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Epigenetic regulation of skeletal muscle differentiation

Régulation épigénétique de la différenciation du muscle squelettique

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Summary

LSD1 and PHF2 are lysine de-methylases that can de-methylate both histone proteins, influencing gene expression and non-histone proteins, affecting their activity or stability. Functional approaches using *Lsd1* or Phf2 inactivation in mouse have demonstrated the involvement of these enzymes in the engagement of progenitor cells into differentiation.

One of the best-characterized examples of how progenitor cells multiply and differentiate to form functional organ is myogenesis. It is initiated by the specific timing expression of the specific regulatory genes; among these factors, MYOD is a key regulator of the engagement into differentiation of muscle progenitor cells. Although the action of MYOD during muscle differentiation has been extensively studied, still little is known about the chromatin remodeling events associated with the activation of *MyoD* expression. Among the regulatory regions of *MyoD* expression, the Core Enhancer region (CE), which transcribes for a non-coding enhancer RNA (CEeRNA), has been demonstrated to control the initiation of *MyoD* expression during myoblast commitment.

We identified LSD1 and PHF2 as key activators of the *MyoD* CE. *In vitro* and *in vivo* ablation of LSD1 or inhibition of LSD1 enzymatic activity impaired the recruitment of RNA PolII on the CE, resulting in a failed expression of the CEeRNA. According to our results, forced expression of the CEeRNA efficiently rescue *MyoD* expression and myoblast fusion in the absence of LSD1. Moreover PHF2 interacts with LSD1 regulating its protein stability. Indeed *in vitro* ablation of PHF2 results in a massive LSD1 degradation and thus absence of CEeRNA expression. However, all the histone modifications occurring on the CE region upon activation cannot be directly attributed to LSD1 or PHF2 enzymatic activity.

These results raise the question of the identity of LSD1 and PHF2 partners, which co-participate to CEeRNA expression and thus to the engagement of myoblast cells into differentiation.

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 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 (**Figure 1**). 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).

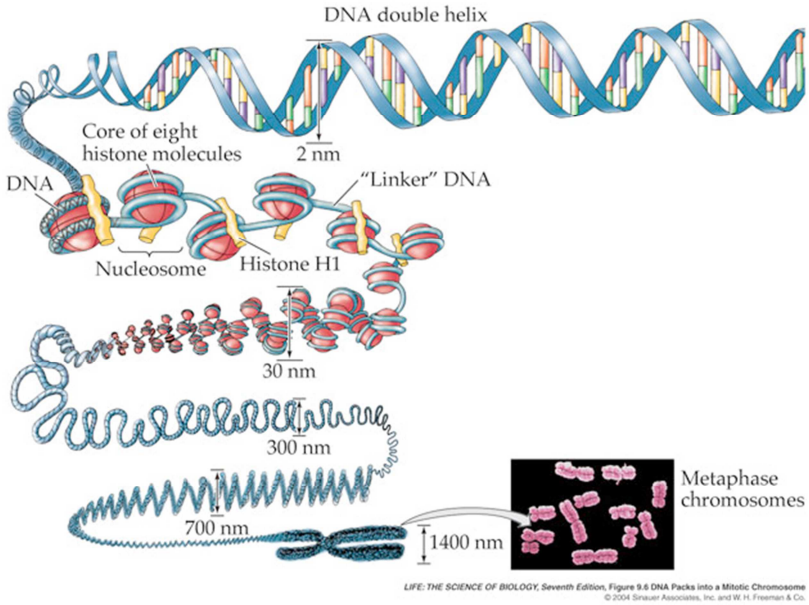


Figure 1: Chromatin structure

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 (**Figure 1**). 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, DNA repair and 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 acetyltransferases (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 generally 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 proteins. 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 mostly 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 H3K4 mono-methylated (H3K4me1) is a chromatin signature for enhancers. Indeed, enhancers are distinguished by robust levels of H3K4me1 and H3K27 acetylation (H3K27ac), as well as recruitment of RNA polymerase II (Pol II) and the histone acetyl-transferase, p300. However, despite extensive studies related to H3K4me1 at enhancers, a clear function for this mark has not yet emerged. H3K36 di- or tri-methylated have been correlated to transcriptional elongation (Kizer et al., 2005; Krogan et al., 2003; Strahl et al., 1999). Nevertheless, loss of the H3K36 methyltransferase Set2 has only minor effects on transcription (Kizer et al., 2005) suggesting that such histone modification may function as regulatory module.

