UNIVERSITÀ DEGLI STUDI
DI MODENA E REGGIO EMILIA

Dottorato di ricerca in Neuroscienze

(nell'ambito della Scuola di dottorato in Neuroscienze)

Ciclo XXVII

STUDY OF THE EPIGENETIC CONTROL OF MUSCLE DIFFERENTIATION: LSD1 REGULATION OF MYOD EXPRESSION

Candidato: SCIONTI Isabella

Relatore (Tutor): Prof. ZOLI Michele

Coordinatore del Dottorato: Prof. ZOLI Michele

Direttore della Scuola di dottorato: Prof. ZOLI Michele

Index

INTRODUCTION **1**
EPIGENETICS AND CELLULAR DIFFERENTIATION 2
CHROMATIN STRUCTURE AND CHROMATIN MODIFICATION FACTORS 3
CHROMATIN REMODELING ENZYMES 4
Histone Acetyltransferases *4*
Histone Deacetylases *5*
Histone methyltransferases and Demethylases *5*
LSD1/KDM1A STRUCTURE *7*
LSD1 as a co-repressor *9*
LSD1 as a co-activator *10*
MYOGENESIS 13
MYOGENIC TRANSCRIPTION FACTORS 15
Paired-homeobox transcription factors *15*
Myogenic regulatory factors *15*
Interaction of MRFs with transcriptional cofactors *18*
MYOD GENE 20
MyoD Structure *21*
MyoD Function *22*
Transcriptional control of MyoD expression *23*
ENHANCER RNAS (ERNAS) 27
Transcription and function of eRNA *27*
Mechanism of eRNAs action *28*

ABSTRACT **32**

RESULTS **35**
LSD1 AND MUSCLE DIFFERENTIATION 36
Lsd1 role during muscle denervation in vivo *36*
Lsd1 inhibition in cultured myoblasts prevents MyoD activation and muscle differentiation ... *37*
Lsd1 is recruited on the MyoD enhancer during myoblast differentiation *39*
Lsd1 regulate CEeRNA expression *41*
LSD1 AND MUSCLE DIFFERENTIATION *IN VIVO* 45
Generation of a pax3 conditional knock out mouse for LSD1 .... *45*
Lsd1 regulates MyoD expression in vivo *48*
Lsd1 inactivation in muscle precursor cells reduces myoblast formation *51*

DISCUSSION **54**

MATERIALS AND METHODS **60**
CELL CULTURES 61
EXPRESSION ANALYSIS 61
RNA extraction *61*
RevertAid H minus RT-PCR system and Real Time PCR *62*
CHIP- CHROMATIN IMMUNOPRECIPITATION 63
WESTERN BLOTTING ANALYSIS 65
Total protein extract preparation *65*
Immunoblot analysis *65*
NUCLEI COUNTING 66
CLONING 66
MOUSE BREEDING AND GENOTYPING 66
EMBRYO HARVESTING 67
Whole Mount In situ Hybridization *67*
IMMUNOFLUORESCENCE 68
STATISTICAL ANALYSIS 69

BIBLIOGRAPHY **71**

Introduction

Epigenetics and cellular differentiation

Numerous studies have indicated that stem cells respond to a combination of intrinsic programs and extracellular cues from the environment that determine which types of progeny they will produce. One of these intrinsic programs is epigenetic modification, which encompasses DNA methylation, chromatin modification and non coding RNA mediated processes. Epigenetics modifications are temporally regulated and reversible, thereby ensuring that stem cells can generate different types of cells from a fixed DNA sequence.

Human embryonic stem cells (hESCs) as well as tissue and organ precursors, named somatic stem cells (SSC) are self-renewing and have the potential to commit into multiple lineages. Lineage commitment, migration, proliferation and differentiation of these cells are regulated by the coordinated activation and repression of several subsets of genes in response to external stimuli.

So far one of the most challenging questions in regenerative medicine is the therapeutic repopulation of diseased organs and tissues by endogenous progenitor cells. Indeed understanding how the epigenetic mechanisms control gene expression at different differentiation stages would be critical to devise strategies and tools aimed at manipulating stem cells for therapeutic regeneration of tissue and organs.

Chromatin structure and chromatin modification factors

Within all eukaryotic nuclei, DNA is organized into a highly dynamic and regulated structural polymer termed chromatin. Nucleosomes are the basic structural unit of chromatin and they represent two turns of genomic DNA (147 base pairs) wrapped around an octamer of two subunits of each of the core histones H2A, H2B, H3 and H4 (figure1). The histones within the nucleosome core interact via a three α-helical "hand shake" motif termed the histone fold (Rhodes, 1997). The core histones are structurally similar highly basic proteins consisting of the histone fold motif and a N-terminal structurally undefined tail. The histone tails are subjected to significant posttranslational modifications and, in part, determine the level of chromatin condensation (Zheng and Hayes, 2003).

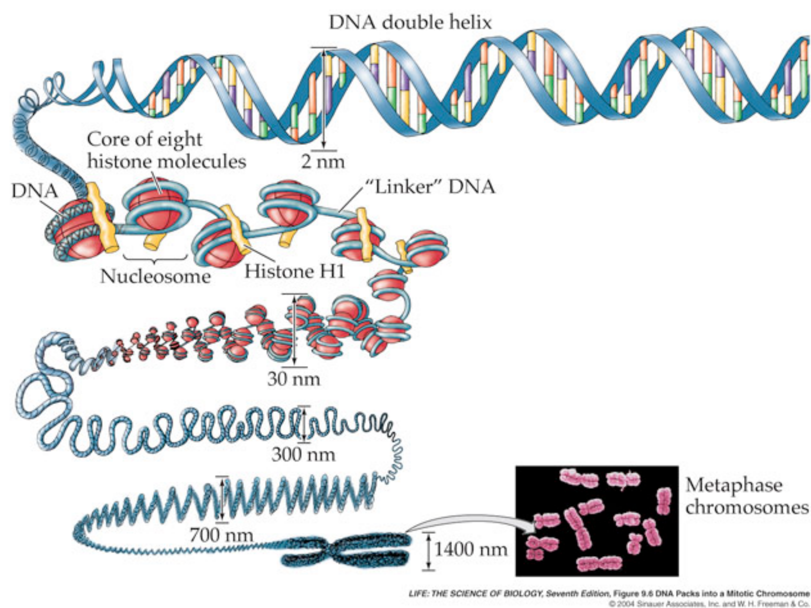


Figure 1: Chromatin structure

Individual nucleosomes are separated from neighboring nucleosomes by a short segment of linker DNA between 10 and 80 bp in length. This chromatin fashion is referred to as "beads on a string" and results in an approximately 10-fold compaction of DNA (Felsenfeld and Groudine, 2003). "Beads on a string" chromatin is compacted a further 5-fold into a 30 nm chromatin fiber by the binding of the linker histone, H1 (figure1). Within the interphase nucleus of a cell, the condensation of the DNA into chromatin fiber is heterogeneous with regions of 30 nm chromatin fiber between more highly condensed regions of 100nm (Horn and Peterson, 2002). Chromatin is the physiological substrate for most DNA-dependent process including transcription and DNA replication. As a result, changes in its structure

have significant effects on these processes. The condensation of chromatin is refractory to DNA replication and transcription therefore mechanisms exist within the cell to locally de-condense the chromatin.

Chromatin modifications can occur through covalent additions to histones. Histones amino-terminal tails are targets of modifications including acetylation, ADP-ribosylation, methylation, phosphorylation, sumoylation and ubiquitination at numerous residues. The biological role of these modifications depends not only on the type of modification but also the location of the modified site within the histone protein. Moreover several reports raised the possibility that all these modifications are combinatorial and interdependent and therefore may form the “histone code”, which means that combination of different modifications may result in several and consistent cellular outcomes (Jenuwein and Allis, 2001; Strahl and Allis, 2000).

Chromatin Remodeling enzymes

Histone Acetyltransferases

Histone acetyltransferase (HATs) are divided into two classes, the nuclear HATs or type A, which include gen5, PCAF, p300 and TAFII250 and are involved in transcriptional regulation; the cytoplasmic HATs or type B, which includes Hat1 that acetylates newly synthesized histones in the cytoplasm prior to nuclear import (Roth et al., 2001). Based on sequence similarities, type A can be further subdivided into the Gen5-related family, including PCAF, the MYST family, the TFII250 family and the p300/CBP family.

All HATs can catalyze the transfer of an acetyl group from acetyl coenzyme A to the ε-NH_3^+ groups of lysine residues within a histone substrate. However, individual HATs have different substrate preferences. In addition to acetylating histones, several HATs including p300/CBP and PCAF target non-histone substrates such as E2F1, p53 and MyoD, modulating their activity.

Hyperacetylation of histone is considered a mark of transcriptionally active regions (Allfrey et al., 1964). Moreover, studies have revealed that the role of acetylation may also affect other DNA-based cellular processes such as DNA repair and replication (Hasan and Hottiger, 2002).

Histone Deacetylases

Histone deacetylases (HDACs) are a group of proteins, which catalyze the removal of acetyl residues from both histone and non-histone substrates. Based on sequence similarities HDACs can be classified into three groups, Class I include HDAC1, HDAC2, HDAC3 and HDAC8, Class II include HDAC4, HDAC5, HDAC6, HDAC7, HDAC9 and HDAC10 and Class III include the SIR2 related proteins SIRT1 to SIRT7 (Khochbin et al., 2001; Thiagalingam et al., 2003). Similar to HATs, HDACs are involved in several signaling pathways such as cycle progression, transcriptional regulation, DNA replication and damage response.

Class I HDACs share a common catalytic domain and are expressed ubiquitously. Members of this family are required for appropriate cell cycle progression (Zhang et al., 2000). Moreover these HDACs are components of several co-repressor complexes like the N-CoR complexes. Except for HDAC3, class I HDACs are strictly nuclear proteins (Takami and Nakayama, 2000). Unlike Class I HDACs, class II HDACs are expressed in tissue specific and are shuttled between the cytoplasm and nucleus.

The Class III group is considered an atypical category of its own, which is NAD^{+}-dependent, whereas other groups require Zn^{2+} as a cofactor.

Histone methyltransferases and Demethylases

Protein methylation is a covalent modification that represents the addition of a methyl group from the donor S-adenosylmethionine (SAM) on a carboxyl groups of glutamate, leucine and isoprenylated cysteine, or on the side-chain nitrogen atoms of lysine, arginine and histidine residue (Clarke, 1993). However histone methylation occurs only on lysine and arginine residues. Arginine can be mono- or di-methylated whereas lysine can be mono- di- or tri-methylated (Kouzarides, 2007).

The enzymes responsible for histone methylation are grouped into three different classes: the lysine-specific SET domain-containing histone methyltransferases (HMTs) involved in the methylation of lysines 4, 9, 27 and 36 of histone 3 and lysine 20 of histone 4; the lysine-specific non-SET domain-containing lysine methyltransferases involved in the methylation of lysine 79 of histone 3; and arginine methyltransferases.

Lysine methyltransferases have enormous specificity compared to HATs. They usually modify one single lysine on a single histone and their output can be related to activation, elongation or repression of gene expression.

In particular H3K4 mono-, di or tri-methylated are methylation marks of transcription initiation and elongation (Hon et al., 2009; Krogan et al., 2003; Noma et al., 2001; Strahl et al., 1999). Indeed, depletion of H3K4 methyltransferase complexes causes drastic reductions in global H3K4me3 amounts. However, impairment of these complexes, results in minimal transcriptional effects (Jiang et al., 2011; Kizer et al., 2005), raising the possibility that direct transcriptional regulation is not the primary function of H3K4me3. Moreover H3K36 di- or tri-methylated have been correlated to transcriptional elongation (Kizer et al., 2005; Krogan et al., 2003; Strahl et al., 1999). Indeed, loss of the H3K36 methyltransferase Set2 has only minor effects on transcription (Kizer et al., 2005). These findings have suggested that such histone modifications may function as regulatory modules. For example, H3K4me3 inhibits tri-methylation of H3K27 by the Polycomb repressive complex 2 (PRC2) (Schmitges et al., 2011).
On the other hand, three lysine methylation sites are connected to transcriptional repression: H3K9, H3K27, and H4K20 (Lachner et al., 2001; Nakayama et al., 2001; Noma et al., 2001). Very little is known regarding the repression functions of H4K20 methylation compared to the other two. Methylation at H3K9 is implicated in the silencing of genes as well as forming silent heterochromatin. Consisted with this, methylation of H3K9 is carried out by SUV39H1 and SUV39H2. These HMTs have been found to contain a SET domain, which consists of 130-140 amino acids commonly present in Trithorax (Thx) and Polycomb (PcG) group proteins, which are respectively involved in activation and repression of the gene expression. H3K27 methylation is involved in silencing of the inactive X chromosome and during genomic imprinting.
Whereas most covalent histone modifications are reversible, until recently it was unknown how methyl groups could be actively removed from histones and thus thinly regulates gene expression.
In 2004, Shi et al (Shi et al., 2004) have characterized the first histone demethylase, LSD1 (**L**ysine-**S**pecific **D**emethylase-**1; KDM1A**) a nuclear amine oxidase homolog. After the LSD1 discovery researchers have focused on the identification of new demethylases with a mechanism based on the one used by *Escherichia coli* DNA repair AlkB demethylase (Trewick et al., 2002).
In 2006, Yamane et al (Yamane et al., 2006) have reported a new class of demethylases JHDM (**J**mjC domain-containig **H**istone **D**emethylase). Subsequently it has been shown that JHDM enzymes form a large and evolutionarily conserved histone demethylase family.

LYSINE-SPECIFIC DEMETHYLASE-1 (LSD1/KDM1A)

LSD1/KDM1A STRUCTURE

Lysine (k)-specific histone demethylase (LSD1/KDM1A) is an amino-oxidase, which demethylates histones through a NAD-dependent reaction (Shi et al., 2004). LSD1 has been identified (Shi et al., 2004) as part of a multiprotein corepressor complex that contains both HDAC1 and 2 and demethylase activity (Lee et al., 2005). It is highly conserved in organisms ranging from *S. pombe* to human.

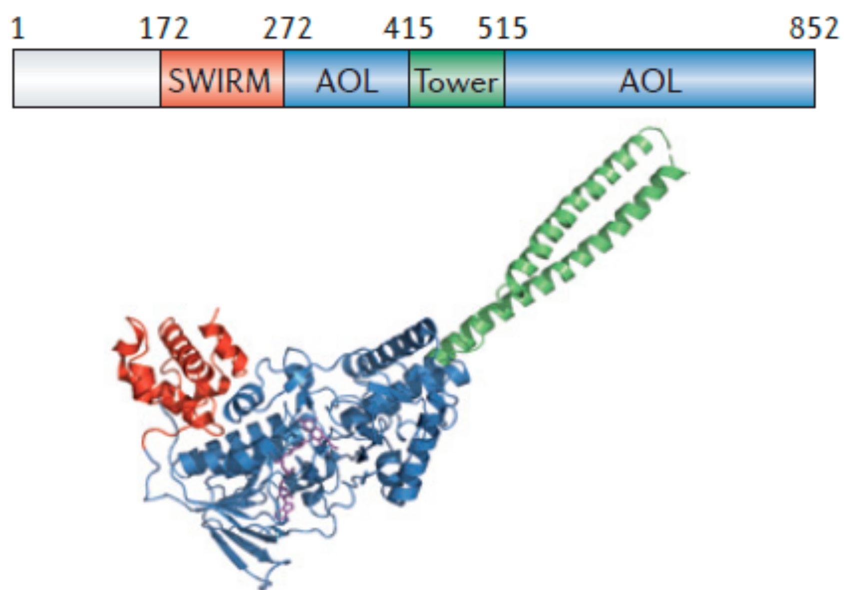


Figure 2: LSD1 structure

The structure of LSD1 contains three domains: the SWIRM, the AOL and Tower domains (figure 2).

- SWIRM domain

The SWIRM domain consists mostly of α-helices and is a structural module often found in chromatin-associated proteins (Da et al., 2006; Qian et al., 2005; Tochio et al., 2006). It is named after the proteins SWI3, RSC8 and MOIRA in which it was first described. SWIRM domains from other proteins have been shown to bind DNA and have been proposed to anchor and properly present their associated protein or protein complexes to nucleosomal substrates (Da et al., 2006; Qian et al., 2005; Yang et al., 2006). However gel-mobility shift assay clearly demonstrated that the SWIRM domain of LSD1 did not shift DNA indicating that it is not a DNA- binding motif (Chen et al., 2006). Moreover, although the structure and the biochemical data suggest that the SWIRM domain of LSD1 is important for the stability of LSD1, the exact function of this domain within LSD1 need to be more investigated.

- AOL domain

The AOL domain folds into a compact structure that exhibits a topology found in several flavin dependent oxidases (Fraaije and Mattevi, 2000). In particular it contains two subdomains, a FAD binding subdomain and a substrate-binding subdomain. The two subdomains together form a large cavity creating a catalytic center at the interface of the two subdomains.

Trough a demethylase assay in vitro it has been demonstrated that LSD1 can catalyze the demethylation of lysine 4 residue of histone 3 (Shi et al., 2004) by cleavage of the α-carbon bond of the substrate to generate an imine intermediate. The intermediate is subsequently hydrolyzed and the carbinolamine produced degrades releasing formaldehyde and amine. The formation of the imine intermediate requires a protonated lysine, thus LSD1 can only demethylate mono- or di-methylated lysine residues because tri-methyl-lysine residues are not protonated. The AOL domain contains a large insertion that forms an additional domain and adopts a tower-like structure (tower domain).

- TOWER domain

The tower domain directly interacts with one of the LSD1-interacting proteins, CoREST. In particular the two CoREST SANT (**S**wi3/**A**da2/**N**CoR/**T**ranscription factor **I**IIB) domains have been proposed to be a histone-tail-presenting module (Boyer et al., 2002). Indeed it

has been demonstrated that the SANT 2 domain of CoREST is sufficient to provide LSD1 the ability to demethylate nucleosomal substrates (Shi et al., 2004). Consistent with this, the interaction between the tower domain and SANT2 domain acts as a molecular bridge connecting LSD1 with its substrates.

Moreover it has been shown that the tower domain is essential for the demethylase activity of LSD1 (Chen et al., 2006). However, how the tower domain can affect the activity of LSD1 still needs to be deeply investigated.

LSD1 can be recruited in different chromatin complexes thus, depending on chromatin context and protein partners, with which it is associated, it can act as a transcriptional co-activator or co-repressor.

LSD1 as a co-repressor

LSD1 is the first histone demethylase identified and it has been originally described as a component of the corepressor complex, which contained the REST corepressor (CoREST) and HDAC1/2 (Shi et al., 2005). This transcriptional corepressor complex could be recruited to RE1 element-containing gene promoters by REST and repressed the transcription of neuron specific genes, as muscarinic acetylcholine receptor M4 (M4AchR), SCN1A, SCN2A, SCN3A and p57, in non-neuronal cells (Shi et al., 2004). As previously described, the link between the SANT2 domain of CoREST and LSD1 is necessary to stimulate the binding and activity of LSD1 towards di-methyllysine 4 of histone 3 (figure 3). Consistent with this, mutations in SANT2 domain, which disrupt the DNA-binding activity of CoREST also diminish demethylation of H3K4, leading to the transcription activation (Yang et al., 2006).

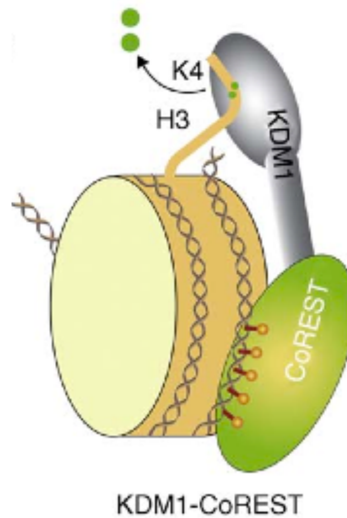


Figure 3: The proposed multivalent interaction between KDM1–CoREST and the nucleosome. Histone H3 tail binds to the active site of KDM1 while CoREST SANT domains bind to DNA (!!! INVALID CITATION !!!).

LSD1 has also been described as part of the SIRT1/HDAC complex where it acts as a transcriptional repressor of Notch target genes (Mulligan et al., 2011). Since the characterization of the LSD1-REST/NRSF complex as a master regulator of neuronal gene expression, many studies have focused on the role of LSD1 in the maintenance of silenced state of several developmental genes in embryonic stem cells (ESCs) (Adamo et al., 2011; Sun et al., 2010). It has been shown that LSD1 inhibits ESC differentiation toward the neural lineage suggesting its importance in the maintenance of pluripotency and specification of neural or neuronal commitment of pluri- multipotent cells (Han et al., 2014). Moreover it has been established that LSD1 is crucial not only for immature hematopoietic stem cell differentiation (Adamo et al., 2011; Whyte et al., 2012) but also for differentiation of mature hematopoietic cells. Indeed, differentiating LSD1 knockout cells aberrantly express hematopoietic stem and progenitor cell's (HSPC) genes normally expressed only in hematopoietic stem cells (HSCs) and progenitors. Thus the failure to silence these genes in LSD1 mutants interferes with proper hematopoietic differentiation (Kerenyi et al., 2013). Due to LSD1 key role in the control of many cell differentiations and the increased interests in the so-called epigenetic therapies, there is a growing interest in LSD1 as a potential drug target (Pollock et al., 2012; Wang et al., 2011a). Very recently it has been also demonstrated that LSD1 controls the osteogenic differentiation of human adipose-derived stem cells (hASCs) negatively regulating the expression of osteogenesis-associated genes, such as OSX and OC genes (Ge et al., 2014). This discovery has

provided a new tool to promote osteogenic differentiation of hASC, which is a critical issue in the bone tissue-engineering field.

LSD1 as a co-activator

One of the first indicators that LSD1 might also function as a transcriptional co-activator came from a study published in the 2005, which suggests that LSD1 interacts with androgen receptor in vitro and in vivo, and stimulates androgen-receptor-dependent transcription (Metzger et al., 2005). Two years after it has been reported that LSD1 is required also for estrogen receptor (ER) –dependent gene transcription (Garcia-Bassets et al., 2007). Therefore, while REST/CoREST-dependent genes clearly employ LSD1 in repression for at least a cohort of the REST-dependent program, these findings have also revealed that LSD1, associated with other complexes is required for a surprisingly broad range of regulated gene activation events. While an in vitro demethylase assay demonstrated the H3K4me2 demethylase activity of LSD1, it failed to do so for the demethylation of H3K9me2, suggesting that the LSD1 activity and substrate specificity may be altered by and required association with other cofactors.

More recently the crucial role of epigenetic mechanisms in tissue differentiation has became more important, thus many studies have focused to unveil the role of LSD1 in this cellular process. In particular it has been reported that LSD1, during adipogenesis, induces the expression of CEBPA gene opposing the function of a KTM SETDB1 (Musri et al., 2010) (figure 4).

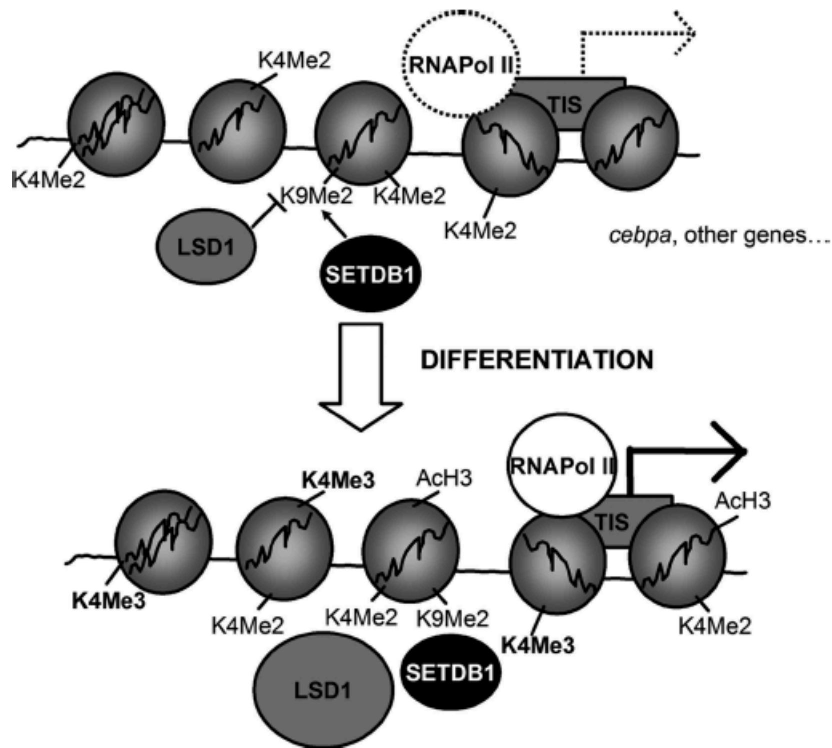


Figure 4: Model showing the interplay between LSD1 and SETDB1 on the regulation of the histone methylation status of the cebpa promoter in 3T3-L1 preadipocytes (Musri et al., 2010).

Moreover it has been proposed a role for LSD1 in skeletal muscle differentiation. Indeed it has been reported that LSD1 in association with MEF2 and MyoD, is recruited onto the Myogenin (MyoG) promoter where it is required for a proper regulation of histone marks during myogenesis (Choi et al., 2010).

Myogenesis

Skeletal muscle is a striated muscle tissue with complicated and heterogeneous features that serves multiple critical functions in the organism. Vertebrate skeletal muscle of the trunk and limbs originates from the somites, which are mesodermal structures that are located on either side of the neural tube in vertebrate embryos (Ordahl and Le Douarin, 1992) In response to the signals from distinct environmental cues, somites differentiate and subdivide into two compartments, the dorsal dermomyotome and the ventral sclerotome. Myogenic precursors in the dermomyotome subsequently give rise to myotomes, which are responsible for the formation of the trunk and deep back muscles (Kalcheim and Ben-Yair, 2005) (figure 5).

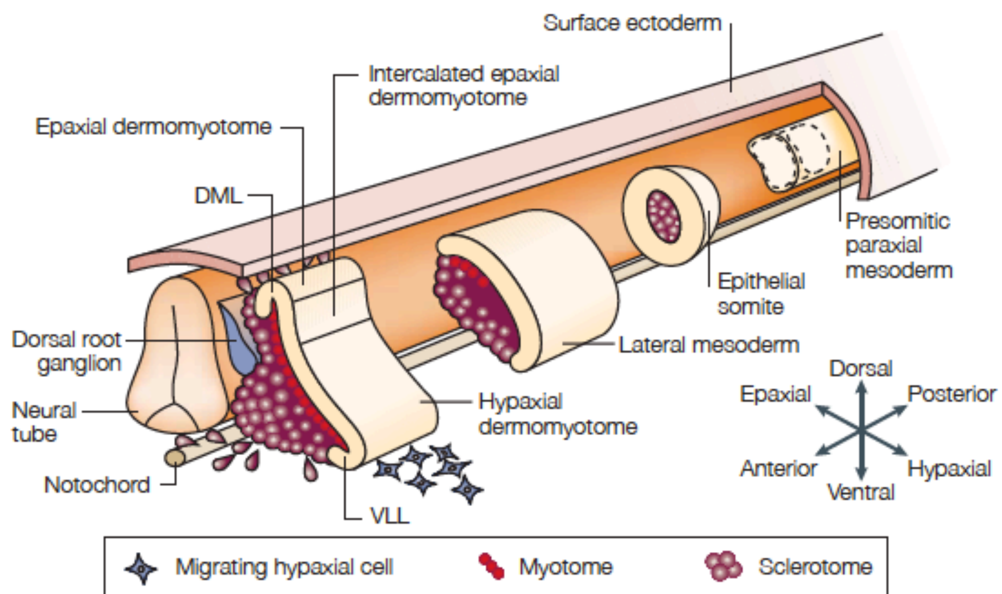


Figure 5: The embryonic origin of limb and trunk skeletal muscle (Parker et al., 2003)

Meanwhile, some of the cells from the lateral edge of the dermomyotome undergo epithelial-mesenchymal transition and delaminate and migrate to the limb buds, where they give rise to limb musculature following sequential steps including myoblast specification, myocyte differentiation and fusion, and mature myofiber formation (Chevallier et al., 1977; Dietrich et al., 1999). During murine skeletal muscle development, myoblasts are derived from two distinct progenitor populations and contribute to two phases of myogenesis (Hutcheson et al., 2009). The first wave of mononucleated myocyte fusion into multinucleated myofibers occurs at approximately embryonic day 11 (E11) and is defined as primary or embryonic myogenesis, in which basic muscle patterning occurs.

