Replicate	GEO Accession	Citation
mus-musculus	C2C12	GM		RNA-seq	#1	GSM2478318	Hamed, et al., 2017
mus-musculus	C2C12	Differentiating / 12h_Ctl		RNA-seq	#1	GSM2478320	Hamed, et al., 2017
mus-musculus	C2C12	Differentiating / 12h_Bex		RNA-seq	#1	GSM2478322	Hamed, et al., 2017
mus-musculus	C2C12	Differentiating / 24h_Ctl		RNA-seq	#1	GSM2478324	Hamed, et al., 2017
mus-musculus	C2C12	Differentiating / 24h_Bex		RNA-seq	#1	GSM2478326	Hamed, et al., 2017
mus-musculus	C2C12	GM		RNA-seq	#2	GSM2478319	Hamed, et al., 2017
mus-musculus	C2C12	Differentiating / 12h_Ctl		RNA-seq	#2	GSM2478321	Hamed, et al., 2017
mus-musculus	C2C12	Differentiating / 12h_Bex		RNA-seq	#2	GSM2478323	Hamed, et al., 2017
mus-musculus	C2C12	Differentiating / 24h_Ctl		RNA-seq	#2	GSM2478325	Hamed, et al., 2017
mus-musculus	C2C12	Differentiating / 24h_Bex		RNA-seq	#2	GSM2478327	Hamed, et al., 2017
mus-musculus	C2C12	Input for p300		ChIP-seq	1	GSM2947736	Khilji, et al., 2018
mus-musculus	C2C12	GM	p300	ChIP-seq	1	GSM2947737	Khilji, et al., 2018
mus-musculus	C2C12	Differentiating / 24h	p300	ChIP-seq	1	GSM2947738	Khilji, et al., 2018
mus-musculus	C2C12	Input for RXRα		ChIP-seq	1	GSM2478303	Hamed, et al., 2017
mus-musculus	C2C12	Differentiating / 24h_Ctl	RXRα	ChIP-seq	1	GSM2478304	Hamed, et al., 2017
mus-musculus	C2C12	Differentiating / 24h_Bex	RXRα	ChIP-seq	1	GSM2478305	Hamed, et al., 2017
mus-musculus	C2C12	Input for histone marks		ChIP-seq	1	GSM2478289	Hamed, et al., 2017
mus-musculus	C2C12	GM	H4K8ac	ChIP-seq	1	GSM2478290	Hamed, et al., 2017
mus-musculus	C2C12	Differentiating / 24h_Ctl	H4K8ac	ChIP-seq	1	GSM2478291	Hamed, et al., 2017
mus-musculus	C2C12	Differentiating / 24h_Bex	H4K8ac	ChIP-seq	1	GSM2478292	Hamed, et al., 2017
mus-musculus	C2C12	GM	H3K9ac	ChIP-seq	1	GSM2478293	Hamed, et al., 2017
mus-musculus	C2C12	Differentiating / 24h_Ctl	H3K9ac	ChIP-seq	1	GSM2478294	Hamed, et al., 2017
mus-musculus	C2C12	Differentiating / 24h_Bex	H3K9ac	ChIP-seq	1	GSM2478295	Hamed, et al., 2017
mus-musculus	C2C12	GM	H3K18ac	ChIP-seq	1	GSM2478296	Hamed, et al., 2017
mus-musculus	C2C12	Differentiating / 24h_Ctl	H3K18ac	ChIP-seq	1	GSM2478297	Hamed, et al., 2017
mus-musculus	C2C12	Differentiating / 24h_Bex	H3K18ac	ChIP-seq	1	GSM2478298	Hamed, et al., 2017
mus-musculus	C2C12	GM	H3K27ac	ChIP-seq	1	GSM2478299	Hamed, et al., 2017
mus-musculus	C2C12	Differentiating / 24h_Ctl	H3K27ac	ChIP-seq	1	GSM2478300	Hamed, et al., 2017
mus-musculus	C2C12	Differentiating / 24h_Bex	H3K27ac	ChIP-seq	1	GSM2478301	Hamed, et al., 2017
mus-musculus	C2C12	GM	H3K27me3	ChIP-seq	1	GSM2478302	Hamed, et al., 2017
mus-musculus	C2C12	Input for RNA Pol II, H3K4me1		ChIP-seq	1	GSM721306	Asp, et al., 2011
mus-musculus	C2C12	GM	RNA Pol II	ChIP-seq	1	GSM721286	Asp, et al., 2011
mus-musculus	C2C12	GM	H3K4me1	ChIP-seq	1	GSM721288	Asp, et al., 2011
mus-musculus	C2C12	Input for H3K4me3, H3K36me3		ChIP-seq	1	GSM918421	Yue et al., 2014
mus-musculus	C2C12	GM	H3K4me3	ChIP-seq	1	GSM918415	Yue et al., 201
mus-musculus	C2C12	GM	H3K36me3	ChIP-seq	1	GSM918417	Yue et al., 2014
mus-musculus	C2C12	GM	MyoD	ChIP-seq	1	GSM915186	Yue et al., 2014
mus-musculus	C2C12	Differentiating / 24h	MyoD	ChIP-seq	1	GSM915183	Yue et al., 2014
mus-musculus	C2C12	Differentiating / 24h	Myogenin	ChIP-seq	1	GSM915159	Yue et al., 2014