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-containing **H**istone **D**e**M**ethylase). Subsequently it has been shown that JHDM enzymes form a large and evolutionarily conserved histone demethylase family (**Figure 2**).

Gene names	Histone targets
KDM2A/FBXL11/JHDM1A	H3K36me1-2
KDM2B/FBXL10/JHDM1B	H3K36me1-2; H3K4me3
KDM3A/JMJD1A/JHDM2A/TSGA	H3K9me1-2
KDM3B/JMJD1B/JHDM2B	H3K9me1
JMJD1C	H3K9me1-2
KDM4A/JMJD2A/JHDM3A	H3K9me2-3;H3K36me2-3; H1.4K26me2-3
KDM4B/JMJD2B/JHDM3B	H3K9me2-3; H3K36me2-3; H1.4K26me2-3
KDM4C/JMJD2C/JHDM3C/GASC1	H3K9me2-3; H3K36me2-3; H1.4K26me2-3
KDM4D/JMJD2D/JHDM3D	H3K9me2-3; H3K36me2-3; H1.4K26me2-3
KDM4E/JMJD2E	H3K9me2-3
KDM5A/JARID1A/RBP2	H3K4me2-3
KDM5B/JARID1B/PLU1	H3K4me2-3
KDM5C/JARID1C/SMCX	H3K4me2-3
KDM5D/JARID1D/SMCY	H3K4me2-3
KDM6A/UTX/MGC141941	H3K27me2-3
KDM6B/JMJD3/KIAA0346	H3K27me2-3
KDM7A/KIAA1718/JHDM1D	H3K9me1-2; H3K27me1-2; H4K20me1
KDM7B/PHF8/JHDM1F/KIAA1111/ZNF422	H3K9me1-2; H4K20me1
KDM7C/PHF2/JHDM1E/CENP35/GRC5	H3K9me1-2; H3K27me1-2; H4K20me3
KDM8/JMJD5	H3K36me2
MINA/MINA53/NO52	H3K9me3
NO66/C14orf169	H3K4me2-3; H3K36me2-3
TYW5	?
UTY	?
HR/Hairless/HAIR	H3K9me1-2
HSPBAP1/HBAP1	?
HIF1AN/HIF1N/FIH	?
JARID2/JARD2	?
JMJD1C/JHD2C	H3K9me
JMJD4	?
JMJD6/PSR	H3R2; H4R3
JMJD7	?
JMJD8	?

Figure 2: List of mammalian lysine histone-demethylases (Park et al., 2016)

Lysine-specific demethylase-1 (LSD1/KDM1A)

LSD1/KDM1A structure

Lysine (k)-specific histone demethylase (LSD1/KDM1A) is an amino-oxidase, which de-methylates histones through a FAD-dependent reaction (Shi et al., 2004). LSD1 has been identified (Shi et al., 2004) as part of a multiprotein co-repressor 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.

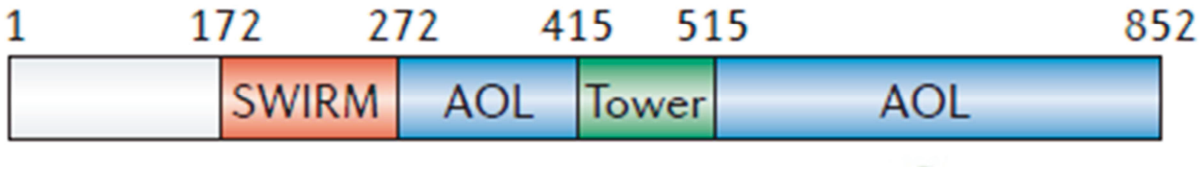


Figure 3: LSD1 structure

The structure of LSD1 contains three domains: the SWIRM, the AOL and Tower domains (**Figure 3**).

- 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 domain from other proteins has been shown to bind DNA and has been proposed to recruit 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 has 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 IIIB) domains have been proposed to be a histone-tail-presenting module (Boyer et al., 2002). Indeed it has been demonstrated that the SANT2 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 allows LSD1 to connect 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, whom it is associated with, it can act as a transcriptional co-repressor or co-activator. Moreover it has been described that LSD1 is able to de-methylate non-histone proteins affecting their activity and stability.

LSD1 as a transcriptional co-repressor

LSD1 is the first histone demethylase identified and it has been originally described as a component of the co-repressor complex, which contained the REST co-repressor (CoREST) and HDAC1/2 (Shi et al., 2005). This transcriptional co-repressor complex is recruited to RE1 element-containing gene promoters by REST and represses 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-methylated lysine 4 of histone 3 (**Figure 4**). 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).