The secondary, or fetal myogenesis that occurs between E14.5 and E17.5 is characterized by fusion of fetal myocytes with each other, or their alignment and fusion with the scaffold-like primary myotubes to form secondary myofibers (Messina and Cossu, 2009). At the end of this phase, each myofiber is coated by basal lamina, underneath which some muscle progenitors termed satellite cells are located (figure 6).

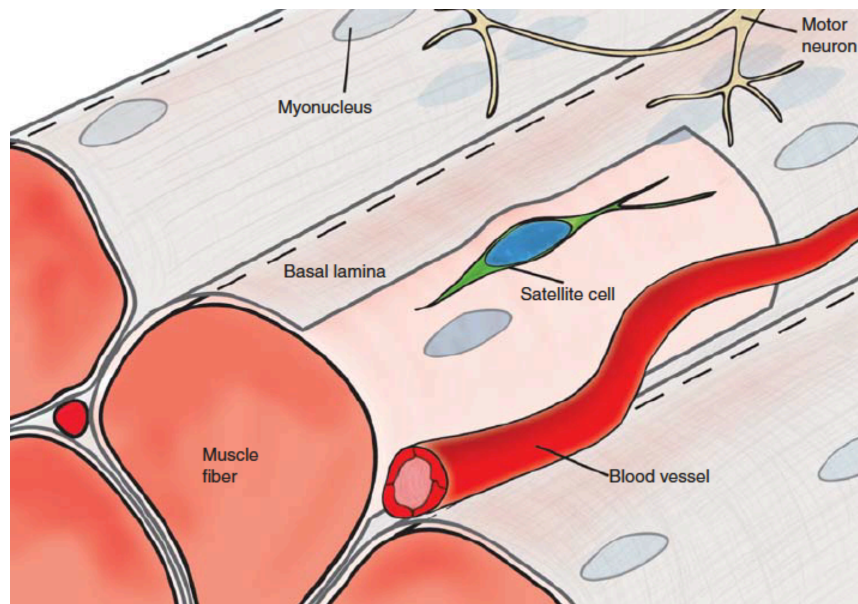


Figure 6: Representation of skeletal muscle and the satellite cell niche (Bentzinger et al., 2012).

Satellite cells normally remain quiescent in adult muscles, but they can be activated upon injury and aid in muscle regeneration. Satellite cell-mediated muscle regeneration is highly similar to developmental myogenesis, as evidenced by common transcription factors and molecular signals that modulate them (Tajbakhsh, 2009; Yin et al., 2013).

Myogenic transcription factors

Paired-homeobox transcription factors

Myogenesis is finely controlled by intrinsic genetic hierarchies of myogenic transcription factors. During mouse muscle development, the precursor cells in the dermomyotome express paired-homeobox transcription factors pax3 and pax7, with preferential expression of pax3 in the dorsalmedial and ventrolateral lips, and pax7 in the central region where satellite cells originate (Gros et al., 2005; Kassar-Duchossoy et al., 2005). Of note, only pax3 is detected in the migrating cells that enter the limb bud. To support this observation, Splotch mice with a pax3 loss-of-function mutation fail to develop limb muscles, and no pax3-positive cells are detected in the limb, indicating a lack of progenitor migration to the site (Daston et al., 1996). Consistently, pax3-deleted mice loose all of their embryonic myofibers (Hutcheson et al., 2009), further supporting that pax3 is required for normal skeletal muscle development. In contrast, pax7 is dispensable for fetal myogenesis because pax7-/- mice do not display skeletal muscle formation defects (Seale et al., 2000). Instead, a complete absence of satellite cells is observed in the mutant mice (Relaix et al., 2006; Seale et al., 2000). However, Hutcheson et al. demonstrated an essential role for pax7 in fetal myogenesis by ablating pax7-expressing cells from mouse embryos (Hutcheson et al., 2009). Pax7 lineage deletion resulted in the loss of fetal (secondary) myofibers, consistent with the observation that pax7 is expressed in fetal myoblasts (Biressi et al., 2007; Horst et al., 2006). These studies suggest different role for pax3 and pax7: the first one is critical for initial myofiber formation and pax7 is required to maintain the satellite cell pool.

Myogenic regulatory factors

Pax3+pax7+ progenitors are mitotically active and cannot differentiate into myotubes (Kassar-Duchossoy et al., 2005), suggesting that molecules other than pax3 and pax7 are responsible for myogenic induction and precursor cell differentiation. The discovery of MyoD, a transcription factor that is able to convert mouse pluripotent mesenchymal C3H10T1/2 cells into fusion-capable myoblasts (Davis et al., 1987) sheds light on the molecular nature of muscle differentiation. Subsequent studies revealed three more transcription factors: Myf5, myogenin and MRF4, which are also able to induce myoblast traits in non-muscle cells (Braun et al., 1990; Braun et al., 1989; Edmondson and Olson,

1989). Characterized by their collective expression in the skeletal muscle lineage, these four transcription factors are termed myogenic regulatory factors (MRFs). MRFs have a conserved basic helix-loop-helix (bHLH) DNA binding domain and relatively variable N-terminal and C-terminal domains to mediate transcriptional activation. The HLH domain also facilitates heterodimerization between MRFs and E-proteins that recognize the E-box consensus sequence CANNTG, which is present in many muscle specific gene promoters (Singh and Dilworth, 2013).

Myf5 is the first MRF expressed within the dermomyotome on the eighth day of mouse embryonic development, and its expression starts to decrease on E11 (Buckingham and Tajbakhsh, 1993). In contrast, MyoD is expressed at approximately E10.5, and myogenin transcripts begin to accumulate immediately after MyoD activation (Braun et al., 1994). Two waves of MRF4 expression have been observed in mouse embryogenesis. The first wave occurs between E9 and E11.5 and the second one starts at E16 and persists through adulthood (Patapoutian et al., 1995). Genetic studies in mice indicate redundant and differential roles of MRFs in myogenesis. Mice lacking Myf5 or MyoD have no major defects in muscle development. Myf5-null mice have normal skeletal muscle morphology and muscle-specific gene expression, while the appearance of myotome cells is delayed until MyoD is expressed (Braun et al., 1992). Indeed, myogenesis in Myf5-null mice is fully restored by a MyoD-expressing lineage (Gensch et al., 2008; Haldar et al., 2008). MyoD deletion results in prolonged and elevated Myf5 expression, which functionally leads to normal skeletal musculature (Rudnicki et al., 1992). Interestingly, Myf5/MyoD double-null mice are completely absent of skeletal myoblasts or myofibers as well as myogenin expression (Rudnicki et al., 1993). These observations indicate that Myf5 and MyoD play partially redundant roles in myogenic cell fate determination and myoblast commitment.

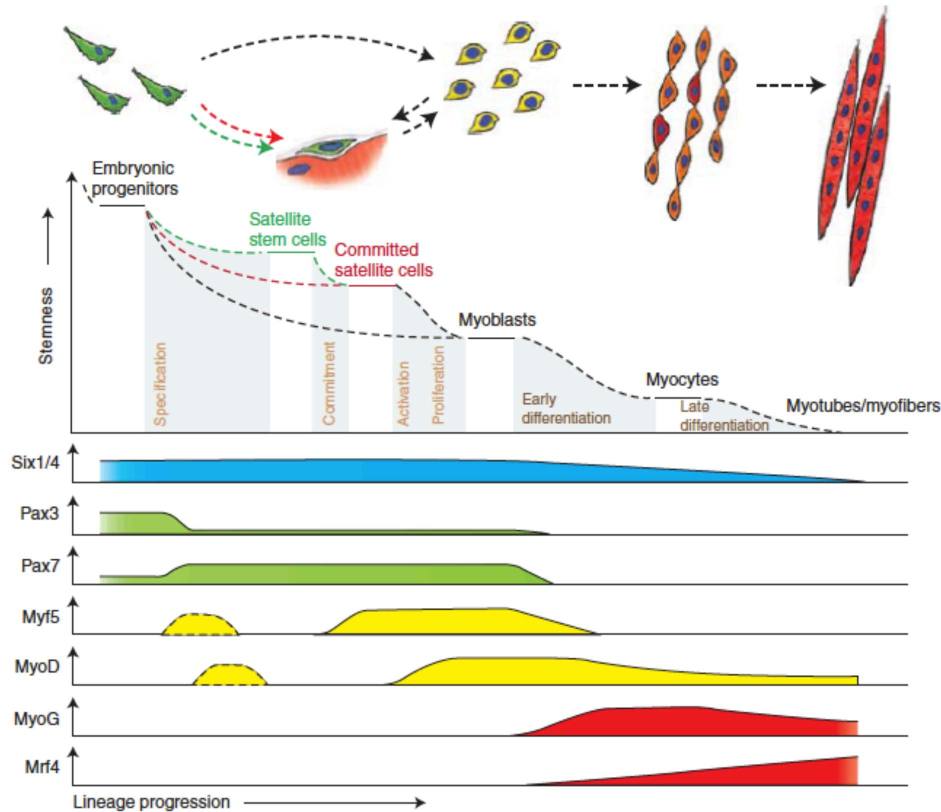


Figure 7: Hierarchy of transcription factors regulating progression through the myogenic lineage (Bentzinger et al., 2012).

Myogenin-mutant mice have severe defects in muscle fiber formation with reduced muscle-specific gene expression such as myosin heavy chain and MRF4. However, Myf5 and MyoD expression appears normal, and mono-nucleated myoblasts are observed in the limbs (Hasty et al., 1993; Nabeshima et al., 1993), suggesting that myogenin is essential for committed myoblast differentiation and acts downstream of Myf5 and MyoD.

MRF4-/- mice have normal skeletal muscle development and demonstrate strong myogenin up-regulation, which may compensate for the absence of MRF4 (Rawls et al., 1998). However, MyoD/MRF4 double mutations result in a severe muscle deficiency that is similar to the myogenin-mutant mice (Lassar et al., 1991). Thus, this up-regulation of myogenin expression was insufficient to induce myogenesis, suggesting both that MRF4 and MyoD have overlapping functions in myoblast differentiation and a potential myogenic determination role for MRF4.

These studies reveal a hierarchical relationship between MRFs whereby Myf5 locates at the top of the hierarchy and collaborates with MyoD in a redundant fashion to specify myoblasts, while myogenin and MRF4 act genetically downstream to induce myoblast differentiation and muscle-specific gene expression (figure 7).

Interaction of MRFs with transcriptional cofactors

A cooperation among MRFs and others molecules has been claimed by many studies to be necessary for myotubes formation and myogenic gene expression. Indeed, during myogenesis, MRFs act as heterodimers together with E proteins (E12, E47 and HEB), which belong to the same transcription factor family, and bind to E boxes in many muscle-specific gene promoters (Christy et al., 1991). In proliferating myoblasts, active Myf5/E protein or MyoD/E protein heterodimers are disrupted by the HLH protein Id (inhibitor of differentiation), which can form complexes with E proteins or MRFs through HLH domain interactions. Id proteins lack the basic DNA binding domain; thus, Id/E or Id/MRF heterodimers fail to bind E boxes in muscle promoters (Jen et al., 1992; Molkentin et al., 1995). Id protein levels are decreased at differentiation onset (Molkentin et al., 1995), allowing the formation of heterodimers Myf5/E protein or MyoD/E protein, and thus myogenic gene expression. Full activation of muscle-specific gene expression by MRFs requires their collaboration with myocyte enhancer factor 2 (MEF2) proteins (MEF2A-D), which belong to the MADS (MCM1, agamous, deficiens, SRF) family of transcription factors (Molkentin and Olson, 1996). MEF2 proteins cannot activate muscle-specific genes on their own, but they potentiate the transcriptional activity of MRFs by interacting with the MRF/E protein complexes (Gossett et al., 1989; Molkentin and Olson, 1996). Consistently, the A/T rich sequence for MEF2 binding is often close to the E-box sequences within muscle genes (Johanson et al., 1999). In addition to activating muscle structural genes, MEF2 proteins mediate myogenic bHLH gene expression in a positive feedback mechanism. Upstream signals activate Myf5 and MyoD, which cooperate with MEF2 proteins to induce myogenin expression (Cserjesi and Olson, 1991; Yee and Rigby, 1993). Myogenin up-regulates MEF2 (Edmondson et al., 1992), which not only acts on the myogenin promoter to amplify gene expression (Wang et al., 2001), but also auto regulates its own promoter (Black et al., 1995). Moreover, MRF4 expression requires synergistic function between MEF2 and MRFs such as myogenin (Naidu et al., 1995). During mouse skeletal muscle development, Mef2c is the first member of the MEF2 family to be expressed followed by Mef2a and Mef2d (Potthoff et al., 2007a). Mef2a or Mef2d homozygous mutant mice display no muscle developmental defects (Potthoff et al., 2007b). However, skeletal muscle-specific Mef2c deletion resulted in disorganized myofibers, disrupted muscle structural gene expression and perinatal lethality, although embryonic and fetal myogenesis appear to be normal (Potthoff et al., 2007b), indicating that MEF2C is required for the skeletal muscle postnatal maturation, but not early development.

Moreover, the condensed nucleosomal organization, which is present on muscle-specific gene promoters, prevents access of transcription factors including MRFs and MEF2 proteins to the regulatory regions of these genes, resulting in transcriptional repression. Therefore, chromatin modification and remodeling are required to relax the chromatin and allow for transcription factor access. Several transcriptional co-activators in muscle including p300, a functional homolog of CREB-binding protein (CBP) and p300/CBP-associated factor (PCAF) have intrinsic histone acetyltransferase (HAT) activity; conversely, histone deacetylases (HDACs) repress transcription in skeletal muscle (McKinsey et al., 2001). MRFs and MEFs interact with HATs and HDACs (figure 8). p300 stimulates muscle-specific gene expression by interacting with MyoD to potentiate its transcriptional activity in human and mouse muscle cell lines (Puri et al., 1997; Sartorelli et al., 1997; Yuan et al., 1996).

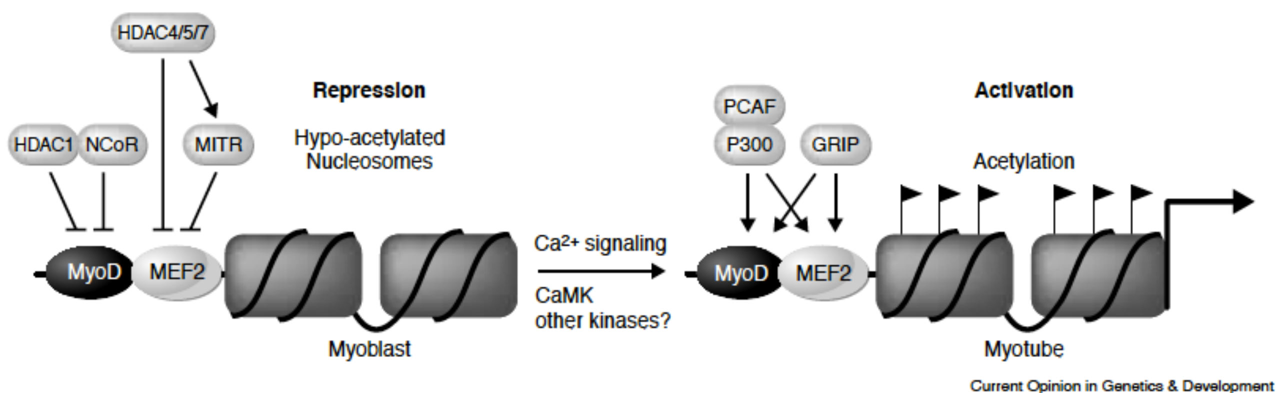


Figure 8: A model for the roles of HATs and HDACs in the control of muscle gene expression (McKinsey et al., 2001).

This enhanced MyoD-mediated gene transcription is not only because of chromatin acetylation and relaxation, but also because of direct acetylation of MyoD by p300 and PCAF (Duquet et al., 2006; Polesskaya et al., 2000; Sartorelli et al., 1999). p300 also interacts with MEF2 and acetylates it in skeletal muscle, resulting in enhanced DNA binding ability, transcriptional activity and myogenic differentiation (Lu et al., 2000; Ma et al., 2005). Conversely, HDACs inhibit muscle-specific gene expression and myogenic differentiation by interacting with MyoD and MEF2 (Mal et al., 2001; Puri et al., 2001) (figure 8).

In addition to acetylation, chromatin remodeling is modulated by factors that loosen histone-DNA interactions using energy from ATP hydrolysis. The SWI/SNF (switching/sucrose non-fermenting) complex is an important chromatin-remodeling enzyme,

which consists of an ATPase subunit brahma-related gene 1 (BRG1) and BRG1-associated factor BAF. Several studies have demonstrated that the SWI/SNF complex plays a crucial role in MyoD and myogenin-mediated muscle gene activation and myogenic differentiation (Forcales et al., 2012; Ohkawa et al., 2006; Ohkawa et al., 2007). On muscle gene regulatory elements, MyoD interacts with SWI/SNF subunit BAF60c, which recruits the catalytic subunit BRG1 to form a functional SWI/SNF complex in differentiating muscle cells, thereby facilitating chromatin remodeling and MyoD-targeted gene expression (Forcales et al., 2012) (figure 9).

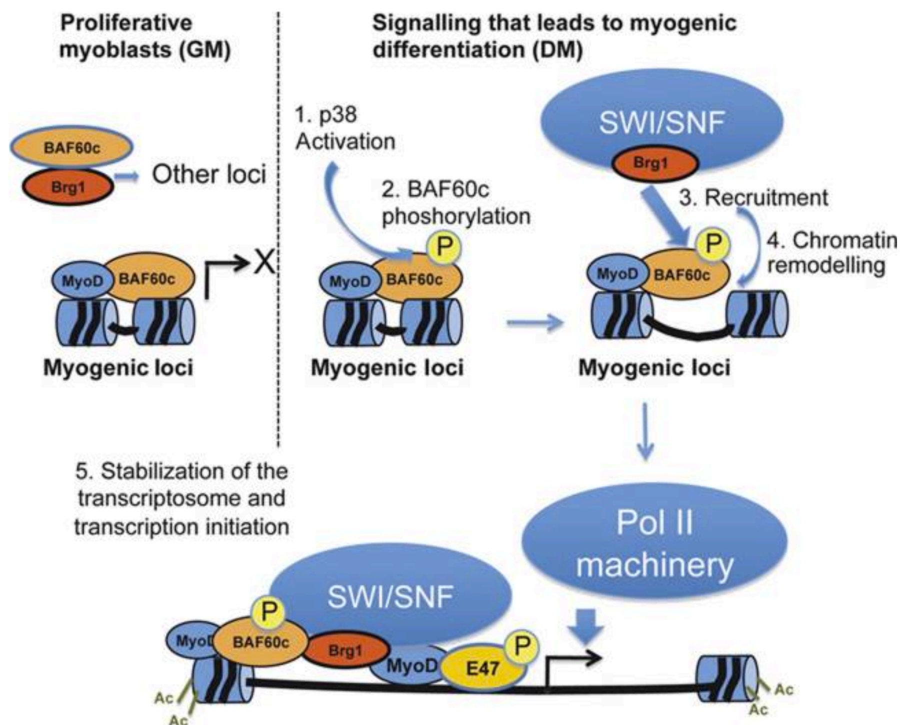


Figure 9: Cartoon of the stepwise recruitment of SWI/SNF to MyoD-target genes by pre-assembled BAF60c–MyoD complex (Forcales et al., 2012).

MYOD GENE

As mentioned previously, forced MyoD expression in non-muscle cells in vitro let them to be converted to muscle (Davis et al., 1987). To achieve this, genes must be silenced, new genes activated, and chromatin remodeled. Thus MyoD has the ability to perform all of these functions. MyoD belongs to a subfamily of bHLH transcription factors, which falls into two broad categories: Class I, broadly expressed in many cell types and contains the E protein family, and Class II expressed in a tissue specific manner and includes the MRFs.

MyoD Structure

MyoD and the other MRFs share two domains with each other and with other bHLH (basic helix-loop-helix) transcription factors, the DNA binding and dimerization domains. Variability among these factors lies in the presence, absence, or combination of activation domains and repressive domains. The common element is comprised of approximately 60 amino acids containing the DNA binding region (basic) followed by two alpha-helices, separated by a variable loop region (HLH) (Ferre-D'Amare et al., 1993). The HLH domain allows for dimerization between two HLH containing factors, either through homo-dimerization, which is uncommon, or through hetero-dimerization (Kadesch, 1993) (figure 10).

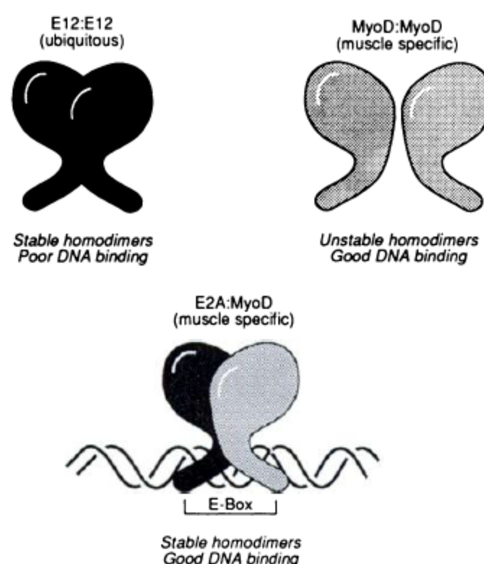


Figure 10: Model of transcriptional regulation by heterodimeric bHLH protein (Kadesch, 1993)

Once dimerized, the basic regions of the two transcription factors bind specific DNA sequences. During myogenesis, the MRF's bind E-boxes, whose consensus sequence is CANNTG, where N can be any nucleotide (figure 10). MyoD has a strong, single transcriptional activation domain (TAD) at the amino terminal end and a histidine-cysteine rich domain containing a tryptophan amino acid necessary for interaction with the Pbx/Meis complex, a known transcriptional activator (Okada et al., 2003; Tapscott, 2005) (figure 11).

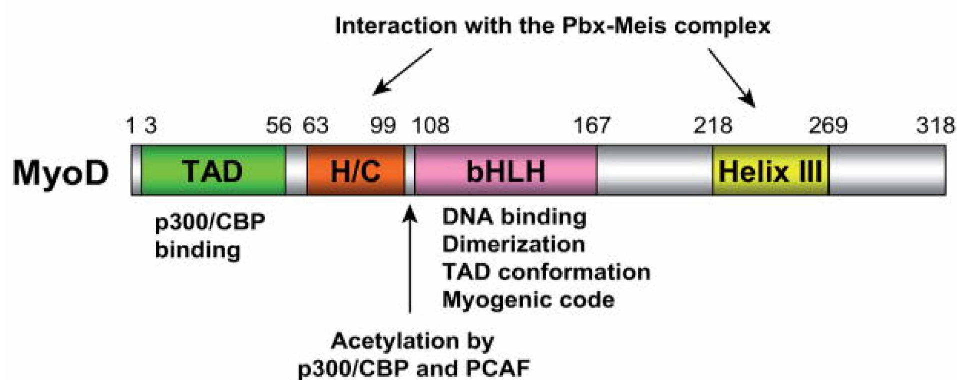


Figure 11: Structure and functional domains of MyoD.

MyoD Function

MyoD is a master transcription factor that remodels chromatin and recruits activating transcriptional complexes to the loci of many genes involved in all aspects of myogenesis. Not all genes are simultaneously expressed in response to MyoD activation (Bergstrom et al., 2002). Some are induced immediately, whereas others are involved in the late stage of differentiation. In addition, some genes are expressed transiently and some are directly decreased. Following the expression of Myod, therefore, the first sets of genes activated might affect cell migration and positioning, followed by the activation of a set of transcription factors; only later in the differentiation program are expressed many of the muscle contractile proteins. MyoD has been shown to directly bind both early and late genomic targets via CHiP data in a fibroblast cell line containing an estrogen induced MyoD allele (Bergstrom et al., 2002). Thus, if Myod initiates the myogenic differentiation program and this program temporally regulates the activity of Myod, it follows that Myod acts on the regulation of its own activity. The proposed cause of this phenomenon is a feed-forward mechanism, where early targets of MyoD are needed to cooperate with MyoD to activate the next temporal level of genes (Penn et al., 2004). Acetylation of the

MyoD protein has also been shown to affect target gene selection (Di Padova et al., 2007). The simplified description of MyoD function is that it heterodimerizes with the structurally similar, but broadly expressed, E-proteins through their shared HLH domains. Specifically, MyoD is shown to heterodimerize with E12 and E47 to activate myogenic genes. p38 phosphorylates E47 at serine 140, and this modification is essential for the association with MyoD (Lluis et al., 2005). Then, through a combination of the activation domain of MyoD and the variable activation/repression domains of the E-proteins, target genes are activated or repressed. Strangely, the target DNA sequence of MyoD is short, (CANNTG) and occurs frequently through out the mammalian genome. A large amount of regulation via protein interactions is therefore required to obtain target gene and temporal specificity. Specificity is achieved either by tandem E boxes, or a combination of E boxes and binding sites for cooperative factors that directly interact with the activation domain of MyoD, such as Mef2, Pbx, Meis, and Sp1 (Sartorelli et al., 1997; Tapscott, 2005). Even though MyoD and the MEF2 family bind different consensus DNA sequences, both sequences are found at almost every skeletal muscle genes promoter region, and efficient transcription of those genes only occurs when both factors are bound (Li and Capetanaki, 1994).

Transcriptional control of MyoD expression

Defining regulatory DNA elements of powerful transcription factors is of ultimate importance in understanding transcriptional pathways. It has been shown that MyoD-/- myoblasts serve as better transplant material than wild type myoblasts in mice (Asakura et al., 2007), which may be applicable to humans with muscle wasting diseases. Very little is known about the transcriptional activation of MyoD expression and all studies concerning the regulation of MyoD gene expression come from the embryonic development. Indeed using upstream regions of MyoD to drive lacZ or CAT expression have revealed two distinct elements, the Core Enhancer (CE) (Goldhamer et al., 1995) and the Distal Regulatory Region (DRR) (Asakura et al., 1995). Transgenic analysis has shown that the CE controls initiation of expression in newly forming myoblasts, while the DRR maintains expression in differentiating muscle. CE-lacZ transgenic embryos exhibit activity in a manner similar to MyoD mRNA detection (Faerman et al., 1995; Goldhamer et al., 1992). DRR-lacZ transgene expression is limited to sites of differentiating muscle (Asakura et al., 1995). In MyoD-/-;Myf-5-/- embryos, where no myoblasts form, the CE transgene is active while the DRR is not (Kablar et al., 1999) indicating the ability of the CE to initiate de novo MyoD expression. The 2.5 kilobases immediately upstream of the transcriptional start site,

including the proximal promoter does not contribute to specificity of expression. Thus the genomic region upstream of MyoD that had the highest activity contains the CE (Goldhamer et al., 1992). However, when the expression profile of the -24lacZ construct, which fully copies endogenous MyoD expression, to a similar construct, which lacks only the CE, there is only a delay in MyoD expression in the hypaxial myotome and limb buds up to E11.5, after which a normal expression profile is restored (Chen et al., 2001) (figure 12). These results suggest that the initial timely activation of MyoD is CE dependent in only a subset of early myogenic cells, and that ultimately, initiation of MyoD expression is CE independent.

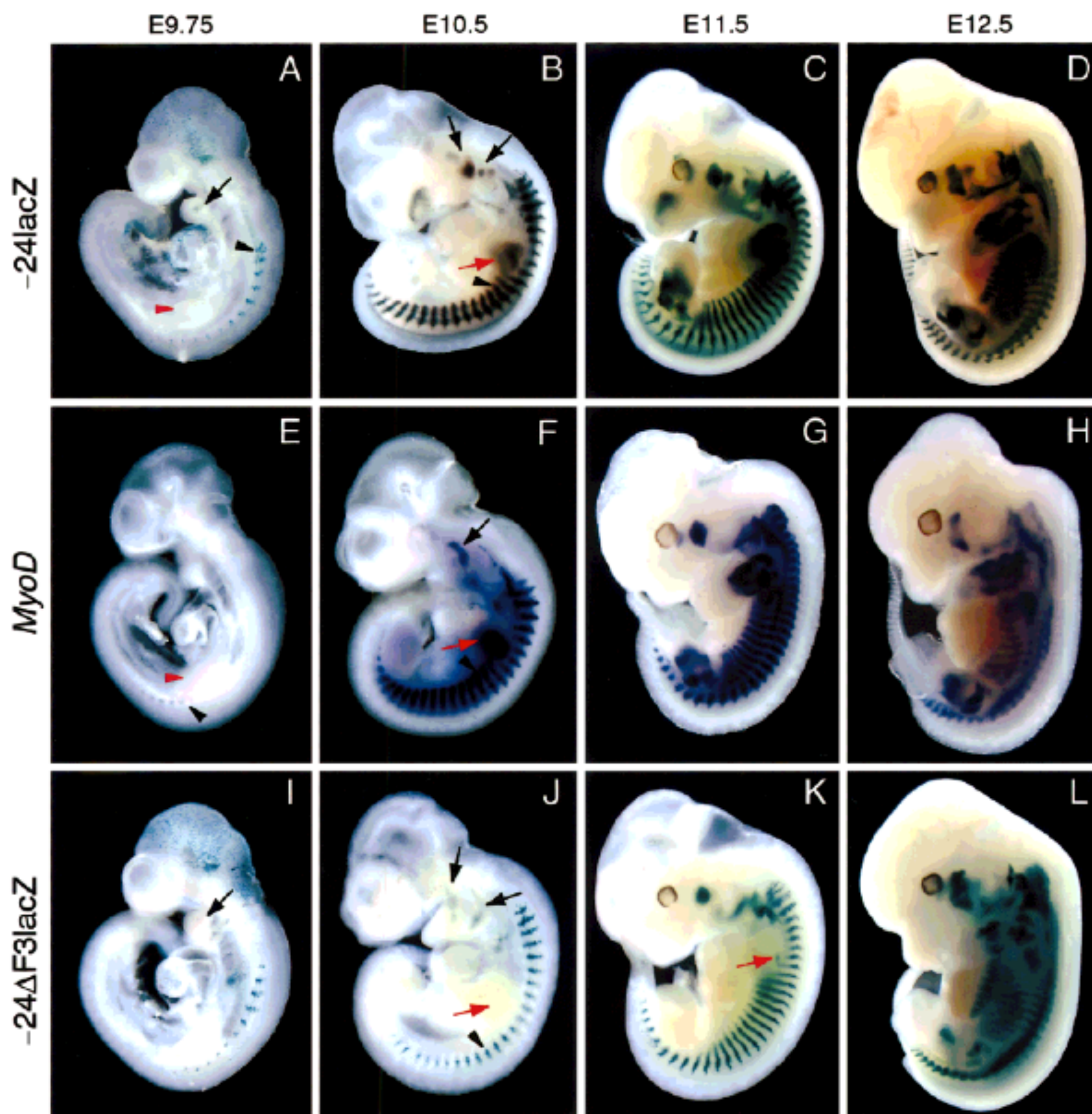


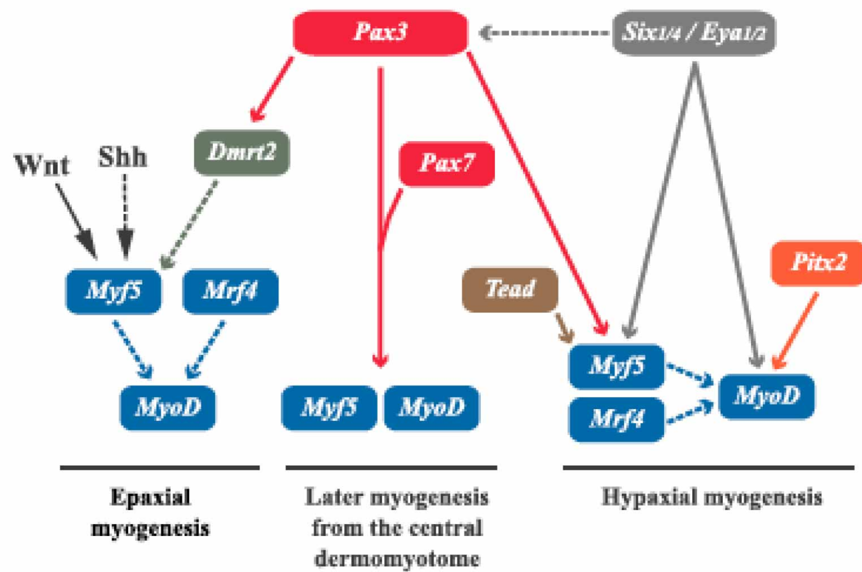
Figure 12: Representative whole mount embryos from E9.75 to E12.5 comparing expression of lacZ in transgenic lines (Chen et al., 2001).

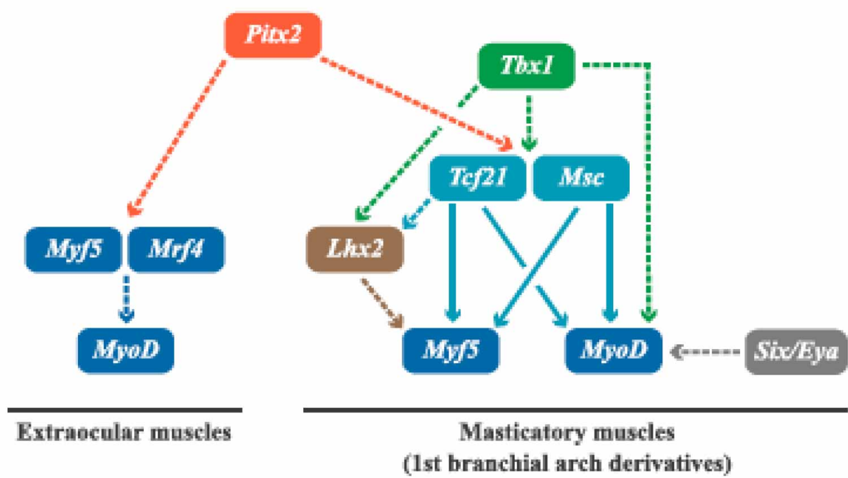
The CE and DRR of human and mice share extremely high sequence similarity and genomic position (Asakura et al., 1995; Goldhamer et al., 1995). The highly conserved

DRR maintains all putative binding sites between the two species, which are four E-boxes (CANNTG) and two MEF-2 sites (Chen et al., 2001). The sequence similarities between the CE's of humans and mice is approximately 90% and all putative binding sites are maintained, including four E-boxes, an AP-1 site, and a H4TF-1 site (Goldhamer et al., 1995).

One of the positive MyoD regulators is SRF (serum response factor), and when inhibited in myoblasts or differentiating myotubes, the MyoD locus is rapidly shut down (Gauthier-Rouviere et al., 1996). Another group of interacting factors is Sp1, YY1 and p300/CBP, which are involved in chromatin remodeling (L'Honore et al., 2003; Roth et al., 2003; Wilson and Rotwein, 2006). Cell-based assays and in vitro studies show a partnership between Fox03, Pax3, and Pax7 in the recruitment of RNA Polymerase II during the formation of the pre-initiation complex at the MyoD locus in myoblast cultures. Fox03 is further implicated as a direct activator of MyoD through Fox03 knock out experiments, where MyoD is down regulated in regenerating muscle (Hu et al., 2008).

Recent findings regarding the transcriptional control of MyoD have shown many factors bind the CE directly. Six1/4 regulates MyoD by binding the CE (Relaix et al., 2013), as does CLOCK and BMAL1, regulators of the circadian rhythm of MyoD expression (Andrews et al., 2010). A limb specific activator of MyoD, Pitx2, has also been shown to bind the CE (L'Honore et al., 2010). Repression of MyoD expression has also been linked to the CE as Sim2 and YB1/p32 bind to the CE and repress the locus by both gain and loss of function experiments (Havis et al., 2012; Song and Lee, 2010) (figure 13). Moreover in vitro cell culture analysis shows the histone variant H3.3, associated with transcriptionally active genes, is required to become associated with the CE for proper expression of MyoD in myoblasts and differentiating myotubes (Yang et al., 2011), showing a role for epigenetic remodeling in the activation of the MyoD locus via the CE.





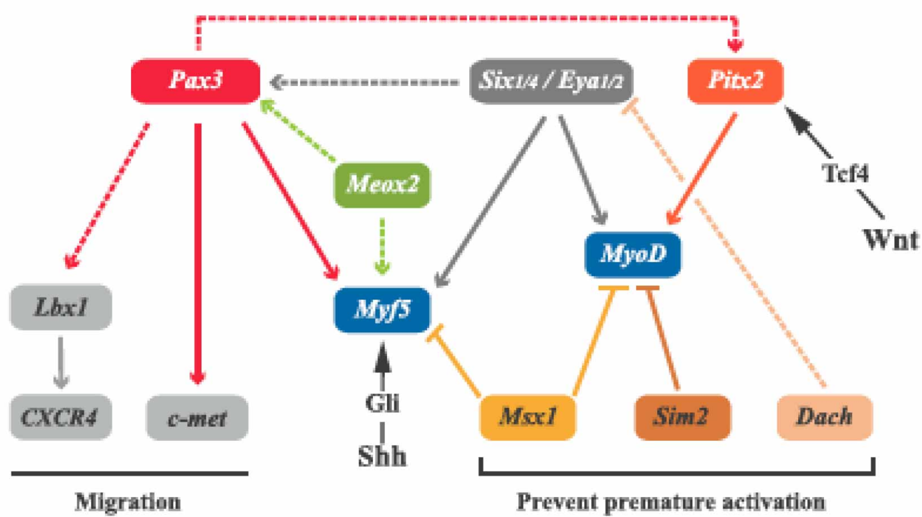


Figure 13: Gene network in myogenesis (Buckingham and Rigby, 2014).

ENHANCER RNAs (eRNAs)

Technological advances in nucleotide sequencing have culminated in a greater understanding of the complexity of the human transcriptome. Indeed through a genome-wide analysis of transcription factor binding sites, their enrichment has been found at distal intragenic and intergenic regions that exhibit features associated with enhancers (Carroll et al., 2006; Nielsen et al., 2008). It is well known that enhancers govern tissue-specific and temporal-specific regulation of gene expression. Thus, the discovery that distal regulatory elements known as enhancers are transcribed and that such enhancer-derived transcripts (eRNAs) serve a critical function in transcriptional activation has added a new dimension to transcriptional regulation.

Transcription and function of eRNA

The first evidence that the majority of the nascent RNA produced in the nucleus is rapidly turned over and does not contribute to mRNAs comes from a Harris et al study (Harris, 1959). The discovery of extensive enhancer transcription suggests that eRNAs, in addition to intronic RNA, are quantitatively important contributors to this rapidly degraded pool of nuclear RNA (Djebali et al., 2012). While enhancers were known to bind sequence-specific transcription factors, the association of RNA polymerase II (RNAPII) and general transcription factors with enhancers came as a surprise (Kim et al., 2010; Koch et al., 2011). However, while transcriptional co-activator CBP and RNAPII are centered at sequence-specific transcription factor sites, eRNAs are transcribed bi-directionally with a strong positive correlation with the expression of nearby protein-coding genes. There is also a group of enhancers that are induced unidirectionally (Hah et al., 2013; Li et al., 2013; Schaukowitch et al., 2014), indeed it remains unclear why some enhancers are transcribed from both strands and others are unidirectional, as well as which is the mechanism by which the primary transcripts of eRNAs proceed to their mature forms. Generally the majority of eRNAs are reported to be monoexonic and not polyadenylated, however, there have been cases where eRNAs are spliced and polyadenylated (Kim et al., 2010; Koch et al., 2011).
However, the exact requirements for eRNAs in activation of their target genes were not determined. Two reports have provided evidence linking the long noncoding RNAs to transcriptional activation. Using knockdown approaches, Orom et al have shown that long noncoding RNAs positively regulate neighboring protein-coding genes (Orom et al., 2010).

Genomic sites analysis for noncoding RNA (termed ncRNA-a3) that regulated the TAL1 gene has revealed its association with enhancers regions defined by enrichment in monomethyl H3K4 and acetylated H3K27 marks (Lai et al., 2013; Orom et al., 2010). This locus produced bi-directional transcripts that were polyadenylated and spliced. A similar approach was used to show that a noncoding RNA termed HOTTIP activates several 50 HOXA genes in vivo (Wang et al., 2011b). HOTTIP also corresponded to a spliced and polyadenylated transcript. While these experiments revealed a role for long noncoding RNAs in activation of neighboring protein-coding genes, they did not assess their specific signaling pathway(s). Moreover genome-wide studies have revealed the p53 association with a large number of distal binding sites (Melo et al., 2013). In particular two distinct extragenic p53 sites have revealed the expression of eRNAs that were stimulated following treatment of cells with Nutlin-3, an inducer of p53. Their depletion abrogated the Nutlin-3 induction of transcription of the targeted protein-coding gene (Melo et al., 2013). A similar scenario was observed following activation of estrogen-responsive genes in MCF7 cells (Li et al., 2013). Treatment of MCF7 cells with estradiol (E2) induced the binding of estrogen receptor alpha (ER-alpha) to a large number of extragenic binding sites. The majority of these E2-induced enhancers produced bidirectional eRNAs and their depletion diminished the E2-induced activation of their target genes. Moreover it was further shown that in the case of one enhancer, involved in activation of FOXC1 gene, only the sense strand of eRNA contained the activating function suggesting that perhaps only one strand of eRNAs confers transcriptional activation (Li et al., 2013). Further evidence to the functionality of eRNAs was provided following studies of nuclear receptor Rev-Erbs in mouse macrophages (Lam et al., 2013). The majority of Rev-Erbs-associated extragenic enhancers transcribed for bi-directional eRNAs. Detailed examination of two distinct sites adjacent to Mmp9 and Cx3cr1 genes revealed that Rev-Erbs binding at these enhancers resulted in repression of eRNAs expression, leading to silencing of the targeted genes (Lam et al., 2013). Significantly, they also show that only one strand of the eRNA is involved in the activating function, which leaving open the question of the functional importance of the other strand.

Taken together these data suggest eRNAs to be part of a genome-wide activity-dependent epigenetics mechanism.

Mechanism of eRNAs action

To date many evidence suggests that eRNA transcripts *per se* and eRNA transcription can both play functional roles, however, it is currently not clear whether these functions are primarily performed *in trans* or *in cis.* The Chromatin interaction studies have demonstrated that enhancers, responsible of looping with promoters of protein-coding genes, possess higher expression of eRNAs (Lin et al., 2012; Sanyal et al., 2012). Thus these studies suggested a potential role of eRNAs in the process of proper formation of chromosomal looping between enhancers and transcription start sites (TSSs). Recent experiments have suggested that two multi-protein complexes, Mediator and Cohesin, play an important role in such stimulus-dependent chromatin looping (Kagey et al., 2010; Phillips-Cremins et al., 2013). More importantly, these experiments have suggested a role for eRNAs in either the establishment or the maintenance of enhancer–promoter contacts (figure 14).

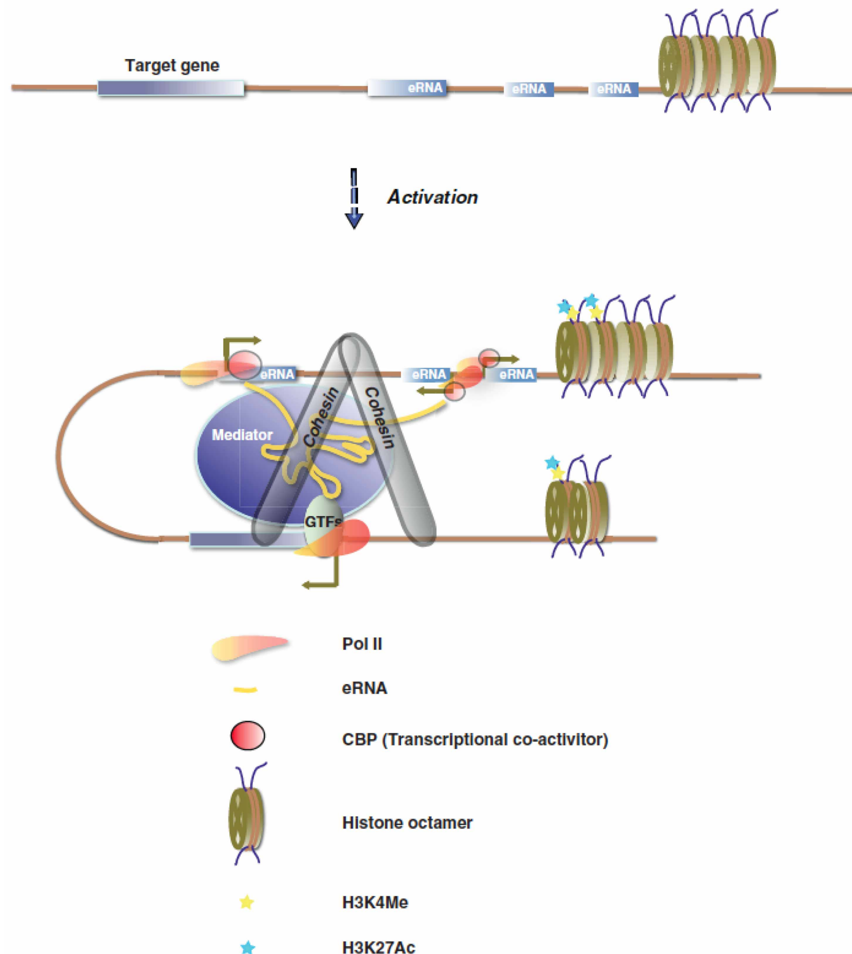


Figure 14: Cartoon of eRNA functional activity *in cis (Lai and Shiekhattar, 2014)*.

On the other hand it has been proposed that eRNA transcripts facilitate RNA PolII recruitment to the promoter of the target gene. Consistent with this hypothesis Mousavi et al. knocked down eRNA transcripts produced in each MyoD-bound enhancer across the MyoD locus, showing that only the eRNA from the CE (CEeRNA) acts *in cis* and was critical for MyoD expression (Mousavi et al., 2013). Thus, knockdown of CEeRNA decreased RNA PolII recruitment at the promoter and gene body of MyoD (figure 15).

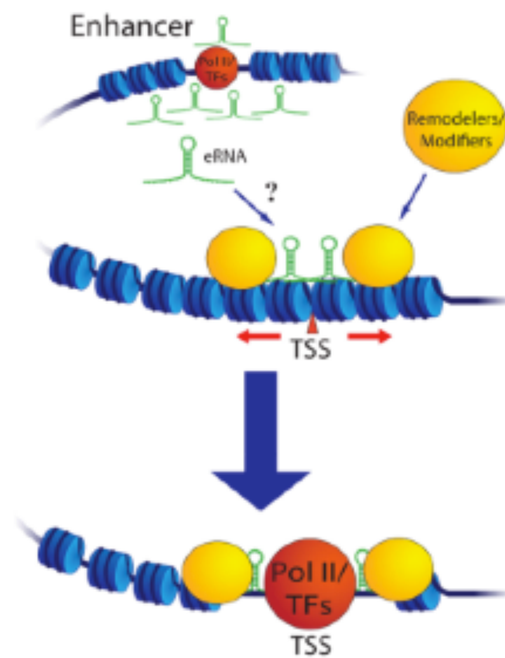


Figure 15: Schematic model of CEeRNA mechanism on MyoD TSS (Mousavi et al., 2013)

Even if eRNAs mediate expression of other genes *in trans* has not been systemically addressed several observations suggest this possibility through the differential recruitment of protein complexes by eRNAs at promoter regions. For instance, in 2006 it has been demonstrated how eRNA Evf-2 can induce the expression of Dlx2, which in turn can increase the activity of the Dlx5 and Dlx6 enhancers (Feng et al., 2006). Moreover very recently knocking down an eRNA transcribed from the DRR region at MyoD locus has resulted in a down-regulation of MyoD target genes, such as myogenin (Mousavi et al., 2013; Mueller et al., 2015).

Taken together these results suggest how eRNAs can also exert their function as co-activator of master regulatory genes (figure 16).

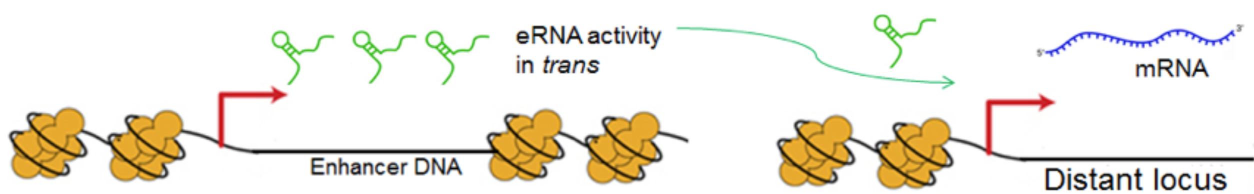


Figure 16: eRNA mechanism of action *in trans*.

Abstract

The transcription of genetic information encoded in DNA is regulated by transcription factors and by post-translational modifications of histone proteins' tails, named histone code. Such "histone code" together with DNA methylation is part of the epigenetic code, which is cell and tissue specific. Many studies have focused on the correlation between transcriptional activation/repression and the histone code. While histone acetylation, regulated by histone acetyl transferases (HATs) and histone deacetylases (HDACs), is commonly associated with transcription activation, histone methylation, which is controlled by histone methyltransferases (KMTs) and histone demethylases (KDMs) is more versatile since lysines methylation can either be linked to transcription activation or repression, depending on the position of the lysine residue involved. Lysine-Specific Demethylase-1 (LSD1), is the first histone demethylase described and is a monoamine oxidase that can de-methylate lysine 4 or 9 of histone 3, and therefore promote transcriptional repression or activation, respectively. Current literature points to a critical role for LSD1 in the regulation of developmental and differentiation genes. The aim of this thesis is the investigation of a possible role of LSD1 in skeletal muscle differentiation. Skeletal muscle is a striated muscle tissue with complicated and heterogeneous features that serves multiple critical functions in the organism. Myogenesis is initiated by complex signaling and transcriptional cascades, which lead to the activation of the lineage-specific regulatory genes MyoD, Myf-5, MRF4, and Myogenin. While MyoD, Myf5, and Mrf4 function as myogenic determination factors, Myogenin, similar to Mrf4 and MyoD, acts as a differentiation factor, controlling the differentiation of myoblasts into skeletal muscle fibers. Since MyoD discovery, it has been widely demonstrated that it is a determination factor and downstream member of the myogenic program. However, the transcriptional control of this important gene remains poorly defined. Goal of my work was to demonstrate if LSD1 could be involved in the transcriptional regulation of MyoD gene. To achieve this aim I have used both *in vivo* and *in vitro* approaches. In particular, I have found that the inactivation of LSD1, by using a pax3-cre transgenic mouse strain, causes a strong reduction of MyoD activation in the somites, and thereby a strong reduction of the number of differentiated muscle cells. Consistent with these results, by using an shRNA in vitro strategy, I have observed during differentiation of C2C12, a mouse model of myoblast cell, a statistically significant decrease of MyoD gene expression and, thus protein in the shLSD1 C2C12 stable clones in comparison with the control one. Together *in vivo* and *in vitro* results suggest that LSD1 might be involved in the transcriptional regulation of MyoD gene. The known genetic regulatory elements controlling MyoD expression are the core enhancer (CE) and distal regulatory region (DRR). By using a Chromatin immunoprecipitation assay (ChIP) I have

shown that LSD1 binds to the CE region where it promotes H3K9 demethylation during myoblast differentiation. Recently, it has been demonstrated that the CE transcribes an RNA enhancer (CEeRNA) that modulates the transcription of MyoD gene during muscle differentiation. Here I have demonstrated that the absence of LSD1 during *in vivo* and *in vitro* muscle differentiation prevents the transcription activation of CEeRNA leading to a delay in the MyoD gene expression and thus in the muscle cell differentiation. Altogether these results indicate that LSD1 participates to the establishment of the muscle lineage and in the muscle differentiation, playing a role in the timing of MyoD activation by controlling the transcriptional activation of the CEeRNA.

Results

LSD1 and muscle differentiation

Lsd1 role during muscle denervation in vivo

Previous laboratory results have shown that in muscle the gene repression by electrical activity is mediated by a global inhibition of histone acetylation in muscle fibers, mediated by the histone deacetylase 9 (HDAC9) (Mejat et al., 2005). Muscle denervation eliminating electrical activity and the associated transcriptional repression, induces the activation of myogenin expression all along the muscle fibers, and provokes a strong histone hyper acetylation in all muscle nuclei. Immunofluorescence experiments with anti H3K9me2 antibodies performed with innervated muscle fibers revealed that this modification, associated to transcription repression, was specifically absent in subsynaptic nuclei whereas it was present in extra synaptic nuclei. The same experiments performed on denervated muscle fibers revealed that in the absence of innervation, H3K9me2 disappeared from extra synaptic nuclei, concomitantly to the activation of myogenin gene expression. To identify the demethylases responsible of this change, Prof. Schaeffer team has used a systematic RT PCR approach to compare the expression of histone demethylases in innervated and denervated muscle and they have observed that the expression of LSD1 was increased in denervated muscle (figure 17A). This result was confirmed at protein level by western blot analysis (figure 17B).