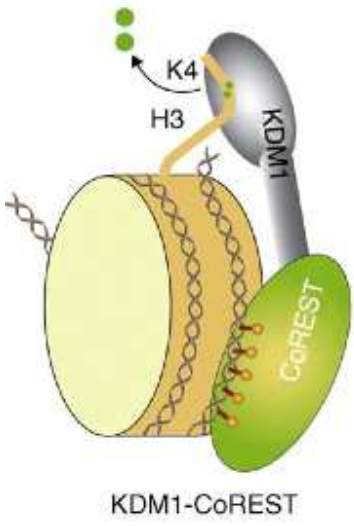


Figure 4: The proposed multivalent interaction between KDM1–CoREST and the nucleosome. Histone H3 tail binds to the active site of KDM1 while CoREST SANT domains bind to DNA.

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). In 2012 Whyte and colleagues by using chromatin

immunoprecipitation sequencing have shown that LSD1 is mostly enriched at enhancer regions of actively transcribed genes in ESCs, where, upon differentiation, it is required to decommission them to allow ESCs proper differentiation (Whyte et al., 2012). Indeed 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). Furthermore LSD1 has been described as a key regulator of brown adipocyte differentiation, where directly repressing some Wnt pathway genes initiates adipogenesis (Chen et al., 2016).
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., 2011). 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 transcriptional 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 a cohort of the REST-dependent programs, these findings have also revealed that LSD1, associated with other complexes is required for a surprisingly wide

range of regulated gene activation events. While an *in vitro* demethylase assay has 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 activation of cells 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 5**).

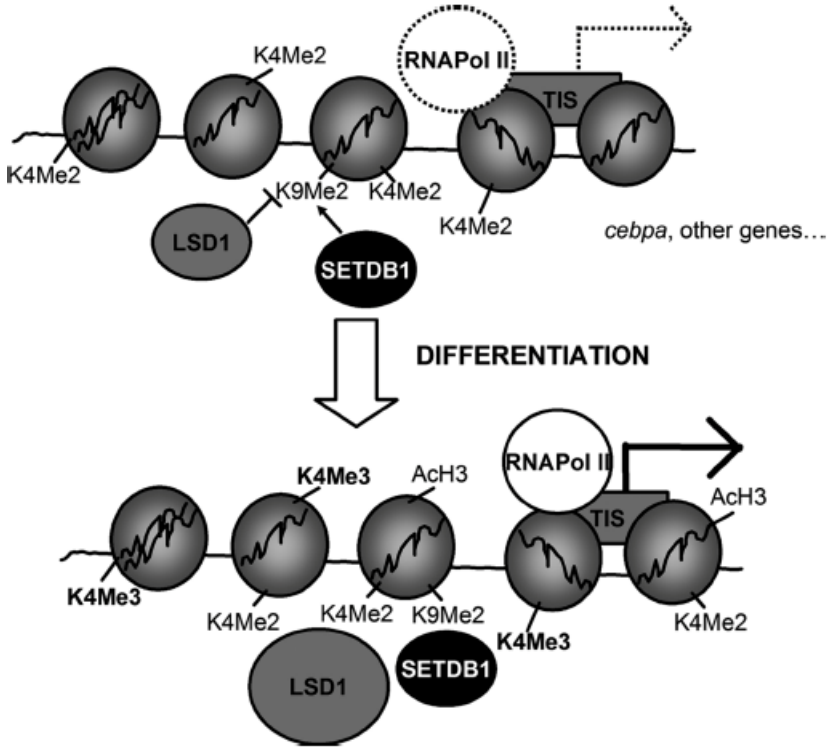


Figure 5: Model showing the interplay between LSD1 and SETDB1 the regulation of the histone methylation status of the cebpa promoter in 3T3-L1 preadipocytes (Musri et al., 2010).

Moreover it has been proposed a role for LSD1 in the skeletal muscle differentiation. Indeed it has been reported that LSD1 has a key role in the early step of myoblast differentiation modulating *MyoD* expression (Scionti et al., 2017) as well as a key role during late myogenesis regulating *Myogenin* promoter (Choi et al., 2010).

LSD1 de-methylates non-histone proteins

Since the initial characterization of LSD1, many evidences have shown that histones are not the only targets of LSD1, opening new lines of research on how LSD1 regulates gene expression.