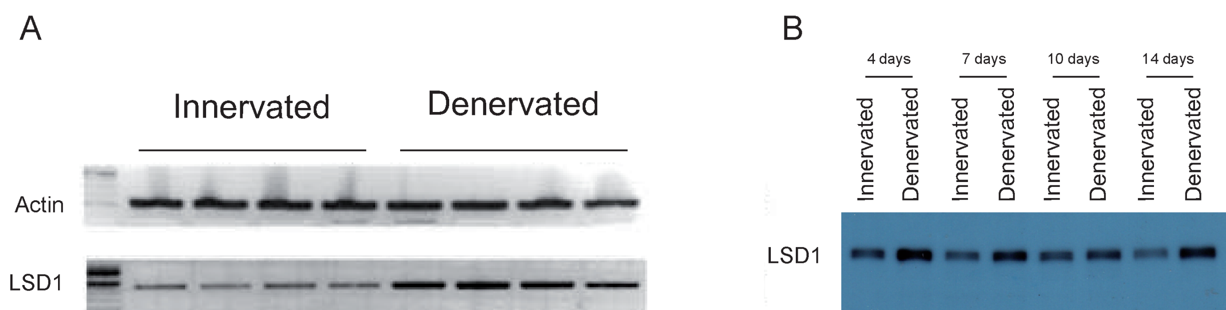


Figure 17: A) Lsd1 expression evaluated by RT PCR on 4 innervated and denervated (48h) tibialis anterior muscles. **B)** Kinetics of Lsd1 protein expression after denervation at tibialis anterior muscle.

Consistent with recent studies, which focus on the crucial role of epigenetic mechanisms in tissue differentiation we have decided to deeply study the role of Lsd1 in muscle differentiation.

Lsd1 inhibition in cultured myoblasts prevents MyoD activation and muscle differentiation

We wanted to test if Lsd1 could be directly involved in muscle differentiation. For this purpose, we generated a stable C2C12 cell line infected with a lentiviral vector containing the mouse Lsd1-targeting sequence pLKO.1-sh-LSD1 (TRCN0000071377, Open biosystem). As a control, the pLKO.1 vector SHC016V purchased from Sigma was used. These cell lines will be designated Sh LSD1 and Sh SCRA respectively. The Sh LSD1 cells are characterized by low LSD1 protein expression compared to Sh SCRA, as shown in figure 18

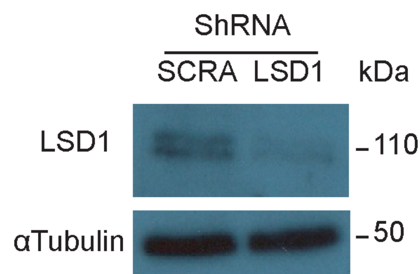


Figure 18: Specific shRNA-mediated knockdown of LSD1 in C2C12 cells.

We then asked whether Lsd1 down-regulation could be involved in myoblast differentiation affecting myotube formation. To test this hypothesis we transiently transfected Sh LSD1 and Sh SCRA cells with the pRNAt vector, which encodes GFP. When these cells were switched from growth medium (GM) to low serum differentiation medium (DM) after 5 days Sh LSD1 cells showed reduced ability to fuse and form myotube (figure 19A). Indeed only 3% of Sh LSD1 cells underwent fusion with the majority of myotubes (figure 19B) with 2-5 nuclei (figure 19C) whereas 63% of Sh SCRA cells formed myotubes containing more than 10 nuclei (figure 19B and C). These results are consistent with the increased Lsd1 expression *in vivo* denervation, suggesting that inhibition of Lsd1 affect myoblast differentiation.

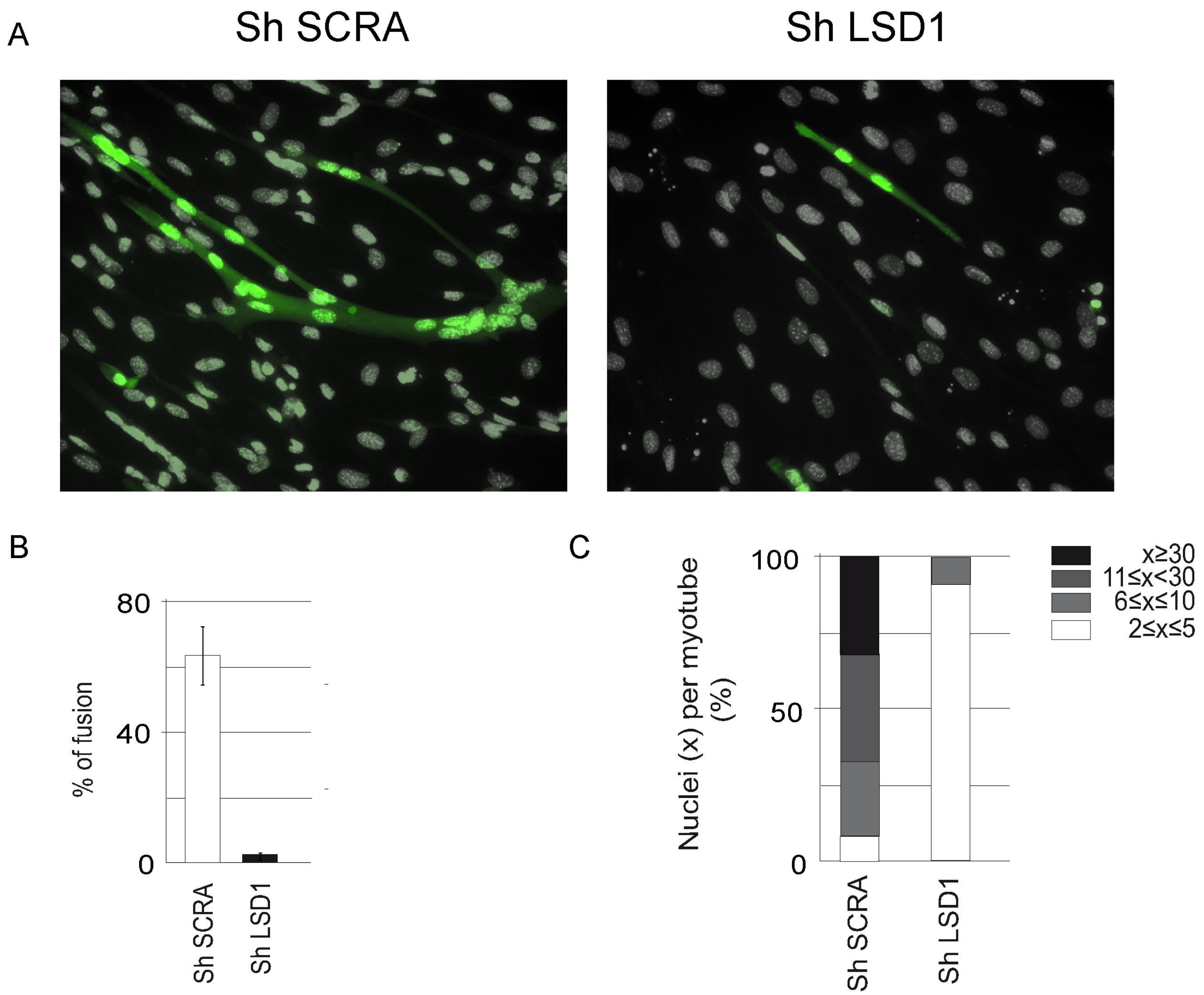


Figure 19: Sh SCRA and Sh LSD1 cells were transfected with pRNAt vector grown in GM for 24 hours and induced to differentiate in DM for 5 days. Cells were fixed, stained with DAPI and analyzed by epifluorescence microscopy. Transfected cells expressed GFP. Nuclei were counted in 100 pRNAt-transfected Sh LSD1 cells and 110 pRNAt-transfected Sh SCRA. The % of fusion is the proportion of cells containing two or more nuclei. All histogram data are means ± s.d. of triplicate results.

As previously described during muscle differentiation there is a hierarchical relationship between MRFs whereby MyoD is implicated in specifing myoblasts, while myogenin and MRF4 act genetically downstream to induce myoblast differentiation.

Considering the effect of Lsd1 on cell fusion and myotube formation we wondered whether Lsd1 could affect the expression of one of these muster regulator genes, such as MyoD. Thus Sh LSD1 and Sh SCRA cells were induced to differentiate and mRNA and protein expression level of MyoD and protein expression of myogenin were monitored during 4 days after DM addition (figure 20).

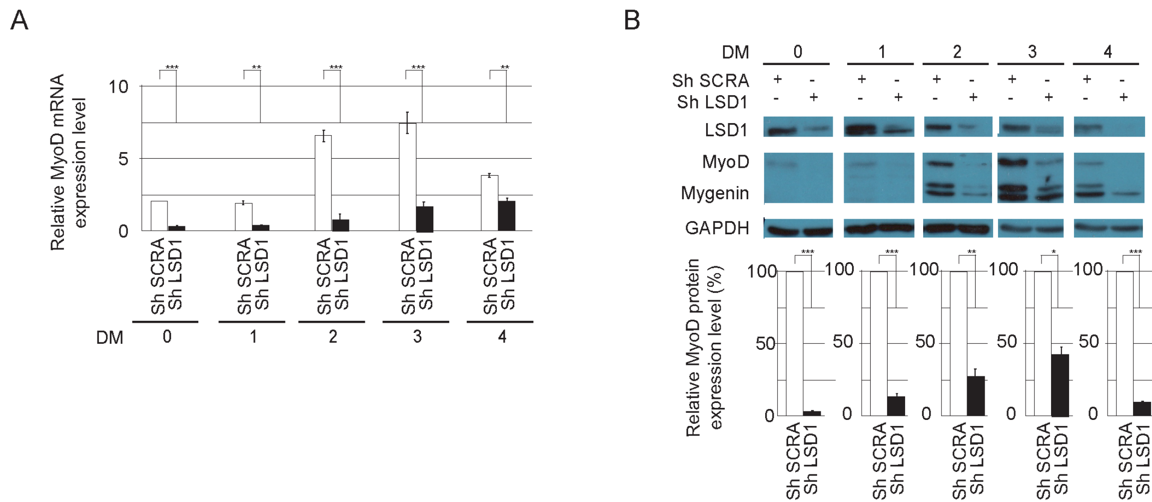


Figure 20: A) mRNA expression levels of MyoD gene under Sh SCRA and sh LSD1 during differentiation. RT–PCR values were normalized to the expression levels of the *cycloB* gene, and are shown as the fold difference against Sh SCRA DM 0. All histogram data are means ± s.d. of triplicate results. ***p* < 0.01, ***p <0,001 (Bonferroni test after ANOVA). **B)** Lsd1, MyoD and Myogenin protein expression was determined by Western blot during differentiation in Sh SCRA and Sh LSD1 cells. MyoD protein expression was normalized to the level of GAPDH protein expression and is shown as fold difference in percentage against each Sh SCRA relative DM time. All histogram data are means ± s.d. of triplicate results. **p* < 0.05, ***p* < 0.01, ***p <0,001 (t-test).

As shown in figure 20 during myoblast differentiation MyoD mRNA and protein level in Sh LSD1 cells is significantly lower compared to Sh SCRA cells. As expected myogenin protein level in SH LSD1 cells does not reach the same level of Sh SCRA while Lsd1 protein level remains low during all the experiment (figure 20B).
These results clearly show that Lsd1 depletion alters MyoD expression and muscle differentiation process.

Lsd1 is recruited on the MyoD enhancer during myoblast differentiation

Given the inhibition of MyoD expression we observed *in vitro* with the inactivation of Lsd1 during muscle differentiation, we investigated a possible direct involvement of Lsd1 in the activation of the MyoD. ChIP experiments were performed with an anti LSD1 antibody to detect the presence of Lsd1 on the core enhancer (CE) and the promoter (TSS) regions, which are directly involved in the control of MyoD expression (Mousavi et al., 2013). While in Sh SCRA myoblasts, Lsd1 was not detected in these regions, 3 days after the DM addition, Lsd1 was strongly enriched on both CE and TSS regions (Figure 21A) compared to negative region 1. The presence of Lsd1 is correlated with a reduction of the H3K9me2

repressive mark (figure 21B) and a high enrichment of H3K4me3 activation mark (figure 21C) in both regions. However, ChIP experiments on Sh LSD1 cells after 3 days in DM showed that the depletion of Lsd1 did not led to H3K4me3 enrichment only in the CE region, suggesting a role of Lsd1 in the direct activation of this region (figure 21C).

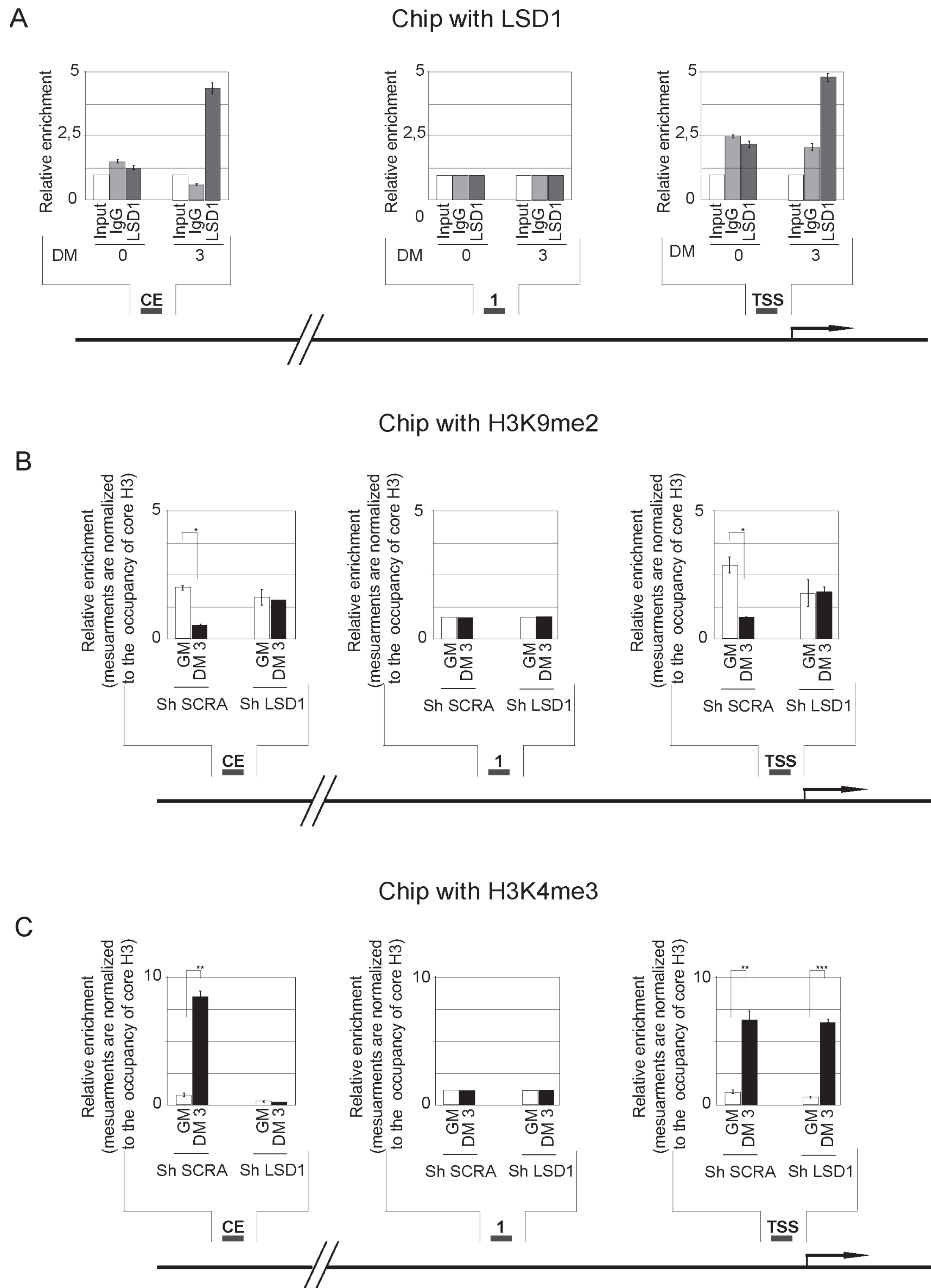


Figure 21: A) Localization of Lsd1 at the MyoD gene locus. ChIP analyses were performed in C2C12 cells with an anti-LSD1 antibody. Three sites (CE; 1; TSS) were tested for qPCR amplification. Enrichment values

were normalized to input, and shown as the fold difference relative to region 1. Input (white bars) control IgG (light grey bars), anti-LSD1 antibody (dark grey bars). **B)** ChIP analyses on Sh SCRA and Sh LSD1 cells in GM and after 3 days in DM of the MyoD gene locus, using antibody against di-methylated H3K9. Enrichment values were normalized to input and to the occupancy of the core H3, data are shown as fold difference relative to region 1. **C)** ChIP analyses on Sh SCRA and Sh LSD1 cells in GM and after 3 days in DM of the MyoD gene locus by using antibody against tri-methylated H3K4. Enrichment values were normalized to input and to the occupancy of the core H3, data are shown as fold difference relative to region 1. All histogram data are means ± s.d. of triplicate results. *$p < 0.05$, **$p < 0.01$. (t-test)

Lsd1 regulates CEeRNA expression

The CE region has recently been shown to transcribe for a RNA enhancer (CEeRNA), and the CEeRNA transcription was shown to be required for the activation of MyoD expression (Mousavi et al., 2013). Thus we checked the level of CEeRNA in Sh LSD1 myotubes and as shown in figure 22, after 3 days in DM the level of the CEeRNA is not increased compared to the level reached in the Sh SCRA myotubes and such difference is statistically different.

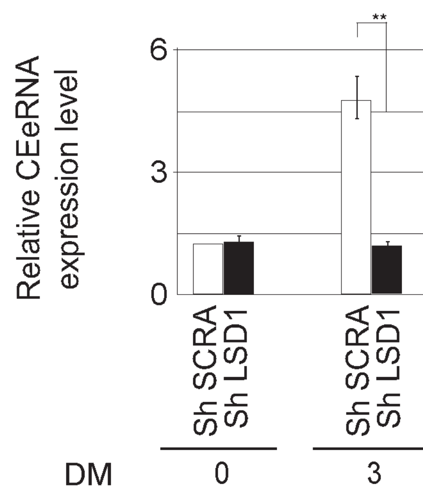


Figure 22: CEeRNA expression in Sh SCRA and Sh LSD1 cells after 3 days of differentiation. RT–PCR values were normalized to the expression levels of the *cycloB* gene, and are shown as the fold difference against Sh SCRA DM 0. All histogram data are means ± s.d. of triplicate results. **$p < 0,01$. (t-test).

Taken together these results strongly support the idea that Lsd1 controls MyoD expression through the activation of the CE region.

To deeply support this hypothesis we cloned the full length CEeRNA in the pRNAt vector that encodes GFP, under the control of the strong H1 promoter (figure 23) in order to rescue the expression of MyoD.

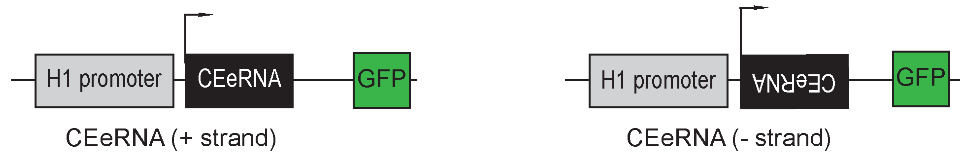


Figure 23: Constructs of CEeRNA used for rescue experiment.

Sh SCRA myoblast were transfected with pRNAt empty vector, Sh LSD1 myoblasts were transfected with pRNAt empty vector and pRNAt – CEeRNA (+ strand or – strand). MyoD mRNA level was monitored after 3 days in DM. The MyoD mRNA and protein level in Sh LSD1 cells transfected with CEeRNA vector (+strand) does not change compared to Sh LSD1 cells transfected with the empty vector. However, the MyoD mRNA and protein level is restored in Sh LSD1 cells transfected with CEeRNA vector (-strand) (figure 24).

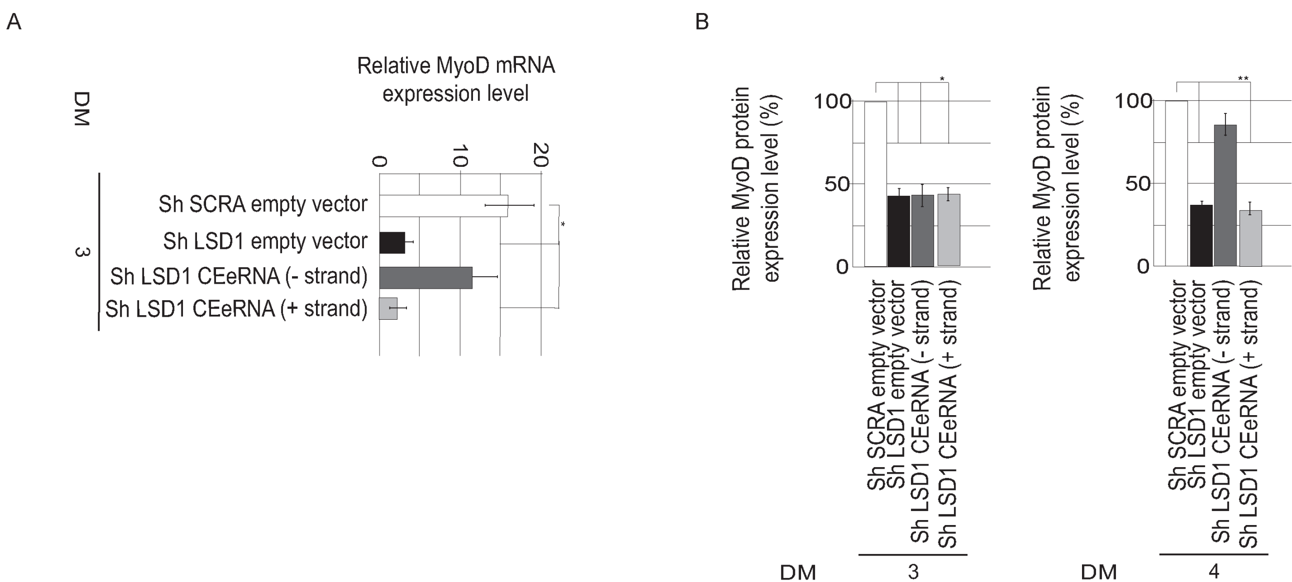


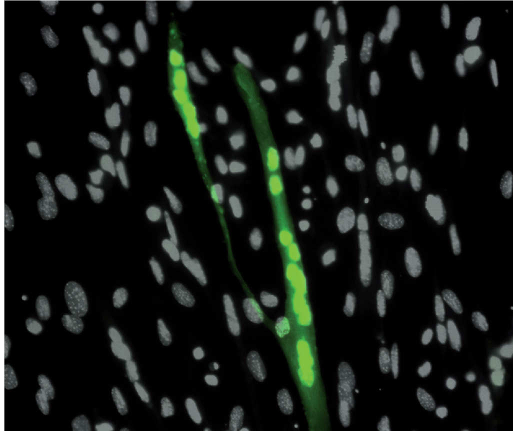
Figure 24: A) mRNA expression levels of MyoD gene in Sh SCRA empty vector, Sh LSD1 empty vector and Sh LSD1 CEeRNA (+ strand) and Sh LSD1 CEeRNA (- strand) cells after 3 days of differentiation. RT–PCR values were normalized to the expression levels of the *cycloB* gene, and are shown as the fold difference against Sh SCRA DM 0. **B)** MyoD protein expression was determined by Western blot after 3 and 4 days of differentiation in Sh SCRA empty vector, Sh LSD1 empty vector and Sh LSD1 CEeRNA (+ strand) and Sh LSD1 CEeRNA (- strand) cells. MyoD protein expression was normalized to the level of GAPDH protein expression and is shown as fold difference in percentage against each Sh SCRA DM. All histogram data are means ± s.d. of triplicate results. *$p < 0.05$, **$p < 0.01$. (Bonferroni test after ANOVA).

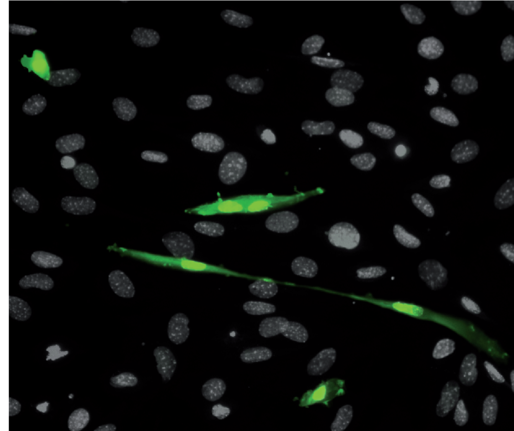
Consistent with the rescue of MyoD expression at mRNA and protein level we asked if the ability of cell fusion and myotube formation is restored in Sh LSD1 transfected cells with CEeRNA vector (-strand). As shown in figure 25B, 30% of Sh LSD1 transfected with CEeRNA vector (-strand) cells underwent fusion with 6-10 nuclei per myotube (figure 25C) whereas only 3% of Sh LSD1 transfected cells with empty vector formed myotubes (figure 25B). However Sh LSD1 transfected with CEeRNA vector (-strand) myotubes are less ramified and thinner compared to the one observed in Sh SCRA cells (figure 25A), suggesting that the LSD1 involvement in muscle differentiation is not only at MyoD level.

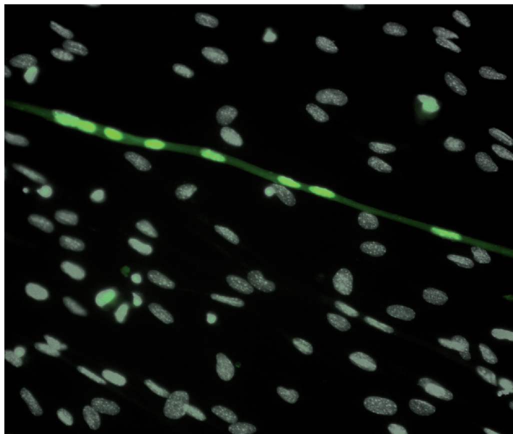
A

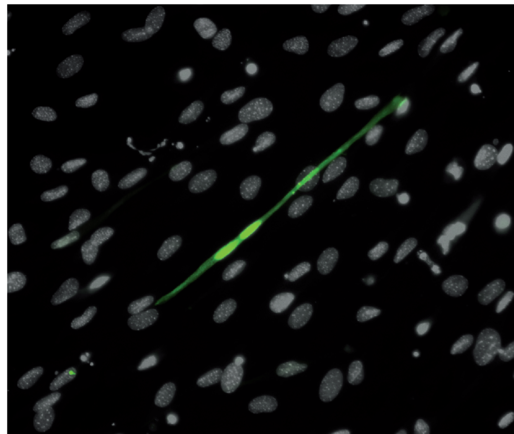
Sh SCRA pRNAt

Sh LSD1 pRNAt





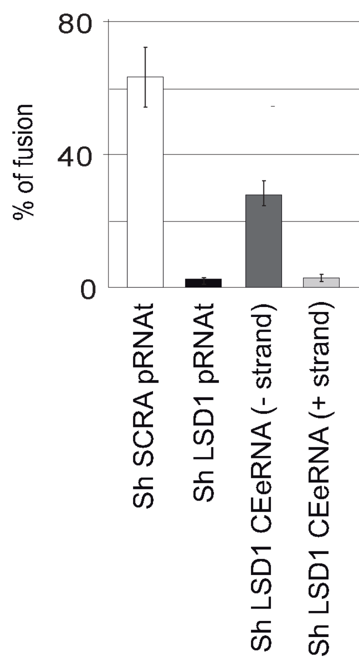




Sh LSD1 CEeRNA (-strand)

Sh LSD1 CEeRNA (+strand)

B



C

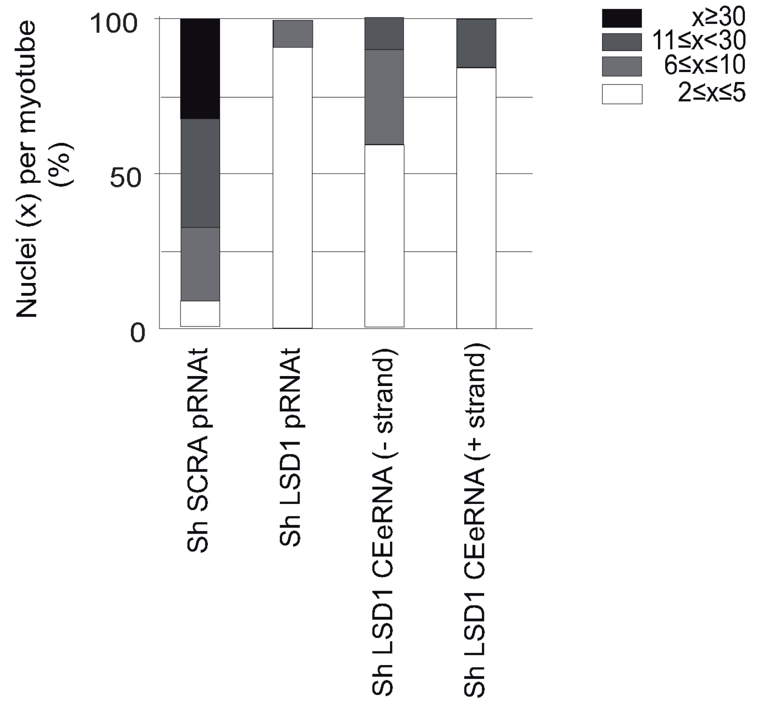


Figure 25: Sh SCRA cells were transfected with pRNAt empty vector and Sh LSD1 were transfected with pRNAt empty vector or CEeRNA (+ strand) vector or CEeRNA (- strand) vector grown in GM for 24 hours and induced to differentiate in DM for 5 days. Cells were fixed, stained with DAPI and analyzed by epifluorescence microscopy. Transfected cells expressed GFP. Nuclei were counted in 180 pRNAt empty vector-transfected Sh LSD1 cells, 110 pRNAt empty vector-transfected Sh SCRA, 132 pRNAt CEeRNA (-strand) Sh LSD1 and 102 pRNAt CEeRNA (+strand) Sh LSD1. The % of fusion is the proportion of cells containing two or more nuclei. All histogram data are means ± s.d. of triplicate results.

Lsd1 and muscle differentiation *in vivo*

Generation of a pax3 conditional knock-out mouse for LSD1

Lsd1 mRNA is early and widely expressed during development. However, the precise distribution of Lsd1 protein during muscle development needed to be determined. In collaboration with Dr Fredéric Relaix, Lsd1 distribution in somites has been determined by immunofluorescence in Embryonic Day (E) 11.5 in $Pax3^{EGFP/+}$ mouse embryos in which Pax3 positive cells express EGFP (Relaix et al., 2005). Differentiating myoblasts were identified with an anti-MyoD antibody (figure 26). Staining revealed that Lsd1 is expressed both in the dermomyotome and myotome and, consistent with *in vitro* results, Lsd1 levels are significantly higher in MyoD positive cells (figure 26).

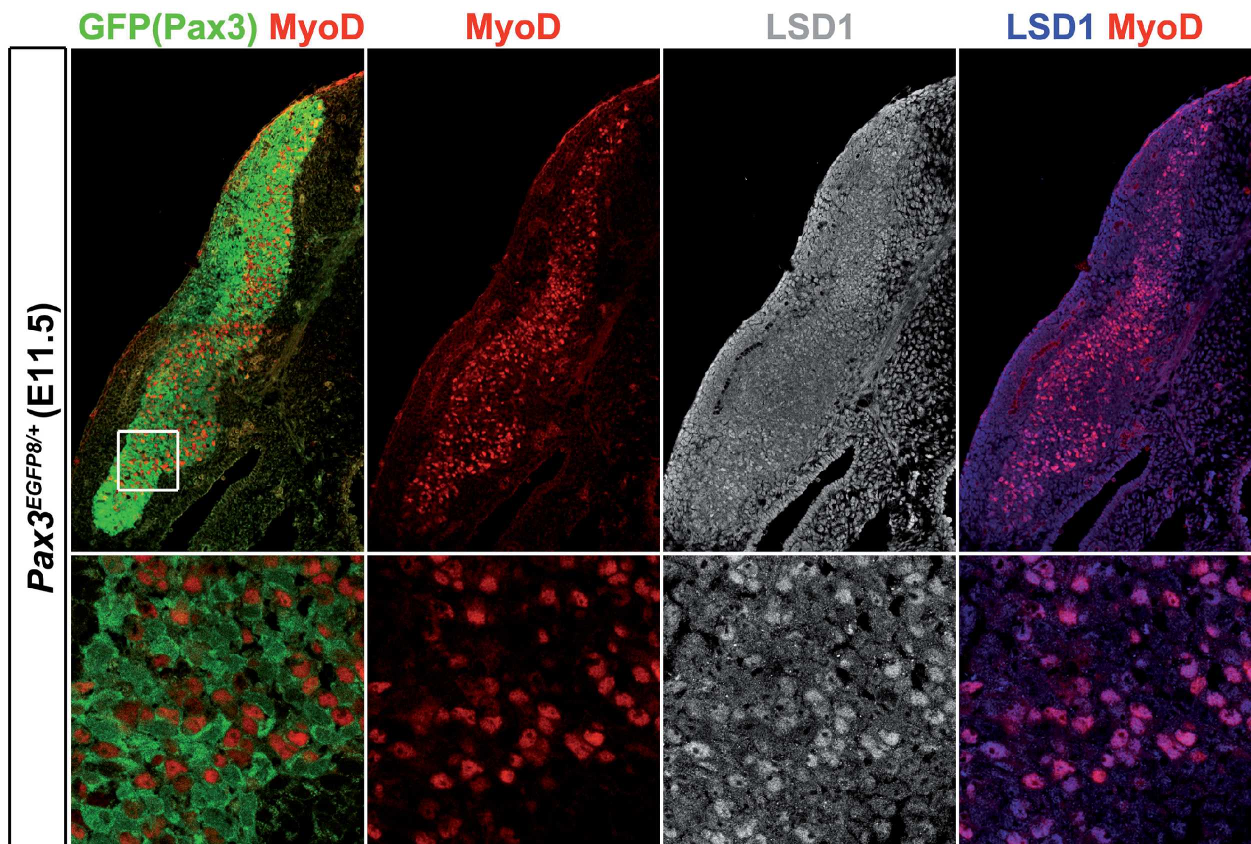


Figure 26: Expression of Pax3, MyoD and Lsd1 in somites.

To further delineate the function of Lsd1 in muscle lineage determination and differentiation, Lsd1 was conditionally inactivated by crossing LSD1 $^{Flox/Flox}$ mice (Zhu et al., 2014) and Pax3 $^{cre/+}$: Lsd1 $^{Flox/+}$ transgenic mice (Li et al., 2000).

Although an expected ratio among genotypes is 1:1:1:1 for Pax3 $^{cre/+}$: Lsd1 $^{Flox/Flox}$; Pax3 $^{+/+}$: Lsd1 $^{Flox/Flox}$; Pax3 $^{cre/+}$: Lsd1 $^{Flox/+}$; Pax3 $^{+/+}$: Lsd1 $^{Flox/+}$, after PCR analysis of 42 offspring, not a single Pax3 $^{cre/+}$: Lsd1 $^{Flox/Flox}$ pup was found. Subsequently, we set up timed-mating experiments and genotyped 92 embryos at embryonic day 9.5 until 18. After finding Pax3 $^{cre/+}$: Lsd1 $^{Flox/Flox}$ alive embryos at E15 and no embryos with the same genotype at E18, we concluded that Lsd1 deficiency was lethal at around E15.5. Conditional deletion of the Lsd1 gene was confirmed by PCR analysis.

In collaboration with Fredéric Relaix, Lsd1 expression in Pax3$^{Cre/+}$:LSD1$^{flox/flox}$ embryos was immunohistochemically examined. Immunofluorescence on transverse sections of E 11.5 embryos has shown that Lsd1 expression was efficiently abrogated in the neural tube and in the somites. Moreover Pax3 staining has revealed a reduction of the size of the somites (Figure 27).

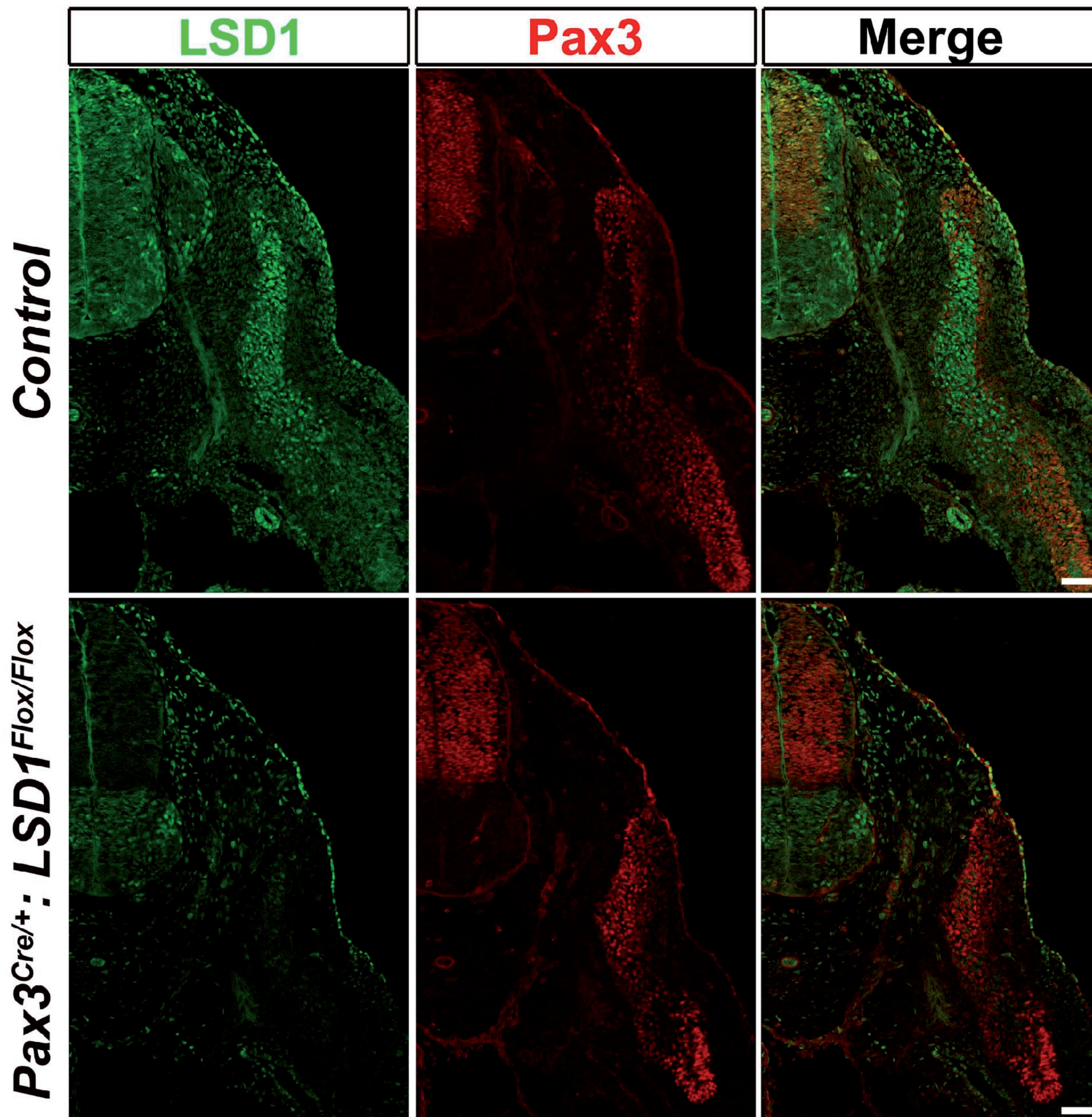


Figure 27: Expression of Pax3 and Lsd1 in somites and in the neural tube. Scale Bar indicates 50µm.

We then asked if ablation of Lsd1 in the neural tube could have any effect. Thus, we performed a whole-mount in situ hybridization analysis for Sox10 gene, which has a key role in the mechanisms triggering neural crest stem cells (NCSC) differentiation. As shown in figure 28, Sox10 expression in Pax3$^{Cre/+}$:LSD1$^{flox/flox}$ embryos at E10.5 is normal.

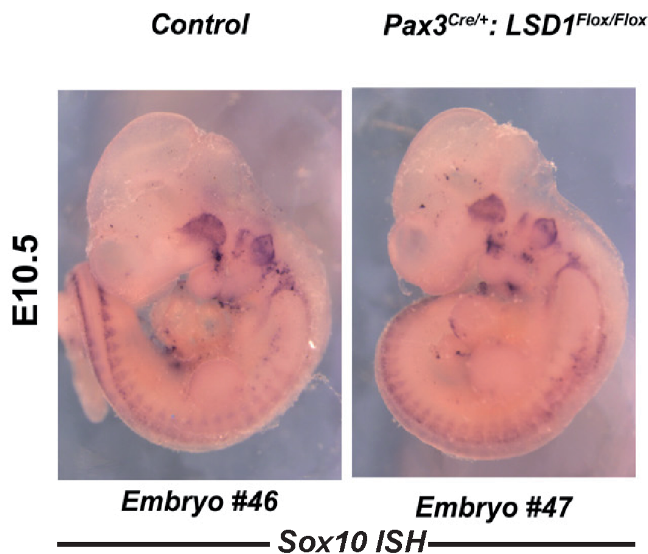


Figure 28: Control (left) and Pax3$^{Cre/+}$:LSD1$^{flox/flox}$ (right) embryos at E10.5 were hybridized with Sox10-specific probe.

Lsd1 regulates MyoD expression in vivo

Murine skeletal muscle development is characterized by two phases of myogenesis (Hutcheson et al., 2009): the first one occurs at approximately E11, the second one occurs between E14.5 and E17.5. MyoD is starting to be expressed at approximately E10.5.

In order to investigate the involvement of Lsd1 in MyoD expression, control and Pax3$^{Cre/+}$:LSD1$^{flox/flox}$ mouse embryos were collected from E11.5 until birth. In situ hybridization experiments, using a specific probe on MyoD gene, revealed that Lsd1 inactivation resulted in strongly reduced levels of MyoD mRNA in both somites and limb buds (Figure 28).

Consisted with the hierarchical relationship between MRFs whereby MyoD expression is necessary for the activation of myogenin gene and thus muscle differentiation, myogenin mRNA expression is strongly reduced in Pax3$^{Cre/+}$:LSD1$^{flox/flox}$ mouse embryos compare to the control one (figure 28).

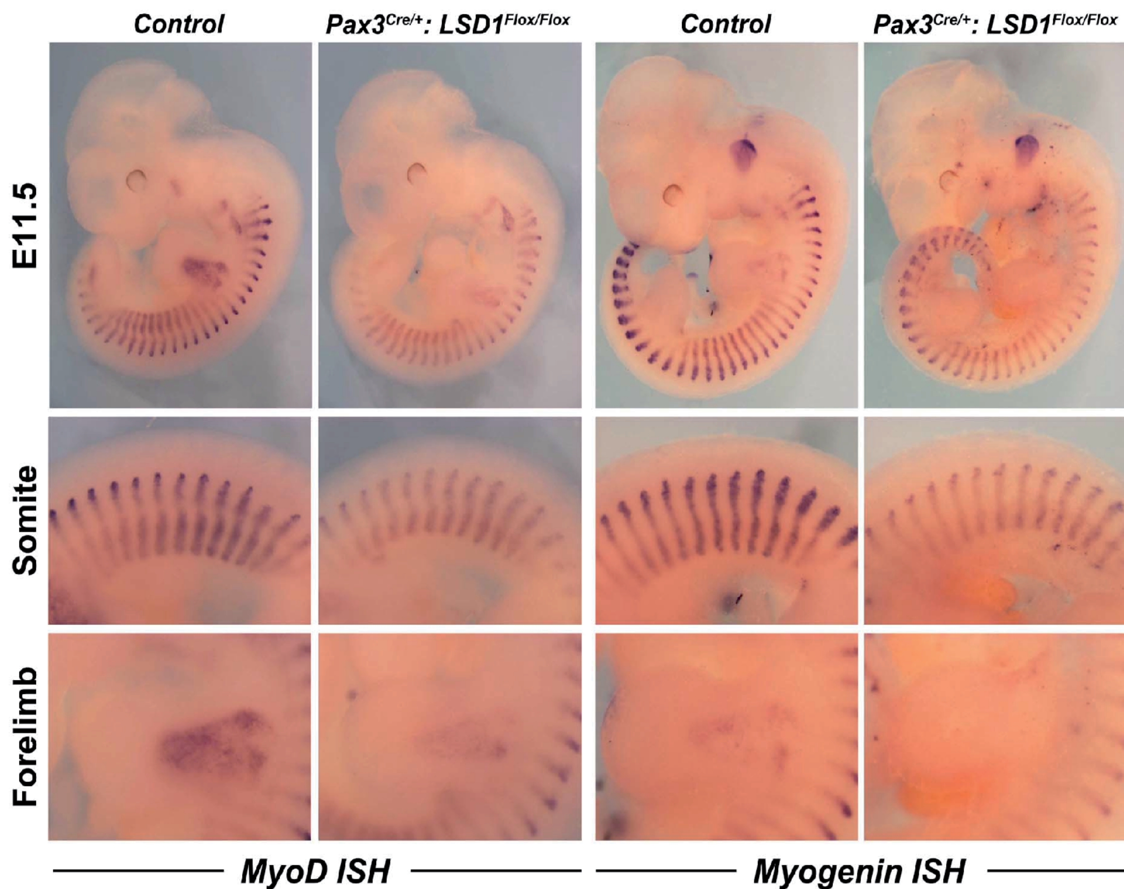


Figure 28: Control and Pax3$^{Cre/+}$:LSD1$^{flox/flox}$ embryos at E11.5 were hybridized with MyoD and a myogenin-specific probes.

To confirm MyoD and Myogenin down-regulation, western blot is performed on Pax3$^{Cre/+}$:LSD1$^{flox/flox}$ and control embryos at E11.5. As expected, expression level of those genes were strongly reduced in the absence of Lsd1 (figure 29).

The strong decrease of MyoD in the forelimb has prompted us to investigate if the migration of muscle precursors is also affected (figure 28). Whole-mount in situ hybridization analysis performed on Pax3$^{Cre/+}$:LSD1$^{flox/flox}$ and control embryos at E10.5 revealed a down regulation of Lbx1 gene both in the forelimb and in the hindlimb (figure 30).

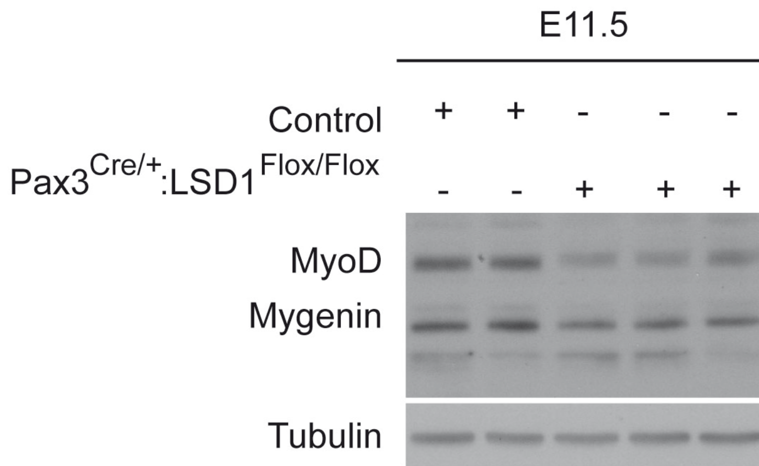


Figure 29: MyoD and Myogenin protein expression was determined by Western blot at E11.5 in 4 Pax3$^{Cre/+}$:LSD1$^{flox/flox}$ and 2 control embryos.

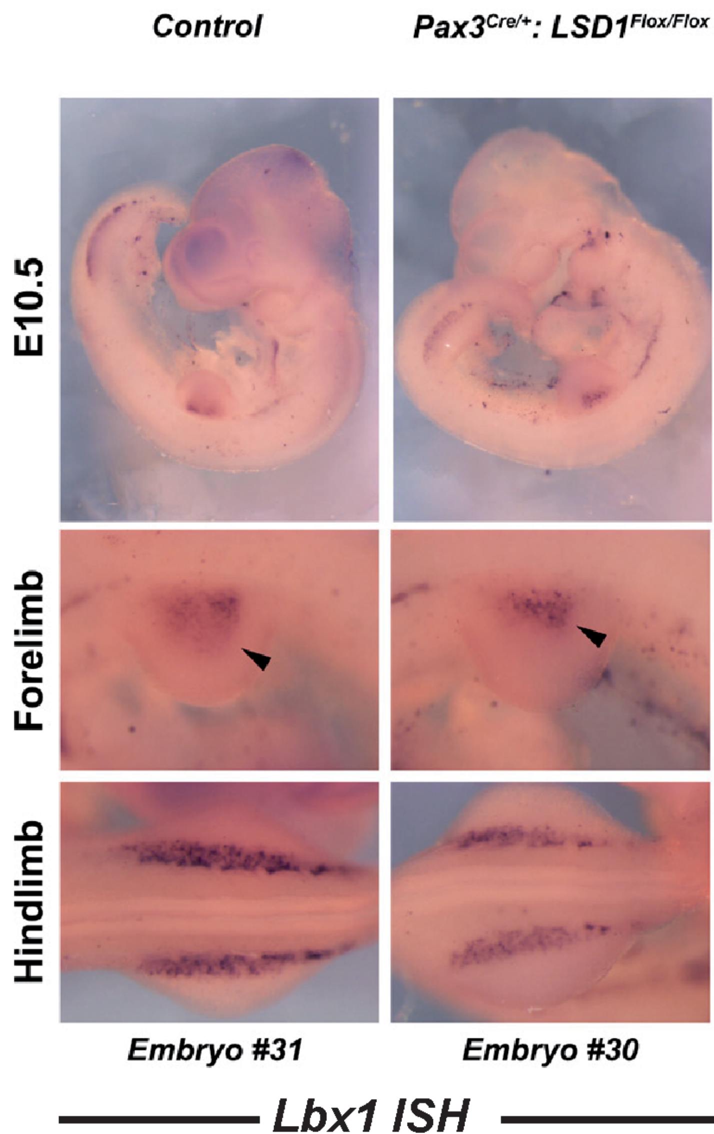


Figure 30: Control (left) and Pax3$^{Cre/+}$:LSD1$^{flox/flox}$ (right) embryos at E10.5 were hybridized with Lbx1-specific probe.

These results suggest that Lsd1 could have not only a role in muscle cell determination but also in the migration of muscle precursor cells.

Lsd1 inactivation in muscle precursor cells reduces myoblast formation

To better understand the role of Lsd1 in cell muscle commitment, immunofluorescence experiments were performed on control and $Pax3^{Cre/+}:LSD1^{flox/flox}$ mice embryos to quantify the proportion of MyoD, myogenin and Pax3 or Pax7 positive cells, in the limbs and the somites, respectively (figure 31 and 32).
At the forelimb level, Lsd1 inactivation resulted in a strong increase of the proportion of pax3+/MyoD- cells together with a decrease in the proportion of Pax3+/MyoD+ and Pax3-/MyoD+ cells (figure 31A and 31B). Moreover the density of migrating cells is decreased in $Pax3^{Cre/+}:LSD1^{flox/flox}$ mouse embryos compared to controls (figure 31A).
In the somites, Pax7 staining was used instead of Pax3 to avoid staining of neural cells. As expected, Lsd1 inactivation increased the proportion of Pax7+/MyoD- cells (figure 32A and 32B). However, in both somites and forelimbs, the proportion of MyoD+/Myogenin-, MyoD+/Myogenin+, MyoD-/Myogenin+ was unaffected by Lsd1 inactivation, suggesting that Lsd1 affect the initiation of the differentiation and not the differentiation per se (figure 31B and 32B).
Altogether these results support the hypothesis that Lsd1 inactivation specifically affects muscle formation by preventing the activation of the MyoD gene, consisted with *in vitro* results.

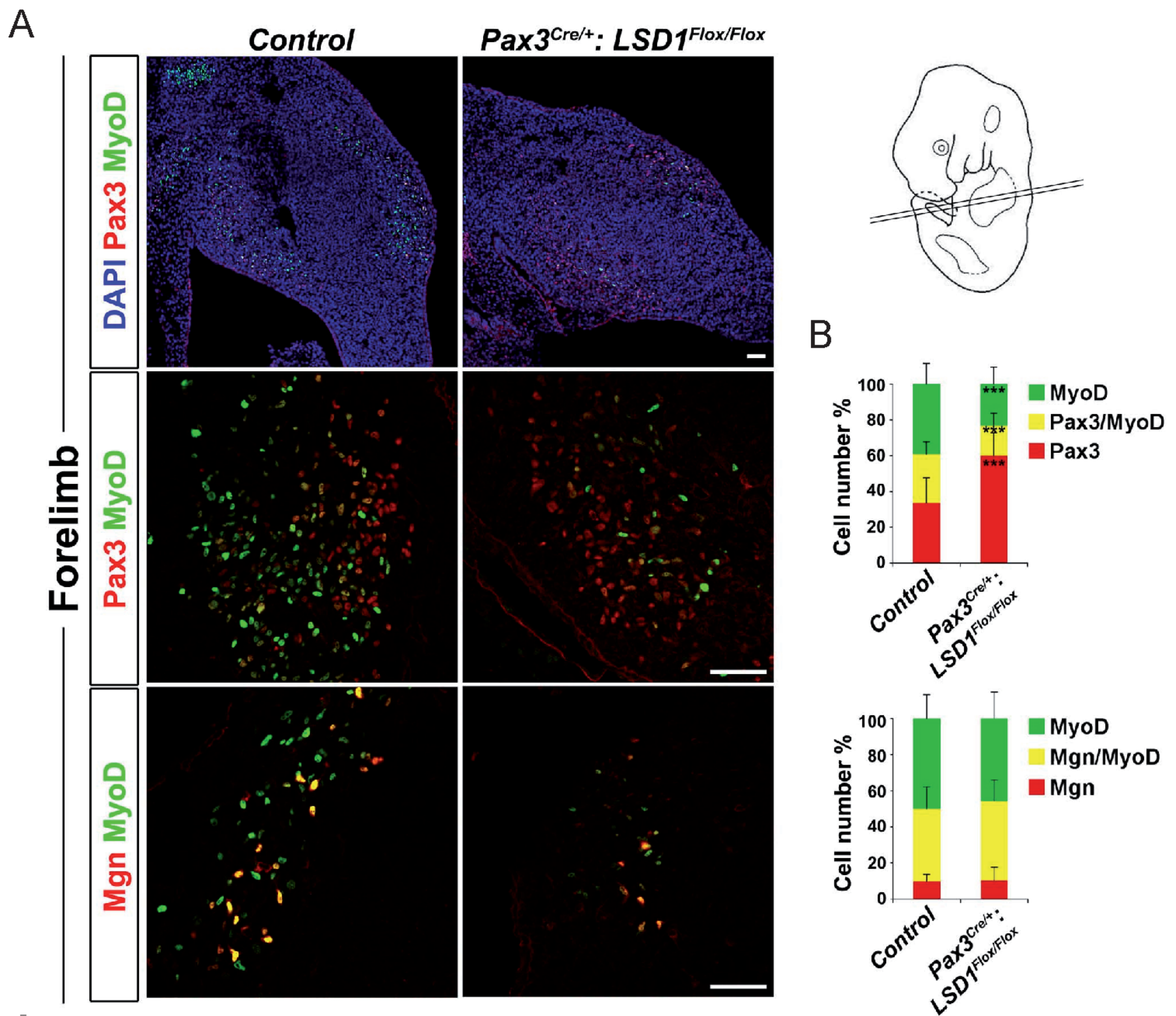


Figure 31: A) Expression of Pax3, MyoD and myogenin in the forelimbs. Scale Bar indicates 50µm. **B)** myogenic precursor cells counting, upper panel, differentiating cells counting, bottom panel. ***p<0,001 (t-test).

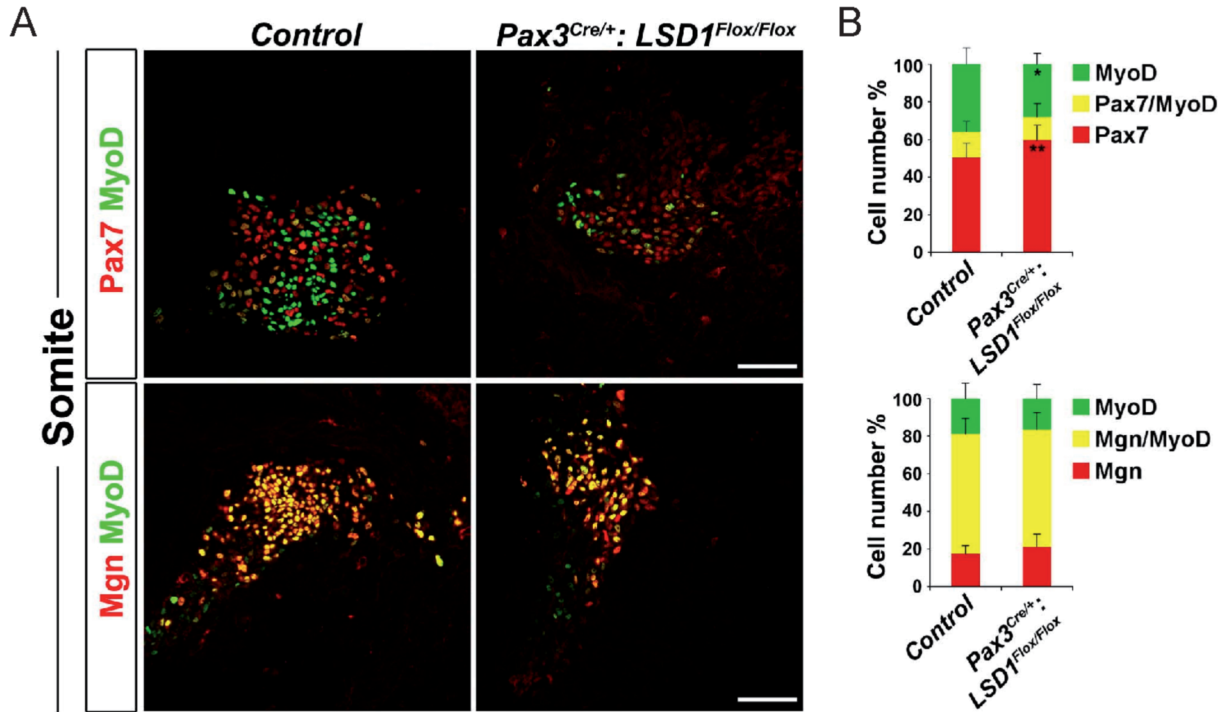


Figure 32: A) Expression of Pax7, MyoD and myogenin in the somites. Scale Bar indicates 50µm **B)** myogenic precursor cells counting, upper panel, differentiating cells counting, bottom panel. *p<0,05, **p<0,01 (t-test).

Discussion

During skeletal myogenesis and muscle regeneration the nucleus of multipotent muscle progenitor cell is sequentially reprogrammed to adopt and maintain the new patter of gene expression. This process is mediated by tissue specific transcription factors on target promoters in response to the activation of signaling cascades. This global genome reprogramming allows the acquisition of the myogenic identity and the proliferation of muscle progenitors and their subsequent differentiation into multinucleated myofibers. For instance, the muscle lineage commitment is determined by a hierarchical activation of genes (Pax3, Pax7, MyoD and Myf5) that establish the myogenic identity. In particular expression of MyoD gene reflects the muscle cells commitment to differentiate, indeed when ectopically introduced in somatic cells, MyoD is able to convert the host cells into skeletal muscle. This potential confers to MyoD a pivotal role in the initiation of skeletal muscle differentiation. Moreover as a master switch transcription factor in skeletal muscle, MyoD has been implicated in the transcriptional regulation of a myriad of genes, most of which are vital in ensuring that the differentiation program is efficiently completed. Epigenetics represents the wide array of changes that regulate gene expression but are not based on the alterations in the primary base sequence of DNA. Moreover, epigenetics has a mayor role in the normal changes in gene expression required in embryogenesis and differentiation. Research regarding the mechanisms that effect these changes has focused predominantly on DNA methylation and modification of histone proteins. However in the last twenty years another mechanism of epigenetic control mediated by non-coding RNA has become of great interest. Thus defining epigenetic regulatory mechanisms of MyoD is of ultimate importance in understanding muscle transcriptional pathway.

Previous results have shown that muscle denervation induces the activation of MRF genes all along the muscle fiber and this phenomenon is associated with a decrease of H3K9me2, transcription repressor mark, on the promoters of these genes. Screening of histone demethylase enzymes expression after denervation points out on the importance of Lsd1 in the activation of these genes.

In this thesis we provide the first *in vivo* and *in vitro* evidence that Lsd1, plays a role in skeletal myogenesis, acting on the regulation of primary MRF gene, MyoD, and is one of the genes responsible of muscle cell determination. Previous *in vitro* studies have already indicated that the histone demethylase, Lsd1, is involved in the control of muscle differentiation. However it has been described that Lsd1 is implicated in the activation of myogenin promoter, allowing terminal differentiation to take place. These results have suggested that Lsd1 mediates the activation of a secondary MRF (myogenin).

Nevertheless, our analysis of skeletal myogenesis in $Pax3^{Cre/+}$:$LSD1^{flox/flox}$ mice embryos provides the first evidence that Lsd1 has a role in the specification of the myogenic cell fate. Indeed using a whole-mount in situ hybridization we show that the expression of MyoD is decreased in $Pax3^{Cre/+}$:$LSD1^{flox/flox}$ mice embryos both in the somite and in the forelimb. Consisted with this evidence, the number of precursor cells (Pax3+/MyoD-) in the somite and forelimb is higher in the $Pax3^{Cre/+}$:$LSD1^{flox/flox}$ mice embryos compared to the control embryos at the same embryonic stage. Thus, these results suggest that Lsd1, acting on MyoD gene expression drives the initiation of muscle differentiation.

The regulation of MyoD gene expression is strictly and timely controlled by several transcription factors, which act independently on three different MyoD regulatory regions (Core Enhancer, Distal regulatory region, and promoter region). In order to deeply dissect the role of Lsd1 in the regulation of MyoD gene our *in vitro* studies on C2C12 myoblast, stably infected with a Sh RNA against Lsd1 and a Sh RNA scrambled, have demonstrated that Lsd1 is mostly involved in the activation of the Core Enhancer. The Core Enhancer region has been shown to be required for the early activation of MyoD gene, in particular the RNA enhancer (CEeRNA) transcribed from this region increases RNAPolII occupancy at the MyoD promoter as well as transcription of the MyoD gene by regulating the chromatin accessibility (figure 33). Therefore, defect in the activation of this region led to a delay in MyoD transcriptional activation. Indeed, it is important to point out that myogenesis is not completely inhibited in the absence of Lsd1 but it is slower than in the control, since MRFs were expressed, although at very low levels, during skeletal muscle differentiation of $Pax3^{Cre/+}$:$LSD1^{flox/flox}$ embryos. Consequently, the number of differentiated cells (MyoD+/myogenin+) does not change in $Pax3^{Cre/+}$:$LSD1^{flox/flox}$ and control embryos, therefore supporting the idea that Lsd1 is more involved in the timing muscle lineage determination than in the differentiation per se.

Importantly we also show that, when Lsd1 is ablated the CEeRNA failed to be transcribed leading to an aberrant and slow activation of MyoD gene (figure 34). Consisted with this, overexpressing the CEeRNA in the Sh LSD1 myoblast after 3 days of differentiation the MyoD mRNA level is restored as well as the protein level. In this scenario, CEeRNA plays a modulatory role in gene induction. CEeRNA is not required for basic transcription, but its role is instead to enhance the transcriptional response by allowing RNA polII to be recruited on MyoD promoter more efficiently, resulting in a larger and precisely timed response. Surprisingly since the discovery of this CEeRNA we provide for the first time that only the – strand of the CEeRNA is active on MyoD promoter.

Our *in vitro* results also show that Lsd1 is involved in the ability of myoblast to form

multinucleated myotubes. Indeed myoblasts with low level of Lsd1 led to the formation of myotubes with very low number of nuclei, and this is improved after restoration of MyoD level, suggesting that Lsd1 can be also involved in the myoblast migration. Consisted with this hypothesis, whole-mount in situ hybridization on $Pax3^{Cre/+}$:$LSD1^{flox/flox}$ mice embryos has shown a decrease of Lbx1 gene expression in the forelimb and in the hindlimb.

In this thesis, by using both molecular and genetic approaches, we have demonstrated that LSD1 promotes myogenic lineage determination, thereby inducing MyoD expression during skeletal muscle differentiation. Thus, LSD1 down-regulation is able to delay but not completely abolish progression along the myogenic lineage. Such epigenetic regulation would be of great interest in the contest of aging or disease where a progressive loss of muscle regenerative capacity has been described. This pathological condition is in part due to a decline in the number and function of satellite cells, the direct cellular contributors to muscle repair. Indeed the choice between self-renewal and differentiation is critical to simultaneously ensure satellite cell pool maintenance and also generating differentiated progeny. Our results suggest that LSD1 might act as a molecular switch between these two states. We could speculate that chronic degenerative stimuli, characteristic of each dystrophy, favor pro-differentiation pathways and ultimately lead to a progressive, functional exhaustion of the satellite cell pool. Thus inhibiting LSD1 activity during dystrophic or aging conditions would slow down but not affect cellular differentiation thus preventing the premature depletion of satellite cell pool.

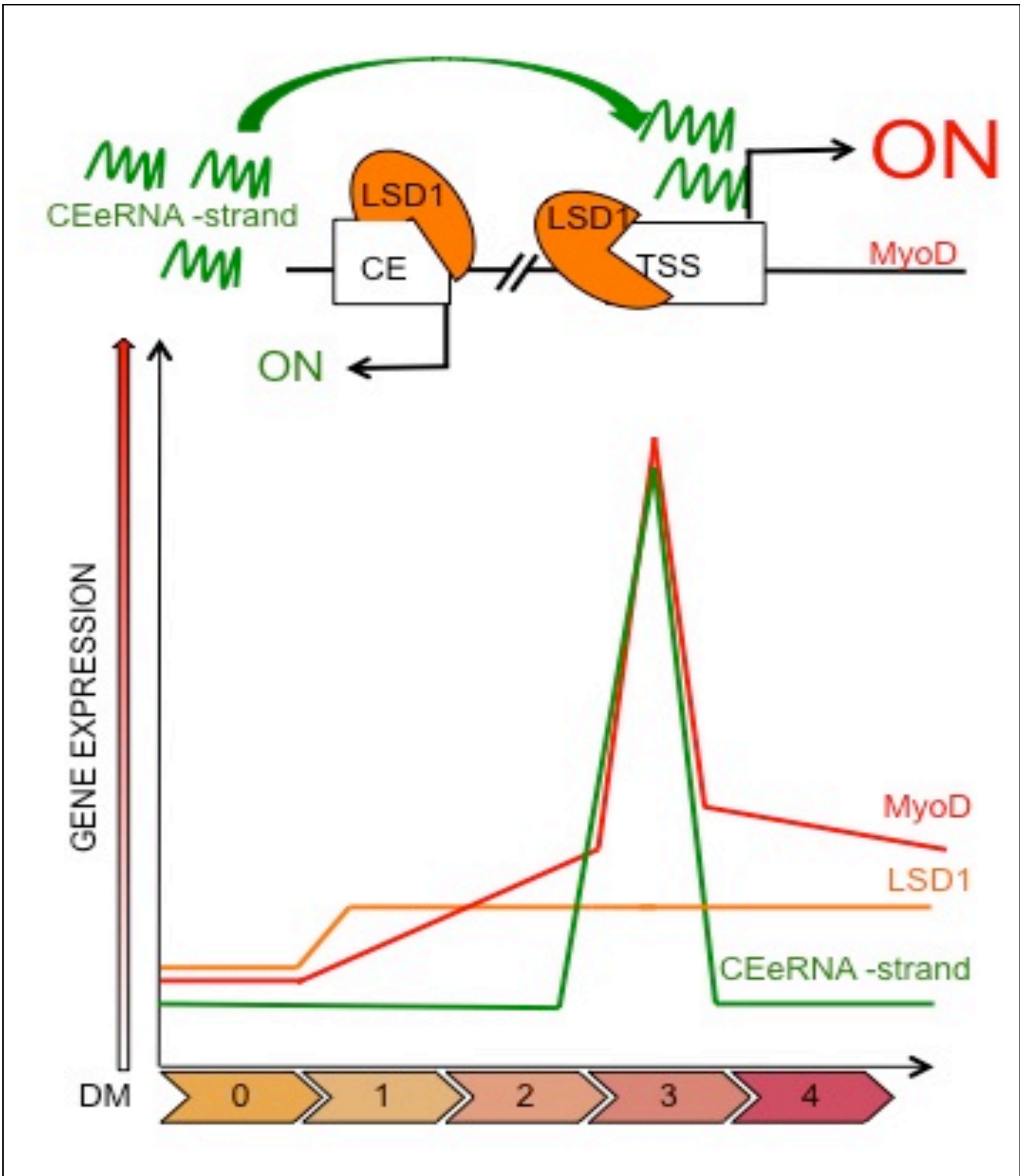


Figure 33: Schematic representation of MyoD, LSD1 and CEeRNA expression during myoblast differentiation.

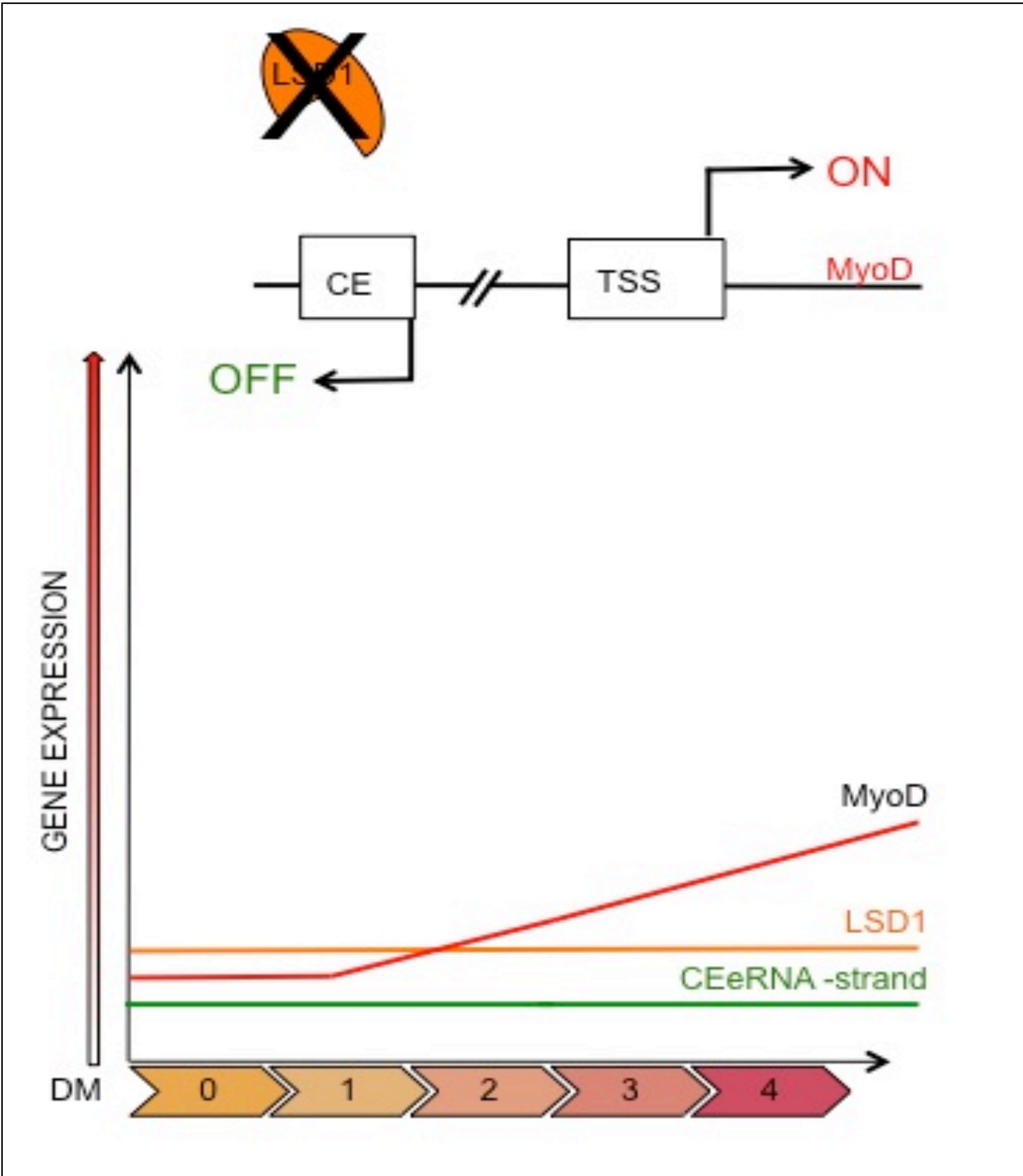


Figure 34: Schematic representation of MyoD, LSD1 and CEeRNA expression during myoblast differentiation in Sh LSD1 cells.

Materials and Methods

CELL CULTURES

C2C12 mouse myoblast cells were cultured at 37°C and 5% CO_2 in DMEM supplemented with 15% fetal bovine serum and antibiotics (growth medium GM). C2C12 were differentiated into myotubes replacing GM with media containing 2% horse serum with antibiotics (differentiation medium, DM).

For knock-down of LSD1 in C2C12 cells (Sh LSD1), lentiviral vectors containing the mouse LSD1-targeting sequence pLKO.1-sh-LSD1 (TRCN0000071377, Sh LSD1) was purchased from Open Biosystem. As a control, the pLKO.1 vector SHC016V purchased from Sigma was used (Sh SCRA). Twenty-four hours after lentiviral infection, C2C12 were selected with puromycin (1µg/ml) for fourteen days and then used for experiments. Cells transfections with plasmids were performed with jetPRIME® (polyplus transfection) according to manufacturer's instructions, briefly 300,000 C2C12 cells were seeded in 35 mm diameter tissue culture dishes and transfected 3 h later. After 24 hours cells were trypsinized and seeded at 150,000 cells per 35 mm dish let in GM for 8 hours and then the GM was changed in DM for 4-5 days.

EXPRESSION ANALYSIS

RNA extraction

The step by step protocol is described for cultured cells grown in one 100-mm dishes. Remove the medium and add slowly 1ml of PBS1X. Wash and remove. Harvest the cells using trypsin treatment and when the cells detach from the culture dish, add 1 volume of fresh medium and transfer the suspension to a tube. Centrifuge for 5 minutes at 1200 rpm, and then remove the supernatant. Add 1ml of TriReagent (Sigma). Pipet gently up and down and incubate for 5 minutes at room temperature. Add 200 µl of chloroform and vortex for 10 seconds. Incubate 15 minutes at room temperature. Centrifuge for 15 minutes at 12000rpm at 4°C. Transfer aqueous phase in a new tube and add 500 µl of isopropyl alcohol. Mix gently and incubate for 10 minutes at room temperature. Centrifuge at 12000rpm for 10 minutes at 4°C. Remove the supernatant and wash the pellet with 1 ml EtOH 75% and centrifuge at 12000 rpm for 5 minutes at 4°C. Remove the supernatant and dry the pellet. Then, dissolve the pellet in 30 µl of DNA/RNAse free water and heat the sample at 55°C for 10 minutes.

RevertAid H minus RT-PCR system and Real Time PCR

The RevertAid H minus RT-PCR was designed for the sensitive and reproducible detection and analysis of RNA molecules in a two-step process. RevertAid H minus Reverse Transcriptase, is an enzyme with RNA-dependent and DNA dependent polymerase activity, but lacks RNase H activity due to a point mutation in the RNase H domain, was engineered to have higher thermal stability, produces higher yields of cDNA, and produce full-length cDNA. cDNA synthesis was performed using total RNA with random hexamer primers.
Combine primer (random hexamer), 500 ng of total RNA and dNTP 25mM mix, adjusting volume to 12 µl with DNA/RNAse free water in a PCR tube. Denature RNA and primers by incubating at 70°C for 5 min and then place on ice. Vortex the 5X cDNA Synthesis buffer for 5 sec just prior to use. Prepare a master reaction mix on ice, with 5X synthesis buffer, RNaseOUT (40U/ ml), DNA/RNAse free water and RevertAid H minus Reverse Transcriptase (200units/µl). Mix gently. Pipet 7 µl of master reaction mix into each reaction tube on ice. Incubate samples 10 min at 25°C followed by 60 min at 42°C. Terminate the reaction by incubating at 70°C for 10 min.
QuantiFast SYBR® Green PCR Kit (Quiagen) for RotorGene is a ready to use cocktail containing all components, except primers and template. For real time PCR, synthesized cDNA and QuantiFast SYBR® Green were run on the RotorGene Realtime PCR System (Quiagen) Relative gene expression was determined using the ΔCt method.
The statistical significance of the differences in the means of experimental groups was determined by Bonferroni test after one-way ANOVA analysis using GRAPH PAD software. The data were presented as the means ± SEM. A p value of <0.05 was considered significant.

ChIP- CHROMATIN IMMUNOPRECIPITATION

The step by step protocol is described for 1 x 10^7 cultured cells, which are used for each immunoprecipitation. In the specific case the protocol is intended for C2C12 myoblast and myotube cells growing adhesively. Minor adjustments have to be introduced for other cell types especially for those growing in suspension. Based on our experience, one of the most critical steps in performing ChIP regards the conditions of chromatin fragmentation, which need to be empirically set up for each cell types employed. Remove the medium and add slowly 1ml of PBS1X. Wash and remove. Harvest the cells using trypsin treatment and when the cells detach from the culture dish, add 1 volume of fresh medium and transfer the suspension to a tube. Centrifuge for 5 minutes at 1200 rpm, and then remove the supernatant. After counting the cells dissolve 1 x 10^7 in 20 ml of medium in a 50ml falcon tube. Add 540 µl of formaldehyde from a 37% stock solution in the tube and mix immediately. Incubate samples on a rotating wheel for 10 minutes at room temperature. Add 1 ml of glycine from a 2,5 M stock solution and mix immediately. Incubate on a rotating wheel for 10 minutes at room temperature. Transfer the tube in ice and centrifuge at 1500 rpm for 5 minutes at 4°C, then keep samples on ice. Remove the supernatant and wash pellet 2 times with ice-cold PBS1X. After each washing, centrifuge at 1500 rpm for 5 minutes at 4°C. Remove the supernatant and dissolve the pellet in 500µl ice-cold Cell Lysis Buffer. Pipet up and down 10-20 times, then incubate on ice for 10-20 minutes. Centrifuge at 3000 rpm for 5 minutes at 4°C. Remove supernatant and dissolve pellet in 600µl ice-cold RIPA buffer. Pipet up and down 10-20 times, then incubate on ice for 10-20 minutes. Cells are sonicated with a Bioruptor® PLUS combined with the Bioruptor® Water cooler (Diagenode) for 1 hour at high power setting. Centrifuge samples at 14000 rpm for 15 minutes at 4°C. Transfer supernatant to a new tube and pre-clear lysate by incubating it with 50 µl of Protein A-Sepharose® 4B fast flow (Sigma) for 15 minutes in the cold room at constant rotation. Centrifuge samples at 3000 rpm for 5 minutes at 4°C. Take the supernatant, after having saved 50 µl aliquot for preparation of INPUT DNA and add 5 µg of specific antibody. Rotate the sample O/N in cold room. Add 50 µl of Protein A-Sepharose® 4B fast flow (Sigma) and incubate with constant rotation for 30 minutes at room temperature. Centrifuge the sample at 4000 rpm for 5 minutes at room temperature. Remove the supernatant and proceed to wash the beads. For each wash, incubate the sample by constant rotation for 3 minutes at room temperature and then centrifuge at 3000 rpm for 2 minutes at room temperature. Wash once with Low Salt Immuno

Complex buffer, once with High Salt Immuno Complex buffer and once with LiCl Immuno Complex buffer. Wash 2 times with 1 ml TE buffer. Remove the supernatant and add 200 µl TE buffer and 10 µg RNAse A and incubate at 37°C for 30 minutes. Add 6 µl Proteinase K (19 mg/ml) and then, incubate at 65°C in a shaker at 950 rpm for 6 hrs. Centrifuge at 14000 rpm for 10 minutes at 4°C, then transfer the supernatant (200µl) to a new tube. Extract once with phenol/chlorophorm/isoamylalcohol. Recover the aqueous phase (200 µl) and transfer to a new tube. Extract once with chlorophorm/isoamylalcohol. Recover the aqueous phase (200 µl) and transfer to a new tube. Add 1 µl glycogen (Glycoblue is 15 mg/ ml stock solution), 1/25 volumes NaCl 5M, and 2.5 volumes of cold ethanol 100% vortex and precipitate at -80°C for 40 minutes. Centrifuge at 14000 rpm for 30 minutes at 4°C. Remove the supernatant and wash pellet with 200 µl EtOH 70%. Dissolve IP-DNA and INPUT samples in 130 µl of 10 mM TrisHCl pH 8. Four µl of IP-DNA were used for Real Time PCR analysis.

Solutions

Cell Lysis Buffer

5mM PIPES pH 8

85mM KCl

0,5% NP40

1mM PMSF

Protease inhibitor cocktail (Sigma)

Phosphatase inhibitor cocktail (Roche)

Water

RIPA Buffer

150 mM NaCl

0,5% NaDoc

1% NP40

1mM PMSF

0,1% SDS

50mM TrisHCl pH8

Protease inhibitor cocktail (Sigma)

Phosphatase inhibitor cocktail (Roche)

Water

Low Salt Immuno Complex buffer

0,1% SDS

1% Triton X-100

2mM EDTA

20mM TrisHCl pH8

150mM NaCl

High Salt Immuno Complex buffer

0,1% SDS

1% Triton X-100

2mM EDTA

20mM TrisHCl pH8

500mM NaCl

LiCl Immuno Complex buffer

1% LiCl

IGEPAL CA630

1% NaDoc

1mM EDTA

10mM TrisHCl pH8

WESTERN BLOTTING ANALYSIS

Total protein extract preparation

The step by step protocol is described for cultured cells grown in one 100-mm dish. Remove the medium and add slowly 1ml of PBS1X. Wash and remove. Harvest the cells using trypsin treatment and when the cells detach from the culture dish, add 1 volume of fresh medium and transfer the suspension to a tube. Centrifuge for 5 minutes at 1200 rpm, and then remove the supernatant. Dissolve cells in 100μl of RIPA buffer, pipet up and down 10-20 times, then incubate on ice for 10 minutes. Cells are sonicated with a Bioruptor® PLUS combined with the Bioruptor® Water cooler (Diagenode) for 10 min at high power setting. Centrifuge at 14000 rpm at 4°C for 20 min and transfer the supernatant, which contains proteins in a new tube.

Immunoblot analysis

Proteins were quantified using the DC^{TM} Protein assay purchased from Bio-Rad following the manufacture protocol. 50 μg of total proteins were separated by 10% SDS-PAGE electrophoresis and transferred onto PVDF Immobilon®-P membranes (Millipore^{TM}). Membranes were blocked with TBS-0,1% tween (TBS-T) containing 5% skimmed milk and incubated overnight at 4°C with primary antibodies. Membranes were washed in TBS-T 3 times for 10min and incubated 1 hour with anti –mouse or –rabbit HRP- conjugated second antibodies. After three washes in TBS-T, membranes were incubated with ECL reagent.

NUCLEI COUNTING

The nuclei counting of myotubes was performed as follows. 300,000 cells were seeded in 35 mm petri dishes and 3 hours later they were transfected with GFP plasmids. After 24 hours 150,000 cells were seeded in 35 mm petri dishes, let them grow for 8 hours and after GM was changed in DM for 5 days. Myotubes were fixed for 20 min in 4% paraformaldehyde (PFA) in PBS and washed 3 times in PBS-0,1% triton-X100 to permeabilize membranes. Myotubes were incubated 20 minutes with DAPI to stain nuclei and finally washed 3 times in PBS. Myotubes were mounted with Vectashield and observed with a fluorescent microscope (AxioImager).

CLONING

Construct were generated with the Phusion Green High-Fidelity DNA Polymerase (Thermo Scientific) and confirmed by DNA sequencing.
Full-length Core enhancer (CE) encoding CEeRNA was subcloned into the pRNAT-H1.1/Neo vector in both directions, CEeRNA + and – strand, using the BAMHI site.

MOUSE BREEDING AND GENOTYPING

All mouse handling, breeding, and sacrificing were done in accordance with European legislations on animal experimentation. All separate lines were maintained by breeding to C57BL/6 mice. Experimental mice ($Pax3^{Cre/+}$; $LSD1^{Flox/Flox}$) were generated by crossing $Pax3^{Cre/+}$;$LSD1^{Flox/+}$ males with $LSD1^{Flox/Flox}$ females. The Cre allele was detected by PCR using a forward primer (5'-CGATGCAACGAGTGATGAGG-3') and a reverse primer (5'-GCATTGCTGTCACTTGGTCGT-3'), generating a product of 288 bp. The LSD1 floxed allele was detected by PCR using a forward primer (5′- ATACGAAGTTATGGATCCAAG-3′) and a reverse primer (5′- GCAGGCGGTTTGAAATGTATTC-3′), generating a product of 371 bp.

EMBRYO HARVESTING

Pregnant female mice were sacrificed following approved protocols. Mice were anesthetized before cervical dislocation. The uterus was removed and placed into dishes filled with PBS. Individual embryos were collected and placed into 4% PFA in PBS overnight at 4°C on a shaker.

Whole Mount In situ Hybridization

Gentle rocking of embryos occurred during all following incubations. Embryos were fixed in 4% paraformaldehyde in PBS at 4°C overnight. Embryos were rinsed and dehydrated in a gradient of methanol mixed with PBS-T (PBS with 0.1% Tween-20) (25%, 50%, 75% and 100% methanol) for 10 minutes each. Embryos were stored at -20°C in 100% methanol until needed.

Embryos were returned to room temperature and rehydrated in a reverse gradient in methanol and PBS-T. For better probe penetration, embryos were digested in 10µg/ml ProteinaseK/PBS-T for 25 minutes, rinsed in PBS-T, then fixed in 0.1% glutaraldehyde/4% paraformaldehyde/PBS-T for 20 minutes. Following rinses in PBS-T, embryos were incubated in a 1:1 mix of PBS-T and hybridization buffer, followed by 100% hybridization buffer for over 2 hours at 68°C. Digoxygenin labeled RNA probe (Sassoon et al., 1989) was then added to a concentration of 1 µg/ml and incubated at 68°C overnight. Embryos were washed in 68°C pre-warmed hybridization mix 2 times, 30 minutes per wash, at 68°C. Embryos were then incubated for 10 minutes as 68°C in a 1:1 mix of hybridization mix and MAB-T (for 50ml of MABT mix 29g maleic acid, 218g NaCl, 5ml 10% Tween20, and H20 to make 50 ml and adjusted pH to 7.5 with NaOH). Embryos were then washed 3 times for 15 minutes in MAB-T at room temperature. Embryos are incubated in 2% Boehringer Blocking Reagent (BBR) in MAB-T for 1 hour at room temperature. Continue with a two hours room temperature block in 2% BBR/20% heat treated Sheep Serum in MAB-T. Anti-Digoxygenin-AP Fab fragment (Roche #11093274910) were then added to a 1:2000 dilution and incubated at least 1 overnight at 4°C. Following incubation with the anti-DIG antibody, embryos were washed three times in MAB-T, followed by three days of washing in MAB-T, all at room temperature. Wash embryos in NTMT two times, ten minutes per wash at room temperature. Replace NTMT with BM Purple AP substrate (cat

#11442074001) and develop color to appropriate level, usually 6-8 hours. After color development level is reached, rinse embryos in PBS-T. Re-fix in 4% PFA and store at 4°C.

Solutions

NTMT
0,1M NaCl
0,1M Tris-HCl (pH9.5!)
50mM $MgCl_2$
1% Tween-20
H_2O

Hybridization Buffer
50% Formamide, Deionized (Sigma #F9037)
1,3 x SSC (pH5)
5 mM EDTA (pH8)
50µg/ml Yeast RNA (Sigma #R6625)
0,2% Tween-20
0,5% CHAPS (Sigma #C3023)
100µg/ml Heparin (Sigma #H4784)
H_2O

IMMUNOFLUORESCENCE

Embryos were isolated and fixed with 4% paraformaldehyde at 4°C overnight. The embryos were rinsed with cold PBS 4 times at 20 minutes each. The fixed muscle was processed through a sucrose gradient of 15% sucrose in PBS overnight, followed by 30% sucrose in PBS overnight. The processed tissue was placed into OCT compound and quickly frozen in dry ice cooled isopentane. The frozen tissue was cryosectioned at 12 microns and washed and then permeabilized with 100% of methanol for 6 min at -20°C.
Slides were saturated in PBS, 0,5% Triton X-100, 5% BSA (PBS-B-T) for 1 hour at room temperature, before being stained at 4°C overnight with primary antibodies diluted in PBS-B-T. After three 10 min washes in PBS, 0,1% Triton X-100, slides were incubated 1 hour at room temperature with secondary antibody diluted in PBS-B-T. After again three washes and counterstained with DAPI, slides were mounted.
Fluorescent images were acquired on a confocal microscope (Leica TCS SP5) and

processed with Photoshop CS4 (Adobe system).

STATISTICAL ANALYSIS

All results are means means ± s.d. To verify if the difference between samples is statistically significant, data were analyzed with Bonferroni test after variance analysis (ANOVA) and with t-test. Data elaboration was carried out with GRAPH PAD Software.

Table of primers:

Gene or region	Application	Forward primer	Reverse primer
MyoD	RT-qPCR	AGCACTACAGTGGCGACTCA	GCTCCACTATGCTGGACAGG
Cyclophillin B	RT-qPCR	GGTCACCAGGGCTGCCATTTG	TTCCAGAGGGGCCATCCACAG
CEeRNA	RT-qPCR	GCCAAGTATCCTCCTCCAGC	AAGCTGAGCACTCTGGGAGA
MyoD TSS	ChIP	AGATAGCCAAGTGCTACCGC	CCAGGGTAGCCTAAAAGCCC
MyoD 1	ChIP	CCCTTCATCCAGGGCACTAC	TTGGGAACCCAGCAGTAAGC
MyoD CE	ChIP	CTAAACACCAGGCATGAGAGG	ACTCACTTTCTCCCAGAGTTGC
CEeRNA	Cloning	CACGTGATGAAAAGTGAGGACA	TGACGTCACCAACAACGGTA

Table of Antibodies:

Name	**Application**	
Anti-LSD1	Western blotting (1/1000)- ChIP (5µg/IP)- IF (1:100)	Abcam
Anti-MyoD	Western blotting (1:500)- IF (1:200)	Santa-cruz Biotecnology®
Anti-Myogenin	Western blotting (1:200)	Santa-cruz Biotecnology®
Anti-Myogenin	IF (1:200)	DAKO
Anti-GAPDH	Western blotting (1:10000)	Cell signaling technology®
Anti-H3K4me3	ChIP (5µg/IP)	Millipore™
Anti-H3K9me2	ChIP (5µg/IP)	Active motif®
Anti-H3	ChIP (5µg/IP)	Active motif®
Anti rabbit-IgG	ChIP (5µg/IP)	Santa-cruz Biotecnology®
Anti-Pax3	IF (1:100)	DSHB
Anti-Pax7	IF (1:100)	DSHB
F(ab')2-Goat anti mouse IgG, Alexa Fluor® 594 coniugate	IF (1:1000)	Life technology
F(ab')2-Goat anti rabbit IgG, Alexa Fluor® 488 coniugate	IF (1:500)	Life technology

BIBLIOGRAPHY

- Adamo, A., Sese, B., Boue, S., Castano, J., Paramonov, I., Barrero, M.J., Izpisua Belmonte, J.C., 2011. LSD1 regulates the balance between self-renewal and differentiation in human embryonic stem cells. Nat Cell Biol 13, 652-659.
- Allfrey, V.G., Faulkner, R., Mirsky, A.E., 1964. Acetylation and Methylation of Histones and Their Possible Role in the Regulation of Rna Synthesis. Proc Natl Acad Sci U S A 51, 786-794.
- Andrews, J.L., Zhang, X., McCarthy, J.J., McDearmon, E.L., Hornberger, T.A., Russell, B., Campbell, K.S., Arbogast, S., Reid, M.B., Walker, J.R., Hogenesch, J.B., Takahashi, J.S., Esser, K.A., 2010. CLOCK and BMAL1 regulate MyoD and are necessary for maintenance of skeletal muscle phenotype and function. Proc Natl Acad Sci U S A 107, 19090-19095.
- Asakura, A., Hirai, H., Kablar, B., Morita, S., Ishibashi, J., Piras, B.A., Christ, A.J., Verma, M., Vineretsky, K.A., Rudnicki, M.A., 2007. Increased survival of muscle stem cells lacking the MyoD gene after transplantation into regenerating skeletal muscle. Proc Natl Acad Sci U S A 104, 16552-16557.
- Asakura, A., Lyons, G.E., Tapscott, S.J., 1995. The regulation of MyoD gene expression: conserved elements mediate expression in embryonic axial muscle. Dev Biol 171, 386-398.
- Bentzinger, C.F., Wang, Y.X., Rudnicki, M.A., 2012. Building muscle: molecular regulation of myogenesis. Cold Spring Harb Perspect Biol 4.
- Bergstrom, D.A., Penn, B.H., Strand, A., Perry, R.L., Rudnicki, M.A., Tapscott, S.J., 2002. Promoter-specific regulation of MyoD binding and signal transduction cooperate to pattern gene expression. Mol Cell 9, 587-600.
- Biressi, S., Tagliafico, E., Lamorte, G., Monteverde, S., Tenedini, E., Roncaglia, E., Ferrari, S., Cusella-De Angelis, M.G., Tajbakhsh, S., Cossu, G., 2007. Intrinsic phenotypic diversity of embryonic and fetal myoblasts is revealed by genome-wide gene expression analysis on purified cells. Dev Biol 304, 633-651.
- Black, B.L., Martin, J.F., Olson, E.N., 1995. The mouse MRF4 promoter is trans-activated directly and indirectly by muscle-specific transcription factors. J Biol Chem 270, 2889-2892.

- Boyer, L.A., Langer, M.R., Crowley, K.A., Tan, S., Denu, J.M., Peterson, C.L., 2002. Essential role for the SANT domain in the functioning of multiple chromatin remodeling enzymes. Mol Cell 10, 935-942.
- Braun, T., Bober, E., Rudnicki, M.A., Jaenisch, R., Arnold, H.H., 1994. MyoD expression marks the onset of skeletal myogenesis in Myf-5 mutant mice. Development 120, 3083-3092.
- Braun, T., Bober, E., Winter, B., Rosenthal, N., Arnold, H.H., 1990. Myf-6, a new member of the human gene family of myogenic determination factors: evidence for a gene cluster on chromosome 12. Embo J 9, 821-831.
- Braun, T., Buschhausen-Denker, G., Bober, E., Tannich, E., Arnold, H.H., 1989. A novel human muscle factor related to but distinct from MyoD1 induces myogenic conversion in 10T1/2 fibroblasts. Embo J 8, 701-709.
- Braun, T., Rudnicki, M.A., Arnold, H.H., Jaenisch, R., 1992. Targeted inactivation of the muscle regulatory gene Myf-5 results in abnormal rib development and perinatal death. Cell 71, 369-382.
- Buckingham, M., Rigby, P.W., 2014. Gene regulatory networks and transcriptional mechanisms that control myogenesis. Dev Cell 28, 225-238.
- Buckingham, M., Tajbakhsh, S., 1993. Expression of myogenic factors in the mouse: myf-5, the first member of the MyoD gene family to be transcribed during skeletal myogenesis. C R Acad Sci III 316, 1032-1046.
- Carroll, J.S., Meyer, C.A., Song, J., Li, W., Geistlinger, T.R., Eeckhoute, J., Brodsky, A.S., Keeton, E.K., Fertuck, K.C., Hall, G.F., Wang, Q., Bekiranov, S., Sementchenko, V., Fox, E.A., Silver, P.A., Gingeras, T.R., Liu, X.S., Brown, M., 2006. Genome-wide analysis of estrogen receptor binding sites. Nat Genet 38, 1289-1297.
- Chen, J.C., Love, C.M., Goldhamer, D.J., 2001. Two upstream enhancers collaborate to regulate the spatial patterning and timing of MyoD transcription during mouse development. Dev Dyn 221, 274-288.
- Chen, Y., Yang, Y., Wang, F., Wan, K., Yamane, K., Zhang, Y., Lei, M., 2006. Crystal structure of human histone lysine-specific demethylase 1 (LSD1). Proc Natl Acad Sci U S A 103, 13956-13961.
- Chevallier, A., Kieny, M., Mauger, A., 1977. Limb-somite relationship: origin of the limb musculature. J Embryol Exp Morphol 41, 245-258.

- Choi, J., Jang, H., Kim, H., Kim, S.T., Cho, E.J., Youn, H.D., 2010. Histone demethylase LSD1 is required to induce skeletal muscle differentiation by regulating myogenic factors. Biochem Biophys Res Commun 401, 327-332.
- Christy, B.A., Sanders, L.K., Lau, L.F., Copeland, N.G., Jenkins, N.A., Nathans, D., 1991. An Id-related helix-loop-helix protein encoded by a growth factor-inducible gene. Proc Natl Acad Sci U S A 88, 1815-1819.
- Clarke, S., 1993. Protein methylation. Curr Opin Cell Biol 5, 977-983.
- Cserjesi, P., Olson, E.N., 1991. Myogenin induces the myocyte-specific enhancer binding factor MEF-2 independently of other muscle-specific gene products. Mol Cell Biol 11, 4854-4862.
- Da, G., Lenkart, J., Zhao, K., Shiekhattar, R., Cairns, B.R., Marmorstein, R., 2006. Structure and function of the SWIRM domain, a conserved protein module found in chromatin regulatory complexes. Proc Natl Acad Sci U S A 103, 2057-2062.
- Daston, G., Lamar, E., Olivier, M., Goulding, M., 1996. Pax-3 is necessary for migration but not differentiation of limb muscle precursors in the mouse. Development 122, 1017-1027.
- Davis, R.L., Weintraub, H., Lassar, A.B., 1987. Expression of a single transfected cDNA converts fibroblasts to myoblasts. Cell 51, 987-1000.
- Di Padova, M., Caretti, G., Zhao, P., Hoffman, E.P., Sartorelli, V., 2007. MyoD acetylation influences temporal patterns of skeletal muscle gene expression. J Biol Chem 282, 37650-37659.
- Dietrich, S., Abou-Rebyeh, F., Brohmann, H., Bladt, F., Sonnenberg-Riethmacher, E., Yamaai, T., Lumsden, A., Brand-Saberi, B., Birchmeier, C., 1999. The role of SF/HGF and c-Met in the development of skeletal muscle. Development 126, 1621-1629.
- Djebali, S., Davis, C.A., Merkel, A., Dobin, A., Lassmann, T., Mortazavi, A., Tanzer, A., Lagarde, J., Lin, W., Schlesinger, F., Xue, C., Marinov, G.K., Khatun, J., Williams, B.A., Zaleski, C., Rozowsky, J., Roder, M., Kokocinski, F., Abdelhamid, R.F., Alioto, T., Antoshechkin, I., Baer, M.T., Bar, N.S., Batut, P., Bell, K., Bell, I., Chakrabortty, S., Chen, X., Chrast, J., Curado, J., Derrien, T., Drenkow, J., Dumais, E., Dumais, J., Duttagupta, R., Falconnet, E., Fastuca, M., Fejes-Toth, K., Ferreira, P., Foissac, S., Fullwood, M.J., Gao, H., Gonzalez, D., Gordon, A., Gunawardena, H., Howald, C., Jha, S., Johnson, R., Kapranov, P., King, B., Kingswood, C., Luo,

O.J., Park, E., Persaud, K., Preall, J.B., Ribeca, P., Risk, B., Robyr, D., Sammeth, M., Schaffer, L., See, L.H., Shahab, A., Skancke, J., Suzuki, A.M., Takahashi, H., Tilgner, H., Trout, D., Walters, N., Wang, H., Wrobel, J., Yu, Y., Ruan, X., Hayashizaki, Y., Harrow, J., Gerstein, M., Hubbard, T., Reymond, A., Antonarakis, S.E., Hannon, G., Giddings, M.C., Ruan, Y., Wold, B., Carninci, P., Guigo, R., Gingeras, T.R., 2012. Landscape of transcription in human cells. Nature 489, 101-108.

- Duquet, A., Polesskaya, A., Cuvellier, S., Ait-Si-Ali, S., Hery, P., Pritchard, L.L., Gerard, M., Harel-Bellan, A., 2006. Acetylation is important for MyoD function in adult mice. EMBO Rep 7, 1140-1146.
- Edmondson, D.G., Cheng, T.C., Cserjesi, P., Chakraborty, T., Olson, E.N., 1992. Analysis of the myogenin promoter reveals an indirect pathway for positive autoregulation mediated by the muscle-specific enhancer factor MEF-2. Mol Cell Biol 12, 3665-3677.
- Edmondson, D.G., Olson, E.N., 1989. A gene with homology to the myc similarity region of MyoD1 is expressed during myogenesis and is sufficient to activate the muscle differentiation program. Genes Dev 3, 628-640.
- Faerman, A., Goldhamer, D.J., Puzis, R., Emerson, C.P., Jr., Shani, M., 1995. The distal human myoD enhancer sequences direct unique muscle-specific patterns of lacZ expression during mouse development. Dev Biol 171, 27-38.
- Felsenfeld, G., Groudine, M., 2003. Controlling the double helix. Nature 421, 448-453.
- Feng, J., Bi, C., Clark, B.S., Mady, R., Shah, P., Kohtz, J.D., 2006. The Evf-2 noncoding RNA is transcribed from the Dlx-5/6 ultraconserved region and functions as a Dlx-2 transcriptional coactivator. Genes Dev 20, 1470-1484.
- Ferre-D'Amare, A.R., Prendergast, G.C., Ziff, E.B., Burley, S.K., 1993. Recognition by Max of its cognate DNA through a dimeric b/HLH/Z domain. Nature 363, 38-45.
- Forcales, S.V., Albini, S., Giordani, L., Malecova, B., Cignolo, L., Chernov, A., Coutinho, P., Saccone, V., Consalvi, S., Williams, R., Wang, K., Wu, Z., Baranovskaya, S., Miller, A., Dilworth, F.J., Puri, P.L., 2012. Signal-dependent incorporation of MyoD-BAF60c into Brg1-based SWI/SNF chromatin-remodelling complex. Embo J 31, 301-316.

- Fraaije, M.W., Mattevi, A., 2000. Flavoenzymes: diverse catalysts with recurrent features. Trends Biochem Sci 25, 126-132.
- Garcia-Bassets, I., Kwon, Y.S., Telese, F., Prefontaine, G.G., Hutt, K.R., Cheng, C.S., Ju, B.G., Ohgi, K.A., Wang, J., Escoubet-Lozach, L., Rose, D.W., Glass, C.K., Fu, X.D., Rosenfeld, M.G., 2007. Histone methylation-dependent mechanisms impose ligand dependency for gene activation by nuclear receptors. Cell 128, 505-518.
- Gauthier-Rouviere, C., Vandromme, M., Tuil, D., Lautredou, N., Morris, M., Soulez, M., Kahn, A., Fernandez, A., Lamb, N., 1996. Expression and activity of serum response factor is required for expression of the muscle-determining factor MyoD in both dividing and differentiating mouse C2C12 myoblasts. Mol Biol Cell 7, 719-729.
- Ge, W., Liu, Y., Chen, T., Zhang, X., Lv, L., Jin, C., Jiang, Y., Shi, L., Zhou, Y., 2014. The epigenetic promotion of osteogenic differentiation of human adipose-derived stem cells by the genetic and chemical blockade of histone demethylase LSD1. Biomaterials 35, 6015-6025.
- Gensch, N., Borchardt, T., Schneider, A., Riethmacher, D., Braun, T., 2008. Different autonomous myogenic cell populations revealed by ablation of Myf5-expressing cells during mouse embryogenesis. Development 135, 1597-1604.
- Goldhamer, D.J., Brunk, B.P., Faerman, A., King, A., Shani, M., Emerson, C.P., Jr., 1995. Embryonic activation of the myoD gene is regulated by a highly conserved distal control element. Development 121, 637-649.
- Goldhamer, D.J., Faerman, A., Shani, M., Emerson, C.P., Jr., 1992. Regulatory elements that control the lineage-specific expression of myoD. Science 256, 538-542.
- Gossett, L.A., Kelvin, D.J., Sternberg, E.A., Olson, E.N., 1989. A new myocyte-specific enhancer-binding factor that recognizes a conserved element associated with multiple muscle-specific genes. Mol Cell Biol 9, 5022-5033.
- Gros, J., Manceau, M., Thome, V., Marcelle, C., 2005. A common somitic origin for embryonic muscle progenitors and satellite cells. Nature 435, 954-958.
- Hah, N., Murakami, S., Nagari, A., Danko, C.G., Kraus, W.L., 2013. Enhancer transcripts mark active estrogen receptor binding sites. Genome Res 23, 1210-1223.

- Haldar, M., Karan, G., Tvrdik, P., Capecchi, M.R., 2008. Two cell lineages, myf5 and myf5-independent, participate in mouse skeletal myogenesis. Dev Cell 14, 437-445.
- Han, X., Gui, B., Xiong, C., Zhao, L., Liang, J., Sun, L., Yang, X., Yu, W., Si, W., Yan, R., Yi, X., Zhang, D., Li, W., Li, L., Yang, J., Wang, Y., Sun, Y.E., Meng, A., Shang, Y., 2014. Destabilizing LSD1 by Jade-2 promotes neurogenesis: an antibraking system in neural development. Mol Cell 55, 482-494.
- Harris, H., 1959. Turnover of nuclear and cytoplasmic ribonucleic acid in two types of animal cell, with some further observations on the nucleolus. Biochem J 73, 362-369.
- Hasan, S., Hottiger, M.O., 2002. Histone acetyl transferases: a role in DNA repair and DNA replication. J Mol Med (Berl) 80, 463-474.
- Hasty, P., Bradley, A., Morris, J.H., Edmondson, D.G., Venuti, J.M., Olson, E.N., Klein, W.H., 1993. Muscle deficiency and neonatal death in mice with a targeted mutation in the myogenin gene. Nature 364, 501-506.
- Havis, E., Coumailleau, P., Bonnet, A., Bismuth, K., Bonnin, M.A., Johnson, R., Fan, C.M., Relaix, F., Shi, D.L., Duprez, D., 2012. Sim2 prevents entry into the myogenic program by repressing MyoD transcription during limb embryonic myogenesis. Development 139, 1910-1920.
- Hon, G.C., Hawkins, R.D., Ren, B., 2009. Predictive chromatin signatures in the mammalian genome. Hum Mol Genet 18, R195-201.
- Horn, P.J., Peterson, C.L., 2002. Molecular biology. Chromatin higher order folding--wrapping up transcription. Science 297, 1824-1827.
- Horst, D., Ustanina, S., Sergi, C., Mikuz, G., Juergens, H., Braun, T., Vorobyov, E., 2006. Comparative expression analysis of Pax3 and Pax7 during mouse myogenesis. Int J Dev Biol 50, 47-54.
- Hu, P., Geles, K.G., Paik, J.H., DePinho, R.A., Tjian, R., 2008. Codependent activators direct myoblast-specific MyoD transcription. Dev Cell 15, 534-546.
- Hutcheson, D.A., Zhao, J., Merrell, A., Haldar, M., Kardon, G., 2009. Embryonic and fetal limb myogenic cells are derived from developmentally distinct progenitors and have different requirements for beta-catenin. Genes Dev 23, 997-1013.

- Jen, Y., Weintraub, H., Benezra, R., 1992. Overexpression of Id protein inhibits the muscle differentiation program: in vivo association of Id with E2A proteins. Genes Dev 6, 1466-1479.
- Jenuwein, T., Allis, C.D., 2001. Translating the histone code. Science 293, 1074-1080.
- Jiang, H., Shukla, A., Wang, X., Chen, W.Y., Bernstein, B.E., Roeder, R.G., 2011. Role for Dpy-30 in ES cell-fate specification by regulation of H3K4 methylation within bivalent domains. Cell 144, 513-525.
- Johanson, M., Meents, H., Ragge, K., Buchberger, A., Arnold, H.H., Sandmoller, A., 1999. Transcriptional activation of the myogenin gene by MEF2-mediated recruitment of myf5 is inhibited by adenovirus E1A protein. Biochem Biophys Res Commun 265, 222-232.
- Kablar, B., Krastel, K., Ying, C., Tapscott, S.J., Goldhamer, D.J., Rudnicki, M.A., 1999. Myogenic determination occurs independently in somites and limb buds. Dev Biol 206, 219-231.
- Kadesch, T., 1993. Consequences of heteromeric interactions among helix-loop-helix proteins. Cell Growth Differ 4, 49-55.
- Kagey, M.H., Newman, J.J., Bilodeau, S., Zhan, Y., Orlando, D.A., van Berkum, N.L., Ebmeier, C.C., Goossens, J., Rahl, P.B., Levine, S.S., Taatjes, D.J., Dekker, J., Young, R.A., 2010. Mediator and cohesin connect gene expression and chromatin architecture. Nature 467, 430-435.
- Kalcheim, C., Ben-Yair, R., 2005. Cell rearrangements during development of the somite and its derivatives. Curr Opin Genet Dev 15, 371-380.
- Kassar-Duchossoy, L., Giacone, E., Gayraud-Morel, B., Jory, A., Gomes, D., Tajbakhsh, S., 2005. Pax3/Pax7 mark a novel population of primitive myogenic cells during development. Genes Dev 19, 1426-1431.
- Kerenyi, M.A., Shao, Z., Hsu, Y.J., Guo, G., Luc, S., O'Brien, K., Fujiwara, Y., Peng, C., Nguyen, M., Orkin, S.H., 2013. Histone demethylase Lsd1 represses hematopoietic stem and progenitor cell signatures during blood cell maturation. Elife 2, e00633.
- Khochbin, S., Verdel, A., Lemercier, C., Seigneurin-Berny, D., 2001. Functional significance of histone deacetylase diversity. Curr Opin Genet Dev 11, 162-166.

- Kim, T.K., Hemberg, M., Gray, J.M., Costa, A.M., Bear, D.M., Wu, J., Harmin, D.A., Laptewicz, M., Barbara-Haley, K., Kuersten, S., Markenscoff-Papadimitriou, E., Kuhl, D., Bito, H., Worley, P.F., Kreiman, G., Greenberg, M.E., 2010. Widespread transcription at neuronal activity-regulated enhancers. Nature 465, 182-187.
- Kizer, K.O., Phatnani, H.P., Shibata, Y., Hall, H., Greenleaf, A.L., Strahl, B.D., 2005. A novel domain in Set2 mediates RNA polymerase II interaction and couples histone H3 K36 methylation with transcript elongation. Mol Cell Biol 25, 3305-3316.
- Koch, F., Fenouil, R., Gut, M., Cauchy, P., Albert, T.K., Zacarias-Cabeza, J., Spicuglia, S., de la Chapelle, A.L., Heidemann, M., Hintermair, C., Eick, D., Gut, I., Ferrier, P., Andrau, J.C., 2011. Transcription initiation platforms and GTF recruitment at tissue-specific enhancers and promoters. Nat Struct Mol Biol 18, 956-963.
- Kouzarides, T., 2007. Chromatin modifications and their function. Cell 128, 693-705.
- Krogan, N.J., Kim, M., Tong, A., Golshani, A., Cagney, G., Canadien, V., Richards, D.P., Beattie, B.K., Emili, A., Boone, C., Shilatifard, A., Buratowski, S., Greenblatt, J., 2003. Methylation of histone H3 by Set2 in Saccharomyces cerevisiae is linked to transcriptional elongation by RNA polymerase II. Mol Cell Biol 23, 4207-4218.
- L'Honore, A., Lamb, N.J., Vandromme, M., Turowski, P., Carnac, G., Fernandez, A., 2003. MyoD distal regulatory region contains an SRF binding CArG element required for MyoD expression in skeletal myoblasts and during muscle regeneration. Mol Biol Cell 14, 2151-2162.
- L'Honore, A., Ouimette, J.F., Lavertu-Jolin, M., Drouin, J., 2010. Pitx2 defines alternate pathways acting through MyoD during limb and somitic myogenesis. Development 137, 3847-3856.
- Lachner, M., O'Carroll, D., Rea, S., Mechtler, K., Jenuwein, T., 2001. Methylation of histone H3 lysine 9 creates a binding site for HP1 proteins. Nature 410, 116-120.
- Lai, F., Orom, U.A., Cesaroni, M., Beringer, M., Taatjes, D.J., Blobel, G.A., Shiekhattar, R., 2013. Activating RNAs associate with Mediator to enhance chromatin architecture and transcription. Nature 494, 497-501.
- Lai, F., Shiekhattar, R., 2014. Enhancer RNAs: the new molecules of transcription. Curr Opin Genet Dev 25, 38-42.
- Lam, M.T., Cho, H., Lesch, H.P., Gosselin, D., Heinz, S., Tanaka-Oishi, Y., Benner, C., Kaikkonen, M.U., Kim, A.S., Kosaka, M., Lee, C.Y., Watt, A., Grossman, T.R.,

Rosenfeld, M.G., Evans, R.M., Glass, C.K., 2013. Rev-Erbs repress macrophage gene expression by inhibiting enhancer-directed transcription. Nature 498, 511-515.

- Lassar, A.B., Davis, R.L., Wright, W.E., Kadesch, T., Murre, C., Voronova, A., Baltimore, D., Weintraub, H., 1991. Functional activity of myogenic HLH proteins requires hetero-oligomerization with E12/E47-like proteins in vivo. Cell 66, 305-315.
- Lee, M.G., Wynder, C., Cooch, N., Shiekhattar, R., 2005. An essential role for CoREST in nucleosomal histone 3 lysine 4 demethylation. Nature 437, 432-435.
- Li, H., Capetanaki, Y., 1994. An E box in the desmin promoter cooperates with the E box and MEF-2 sites of a distal enhancer to direct muscle-specific transcription. Embo J 13, 3580-3589.
- Li, J., Chen, F., Epstein, J.A., 2000. Neural crest expression of Cre recombinase directed by the proximal Pax3 promoter in transgenic mice. Genesis 26, 162-164.
- Li, W., Notani, D., Ma, Q., Tanasa, B., Nunez, E., Chen, A.Y., Merkurjev, D., Zhang, J., Ohgi, K., Song, X., Oh, S., Kim, H.S., Glass, C.K., Rosenfeld, M.G., 2013. Functional roles of enhancer RNAs for oestrogen-dependent transcriptional activation. Nature 498, 516-520.
- Lin, Y.C., Benner, C., Mansson, R., Heinz, S., Miyazaki, K., Miyazaki, M., Chandra, V., Bossen, C., Glass, C.K., Murre, C., 2012. Global changes in the nuclear positioning of genes and intra- and interdomain genomic interactions that orchestrate B cell fate. Nat Immunol 13, 1196-1204.
- Lluis, F., Ballestar, E., Suelves, M., Esteller, M., Munoz-Canoves, P., 2005. E47 phosphorylation by p38 MAPK promotes MyoD/E47 association and muscle-specific gene transcription. Embo J 24, 974-984.
- Lu, J., McKinsey, T.A., Nicol, R.L., Olson, E.N., 2000. Signal-dependent activation of the MEF2 transcription factor by dissociation from histone deacetylases. Proc Natl Acad Sci U S A 97, 4070-4075.
- Ma, K., Chan, J.K., Zhu, G., Wu, Z., 2005. Myocyte enhancer factor 2 acetylation by p300 enhances its DNA binding activity, transcriptional activity, and myogenic differentiation. Mol Cell Biol 25, 3575-3582.
- Mal, A., Sturniolo, M., Schiltz, R.L., Ghosh, M.K., Harter, M.L., 2001. A role for histone deacetylase HDAC1 in modulating the transcriptional activity of MyoD: inhibition of the myogenic program. Embo J 20, 1739-1753.

- McKinsey, T.A., Zhang, C.L., Olson, E.N., 2001. Control of muscle development by dueling HATs and HDACs. Curr Opin Genet Dev 11, 497-504.
- Mejat, A., Ramond, F., Bassel-Duby, R., Khochbin, S., Olson, E.N., Schaeffer, L., 2005. Histone deacetylase 9 couples neuronal activity to muscle chromatin acetylation and gene expression. Nat Neurosci 8, 313-321.
- Melo, C.A., Drost, J., Wijchers, P.J., van de Werken, H., de Wit, E., Oude Vrielink, J.A., Elkon, R., Melo, S.A., Leveille, N., Kalluri, R., de Laat, W., Agami, R., 2013. eRNAs are required for p53-dependent enhancer activity and gene transcription. Mol Cell 49, 524-535.
- Messina, G., Cossu, G., 2009. The origin of embryonic and fetal myoblasts: a role of Pax3 and Pax7. Genes Dev 23, 902-905.
- Metzger, E., Wissmann, M., Yin, N., Muller, J.M., Schneider, R., Peters, A.H., Gunther, T., Buettner, R., Schule, R., 2005. LSD1 demethylates repressive histone marks to promote androgen-receptor-dependent transcription. Nature 437, 436-439.
- Molkentin, J.D., Black, B.L., Martin, J.F., Olson, E.N., 1995. Cooperative activation of muscle gene expression by MEF2 and myogenic bHLH proteins. Cell 83, 1125-1136.
- Molkentin, J.D., Olson, E.N., 1996. Combinatorial control of muscle development by basic helix-loop-helix and MADS-box transcription factors. Proc Natl Acad Sci U S A 93, 9366-9373.
- Mousavi, K., Zare, H., Dell'orso, S., Grontved, L., Gutierrez-Cruz, G., Derfoul, A., Hager, G.L., Sartorelli, V., 2013. eRNAs promote transcription by establishing chromatin accessibility at defined genomic loci. Mol Cell 51, 606-617.
- Mueller, A.C., Cichewicz, M.A., Dey, B.K., Layer, R., Reon, B.J., Gagan, J.R., Dutta, A., 2015. MUNC, a Long Noncoding RNA That Facilitates the Function of MyoD in Skeletal Myogenesis. Mol Cell Biol 35, 498-513.
- Mulligan, P., Yang, F., Di Stefano, L., Ji, J.Y., Ouyang, J., Nishikawa, J.L., Toiber, D., Kulkarni, M., Wang, Q., Najafi-Shoushtari, S.H., Mostoslavsky, R., Gygi, S.P., Gill, G., Dyson, N.J., Naar, A.M., 2011. A SIRT1-LSD1 corepressor complex regulates Notch target gene expression and development. Mol Cell 42, 689-699.
- Musri, M.M., Carmona, M.C., Hanzu, F.A., Kaliman, P., Gomis, R., Parrizas, M., 2010. Histone demethylase LSD1 regulates adipogenesis. J Biol Chem 285, 30034-30041.

- Nabeshima, Y., Hanaoka, K., Hayasaka, M., Esumi, E., Li, S., Nonaka, I., 1993. Myogenin gene disruption results in perinatal lethality because of severe muscle defect. Nature 364, 532-535.
- Naidu, P.S., Ludolph, D.C., To, R.Q., Hinterberger, T.J., Konieczny, S.F., 1995. Myogenin and MEF2 function synergistically to activate the MRF4 promoter during myogenesis. Mol Cell Biol 15, 2707-2718.
- Nakayama, J., Rice, J.C., Strahl, B.D., Allis, C.D., Grewal, S.I., 2001. Role of histone H3 lysine 9 methylation in epigenetic control of heterochromatin assembly. Science 292, 110-113.
- Nielsen, R., Pedersen, T.A., Hagenbeek, D., Moulos, P., Siersbaek, R., Megens, E., Denissov, S., Borgesen, M., Francoijs, K.J., Mandrup, S., Stunnenberg, H.G., 2008. Genome-wide profiling of PPARgamma:RXR and RNA polymerase II occupancy reveals temporal activation of distinct metabolic pathways and changes in RXR dimer composition during adipogenesis. Genes Dev 22, 2953-2967.
- Noma, K., Allis, C.D., Grewal, S.I., 2001. Transitions in distinct histone H3 methylation patterns at the heterochromatin domain boundaries. Science 293, 1150-1155.
- Ohkawa, Y., Marfella, C.G., Imbalzano, A.N., 2006. Skeletal muscle specification by myogenin and Mef2D via the SWI/SNF ATPase Brg1. Embo J 25, 490-501.
- Ohkawa, Y., Yoshimura, S., Higashi, C., Marfella, C.G., Dacwag, C.S., Tachibana, T., Imbalzano, A.N., 2007. Myogenin and the SWI/SNF ATPase Brg1 maintain myogenic gene expression at different stages of skeletal myogenesis. J Biol Chem 282, 6564-6570.
- Okada, Y., Nagai, R., Sato, T., Matsuura, E., Minami, T., Morita, I., Doi, T., 2003. Homeodomain proteins MEIS1 and PBXs regulate the lineage-specific transcription of the platelet factor 4 gene. Blood 101, 4748-4756.
- Ordahl, C.P., Le Douarin, N.M., 1992. Two myogenic lineages within the developing somite. Development 114, 339-353.
- Orom, U.A., Derrien, T., Beringer, M., Gumireddy, K., Gardini, A., Bussotti, G., Lai, F., Zytnicki, M., Notredame, C., Huang, Q., Guigo, R., Shiekhattar, R., 2010. Long noncoding RNAs with enhancer-like function in human cells. Cell 143, 46-58.
- Parker, M.H., Seale, P., Rudnicki, M.A., 2003. Looking back to the embryo: defining transcriptional networks in adult myogenesis. Nat Rev Genet 4, 497-507.

- Patapoutian, A., Yoon, J.K., Miner, J.H., Wang, S., Stark, K., Wold, B., 1995. Disruption of the mouse MRF4 gene identifies multiple waves of myogenesis in the myotome. Development 121, 3347-3358.
- Penn, B.H., Bergstrom, D.A., Dilworth, F.J., Bengal, E., Tapscott, S.J., 2004. A MyoD-generated feed-forward circuit temporally patterns gene expression during skeletal muscle differentiation. Genes Dev 18, 2348-2353.
- Phillips-Cremins, J.E., Sauria, M.E., Sanyal, A., Gerasimova, T.I., Lajoie, B.R., Bell, J.S., Ong, C.T., Hookway, T.A., Guo, C., Sun, Y., Bland, M.J., Wagstaff, W., Dalton, S., McDevitt, T.C., Sen, R., Dekker, J., Taylor, J., Corces, V.G., 2013. Architectural protein subclasses shape 3D organization of genomes during lineage commitment. Cell 153, 1281-1295.
- Polesskaya, A., Duquet, A., Naguibneva, I., Weise, C., Vervisch, A., Bengal, E., Hucho, F., Robin, P., Harel-Bellan, A., 2000. CREB-binding protein/p300 activates MyoD by acetylation. J Biol Chem 275, 34359-34364.
- Pollock, J.A., Larrea, M.D., Jasper, J.S., McDonnell, D.P., McCafferty, D.G., 2012. Lysine-specific histone demethylase 1 inhibitors control breast cancer proliferation in ERalpha-dependent and -independent manners. ACS Chem Biol 7, 1221-1231.
- Potthoff, M.J., Arnold, M.A., McAnally, J., Richardson, J.A., Bassel-Duby, R., Olson, E.N., 2007a. Regulation of skeletal muscle sarcomere integrity and postnatal muscle function by Mef2c. Mol Cell Biol 27, 8143-8151.
- Potthoff, M.J., Wu, H., Arnold, M.A., Shelton, J.M., Backs, J., McAnally, J., Richardson, J.A., Bassel-Duby, R., Olson, E.N., 2007b. Histone deacetylase degradation and MEF2 activation promote the formation of slow-twitch myofibers. J Clin Invest 117, 2459-2467.
- Puri, P.L., Avantaggiati, M.L., Balsano, C., Sang, N., Graessmann, A., Giordano, A., Levrero, M., 1997. p300 is required for MyoD-dependent cell cycle arrest and muscle-specific gene transcription. Embo J 16, 369-383.
- Puri, P.L., Iezzi, S., Stiegler, P., Chen, T.T., Schiltz, R.L., Muscat, G.E., Giordano, A., Kedes, L., Wang, J.Y., Sartorelli, V., 2001. Class I histone deacetylases sequentially interact with MyoD and pRb during skeletal myogenesis. Mol Cell 8, 885-897.

- Qian, C., Zhang, Q., Li, S., Zeng, L., Walsh, M.J., Zhou, M.M., 2005. Structure and chromosomal DNA binding of the SWIRM domain. Nat Struct Mol Biol 12, 1078-1085.
- Rawls, A., Valdez, M.R., Zhang, W., Richardson, J., Klein, W.H., Olson, E.N., 1998. Overlapping functions of the myogenic bHLH genes MRF4 and MyoD revealed in double mutant mice. Development 125, 2349-2358.
- Relaix, F., Demignon, J., Laclef, C., Pujol, J., Santolini, M., Niro, C., Lagha, M., Rocancourt, D., Buckingham, M., Maire, P., 2013. Six homeoproteins directly activate Myod expression in the gene regulatory networks that control early myogenesis. PLoS Genet 9, e1003425.
- Relaix, F., Montarras, D., Zaffran, S., Gayraud-Morel, B., Rocancourt, D., Tajbakhsh, S., Mansouri, A., Cumano, A., Buckingham, M., 2006. Pax3 and Pax7 have distinct and overlapping functions in adult muscle progenitor cells. J Cell Biol 172, 91-102.
- Relaix, F., Rocancourt, D., Mansouri, A., Buckingham, M., 2005. A Pax3/Pax7-dependent population of skeletal muscle progenitor cells. Nature 435, 948-953.
- Rhodes, D., 1997. Chromatin structure. The nucleosome core all wrapped up. Nature 389, 231, 233.
- Roth, J.F., Shikama, N., Henzen, C., Desbaillets, I., Lutz, W., Marino, S., Wittwer, J., Schorle, H., Gassmann, M., Eckner, R., 2003. Differential role of p300 and CBP acetyltransferase during myogenesis: p300 acts upstream of MyoD and Myf5. Embo J 22, 5186-5196.
- Roth, S.Y., Denu, J.M., Allis, C.D., 2001. Histone acetyltransferases. Annu Rev Biochem 70, 81-120.
- Rudnicki, M.A., Braun, T., Hinuma, S., Jaenisch, R., 1992. Inactivation of MyoD in mice leads to up-regulation of the myogenic HLH gene Myf-5 and results in apparently normal muscle development. Cell 71, 383-390.
- Rudnicki, M.A., Schnegelsberg, P.N., Stead, R.H., Braun, T., Arnold, H.H., Jaenisch, R., 1993. MyoD or Myf-5 is required for the formation of skeletal muscle. Cell 75, 1351-1359.
- Sanyal, A., Lajoie, B.R., Jain, G., Dekker, J., 2012. The long-range interaction landscape of gene promoters. Nature 489, 109-113.

- Sartorelli, V., Huang, J., Hamamori, Y., Kedes, L., 1997. Molecular mechanisms of myogenic coactivation by p300: direct interaction with the activation domain of MyoD and with the MADS box of MEF2C. Mol Cell Biol 17, 1010-1026.
- Sartorelli, V., Puri, P.L., Hamamori, Y., Ogryzko, V., Chung, G., Nakatani, Y., Wang, J.Y., Kedes, L., 1999. Acetylation of MyoD directed by PCAF is necessary for the execution of the muscle program. Mol Cell 4, 725-734.
- Sassoon, D., Lyons, G., Wright, W.E., Lin, V., Lassar, A., Weintraub, H., Buckingham, M., 1989. Expression of two myogenic regulatory factors myogenin and MyoD1 during mouse embryogenesis. Nature 341, 303-307.
- Schaukowitch, K., Joo, J.Y., Liu, X., Watts, J.K., Martinez, C., Kim, T.K., 2014. Enhancer RNA facilitates NELF release from immediate early genes. Mol Cell 56, 29-42.
- Schmitges, F.W., Prusty, A.B., Faty, M., Stutzer, A., Lingaraju, G.M., Aiwazian, J., Sack, R., Hess, D., Li, L., Zhou, S., Bunker, R.D., Wirth, U., Bouwmeester, T., Bauer, A., Ly-Hartig, N., Zhao, K., Chan, H., Gu, J., Gut, H., Fischle, W., Muller, J., Thoma, N.H., 2011. Histone methylation by PRC2 is inhibited by active chromatin marks. Mol Cell 42, 330-341.
- Seale, P., Sabourin, L.A., Girgis-Gabardo, A., Mansouri, A., Gruss, P., Rudnicki, M.A., 2000. Pax7 is required for the specification of myogenic satellite cells. Cell 102, 777-786.
- Shi, Y., Lan, F., Matson, C., Mulligan, P., Whetstine, J.R., Cole, P.A., Casero, R.A., 2004. Histone demethylation mediated by the nuclear amine oxidase homolog LSD1. Cell 119, 941-953.
- Shi, Y.J., Matson, C., Lan, F., Iwase, S., Baba, T., Shi, Y., 2005. Regulation of LSD1 histone demethylase activity by its associated factors. Mol Cell 19, 857-864.
- Singh, K., Dilworth, F.J., 2013. Differential modulation of cell cycle progression distinguishes members of the myogenic regulatory factor family of transcription factors. Febs J 280, 3991-4003.
- Song, Y.J., Lee, H., 2010. YB1/p32, a nuclear Y-box binding protein 1, is a novel regulator of myoblast differentiation that interacts with Msx1 homeoprotein. Exp Cell Res 316, 517-529.
- Strahl, B.D., Allis, C.D., 2000. The language of covalent histone modifications. Nature 403, 41-45.

- Strahl, B.D., Ohba, R., Cook, R.G., Allis, C.D., 1999. Methylation of histone H3 at lysine 4 is highly conserved and correlates with transcriptionally active nuclei in Tetrahymena. Proc Natl Acad Sci U S A 96, 14967-14972.
- Sun, G., Alzayady, K., Stewart, R., Ye, P., Yang, S., Li, W., Shi, Y., 2010. Histone demethylase LSD1 regulates neural stem cell proliferation. Mol Cell Biol 30, 1997-2005.
- Tajbakhsh, S., 2009. Skeletal muscle stem cells in developmental versus regenerative myogenesis. J Intern Med 266, 372-389.
- Takami, Y., Nakayama, T., 2000. N-terminal region, C-terminal region, nuclear export signal, and deacetylation activity of histone deacetylase-3 are essential for the viability of the DT40 chicken B cell line. J Biol Chem 275, 16191-16201.
- Tapscott, S.J., 2005. The circuitry of a master switch: Myod and the regulation of skeletal muscle gene transcription. Development 132, 2685-2695.
- Thiagalingam, S., Cheng, K.H., Lee, H.J., Mineva, N., Thiagalingam, A., Ponte, J.F., 2003. Histone deacetylases: unique players in shaping the epigenetic histone code. Ann N Y Acad Sci 983, 84-100.
- Tochio, N., Umehara, T., Koshiba, S., Inoue, M., Yabuki, T., Aoki, M., Seki, E., Watanabe, S., Tomo, Y., Hanada, M., Ikari, M., Sato, M., Terada, T., Nagase, T., Ohara, O., Shirouzu, M., Tanaka, A., Kigawa, T., Yokoyama, S., 2006. Solution structure of the SWIRM domain of human histone demethylase LSD1. Structure 14, 457-468.
- Trewick, S.C., Henshaw, T.F., Hausinger, R.P., Lindahl, T., Sedgwick, B., 2002. Oxidative demethylation by Escherichia coli AlkB directly reverts DNA base damage. Nature 419, 174-178.
- Wang, D.Z., Valdez, M.R., McAnally, J., Richardson, J., Olson, E.N., 2001. The Mef2c gene is a direct transcriptional target of myogenic bHLH and MEF2 proteins during skeletal muscle development. Development 128, 4623-4633.
- Wang, J., Lu, F., Ren, Q., Sun, H., Xu, Z., Lan, R., Liu, Y., Ward, D., Quan, J., Ye, T., Zhang, H., 2011a. Novel histone demethylase LSD1 inhibitors selectively target cancer cells with pluripotent stem cell properties. Cancer Res 71, 7238-7249.
- Wang, K.C., Yang, Y.W., Liu, B., Sanyal, A., Corces-Zimmerman, R., Chen, Y., Lajoie, B.R., Protacio, A., Flynn, R.A., Gupta, R.A., Wysocka, J., Lei, M., Dekker, J.,

Helms, J.A., Chang, H.Y., 2011b. A long noncoding RNA maintains active chromatin to coordinate homeotic gene expression. Nature 472, 120-124.

- Whyte, W.A., Bilodeau, S., Orlando, D.A., Hoke, H.A., Frampton, G.M., Foster, C.T., Cowley, S.M., Young, R.A., 2012. Enhancer decommissioning by LSD1 during embryonic stem cell differentiation. Nature 482, 221-225.
- Wilson, E.M., Rotwein, P., 2006. Control of MyoD function during initiation of muscle differentiation by an autocrine signaling pathway activated by insulin-like growth factor-II. J Biol Chem 281, 29962-29971.
- Yamane, K., Toumazou, C., Tsukada, Y., Erdjument-Bromage, H., Tempst, P., Wong, J., Zhang, Y., 2006. JHDM2A, a JmjC-containing H3K9 demethylase, facilitates transcription activation by androgen receptor. Cell 125, 483-495.
- Yang, J.H., Song, Y., Seol, J.H., Park, J.Y., Yang, Y.J., Han, J.W., Youn, H.D., Cho, E.J., 2011. Myogenic transcriptional activation of MyoD mediated by replication-independent histone deposition. Proc Natl Acad Sci U S A 108, 85-90.
- Yang, M., Gocke, C.B., Luo, X., Borek, D., Tomchick, D.R., Machius, M., Otwinowski, Z., Yu, H., 2006. Structural basis for CoREST-dependent demethylation of nucleosomes by the human LSD1 histone demethylase. Mol Cell 23, 377-387.
- Yee, S.P., Rigby, P.W., 1993. The regulation of myogenin gene expression during the embryonic development of the mouse. Genes Dev 7, 1277-1289.
- Yin, H., Price, F., Rudnicki, M.A., 2013. Satellite cells and the muscle stem cell niche. Physiol Rev 93, 23-67.
- Yuan, W., Condorelli, G., Caruso, M., Felsani, A., Giordano, A., 1996. Human p300 protein is a coactivator for the transcription factor MyoD. J Biol Chem 271, 9009-9013.
- Zhang, H.S., Gavin, M., Dahiya, A., Postigo, A.A., Ma, D., Luo, R.X., Harbour, J.W., Dean, D.C., 2000. Exit from G1 and S phase of the cell cycle is regulated by repressor complexes containing HDAC-Rb-hSWI/SNF and Rb-hSWI/SNF. Cell 101, 79-89.
- Zheng, C., Hayes, J.J., 2003. Structures and interactions of the core histone tail domains. Biopolymers 68, 539-546.
- Zhu, D., Holz, S., Metzger, E., Pavlovic, M., Jandausch, A., Jilg, C., Galgoczy, P., Herz, C., Moser, M., Metzger, D., Gunther, T., Arnold, S.J., Schule, R., 2014.

Lysine-specific demethylase 1 regulates differentiation onset and migration of trophoblast stem cells. Nat Commun 5, 3174.

RINGRAZIAMENTI

Finalmente….. il grande giorno è arrivato…..

Vorrei ringraziare Michele che mi ha dato la possibilità di continuare e portare a termine questo percorso, con sostegno e umanità che non avevo mai visto in nessun capo di laboratorio…..se fossero tutti come te!!!! Non smetterò mai di dirlo ma GRAZIE ancora per avermi presentato Laurent.

Speaking of Laurent….Thank you to have welcomed me in your amazing team, which has allowed me to enjoy again the lab and do science.

A special thank to Evelyne and Veronique, my "gurus", to have been there not only for job reason.

Grazie a Francesca…..senza di te non avrei saputo cosa fare!!!!

E come sempre grazie a tutte quelle persone che mi fanno costantemente capire come non vorrò MAI diventare!

Grazie a tutti gli amici e alla mia famiglia che mi ha sostenuto e continua a sostenermi…..non vi preoccupate prima o poi la smetterò…..

The last but not the least …..

Grazie Samu, hai vissuto intensamente con me questi ultimi tre anni.
Mi hai aiutato a coronare un sogno, anche se questo ci ha tenuto lontani per più di due anni…..e l'hai fatto senza battere ciglio….almeno non con me… ☺
Dopo questo sono sicura che niente può spaventarci!

THANKS!!!!!!!