The first LSD1 non-histone target described was P53 (Huang et al., 2007). P53, like histone proteins, is subject to different post-translational modifications, including acetylation, phosphorylation, methylation, sumoylation and ubiquitination (Bode and Dong, 2004; Huang et al., 2007). While P53 acetylation is commonly associated with activation of its transcriptional activity, methylation can either activates or represses the transcriptional activity depending on the residue involved and on the number of methyl group present. Indeed while di-methylation of P53 at lysine 370 promotes its binding to 53BP1, activating the pro-apoptotic function of P53, LSD1 specifically de-methylating di-methylated lysine 370 of P53, generates mono-methylated lysine 370 which cannot interact with 53BP1 anymore thus repressing P53 transcriptional activity (Huang et al., 2007).

In addition it has been demonstrated that Hypoxia-inducible factor-1α (HIF-1α), which is a key transcriptional regulator responsible for the adaptation of cells and tissues during hypoxia, is finely regulated by LSD1. Indeed LSD1 demethylating HIF-1α stabilizes it and allowed the expression of adaptation to low oxygen genes (Baek and Kim, 2016).

Moreover LSD1 has been shown to influence the global DNA methylation, stabilizing the DNMT1 protein. By using an *in vitro* approach it has been demonstrated that DNMT1, which is methylated by SET7/9 and degraded, upon LSD1 de-methylation is stabilized. Such observation has suggested that LSD1 could regulate gene transcription acting both on histones, on transcription factors and DNA methylation (Wang et al., 2009).

PHD finger protein 2 (PHF2/KDM7C)

PHF2/KDM7C structure

PHD Finger Protein 2 (PHF2/KDM7C) is a **J**mjC domain-containing **H**istone **D**e**M**ethylase, which de-methylates histones using Fe^{2+} and α-ketoglutarate (αKG) as cofactors. PHF2/KDM7C has been firstly identified as a candidate transcriptional regulator gene for hereditary sensory neuropathy type I, *Hsn1* (Hasenpusch-Theil et al., 1999).

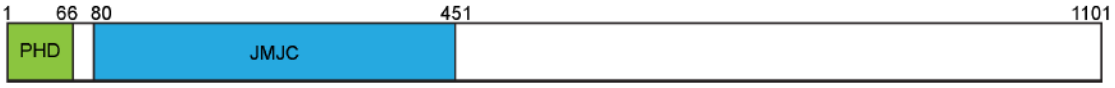


Figure 6: PHF2 structure

The structure of PHF2 contains two main domains: the PHD and the JmjC domains (**Figure 6**).

- Plant HomeoDomain (PHD)

The PHD is an □50-residue module characterized by a conserved Cys4-His-Cys3 motif that coordinates two zinc ions in a cross configuration, where each zinc ion is coordinated by alternate pairs of Cys/His ligands. Proteins containing PHD domain are a subfamily of zinc finger proteins and thus the PHD finger domain is involved in protein/protein or protein/nucleic acid interactions. One of the fundamental mechanisms by which histone methylation regulates chromatin state is to create dynamic signatures at chromatin that are recognized by other proteins named effectors. Proteins containing the PHD domain have been described to strongly bind the H3K4me3 mark and promote both transcription silencing recruiting the histone de-acetylase activity of mSin3a–HDAC1 (Shi et al., 2006) or transcription initiation stabilizing the ATP-dependent chromatin-remodeling complex on promoters regions (Wysocka et al., 2006).

- JmjC domain

The evidence that the LSD1 family de-methylases were unable to de-methylate tri-methyl-lysine residues, raises the possibility that additional de-methylases that use a different reaction mechanism exist. The JmjC domain was first defined based on the amino-acid

similarities in the Jarid2 (Jumonji), Jarid1C (Smcx), and Jarid1A (RBP2) proteins (Clissold and Ponting, 2001; Takeuchi et al., 1995). The JmjC domain is structurally conserved, each of the two-layered β-sheets containing four antiparellel β-strands that produce the typical jellyroll-like structure. A highly conserved His-Y-ASP/GLU-Yn-His motif (Y: any amino-acid; Yn: various number of amino-acid in between) provides three chelating positions for the Fe^{2+}. αKG interacts with the iron and is further stabilized by the interaction with two additional conserved residues (Phe/Thr/Tyr for the first and Lys for the second amino acid). To catalyze histone de-methylation, the cofactor-bound JmjC domain produces a highly reactive oxoferryl species that hydroxylates the methylated substrate, allowing spontaneous loss of the methyl group as formaldehyde (Clifton et al., 2006).
However, the Fe^{2+} binding site in PHF2 differ from the other Jumonji domains examined, indeed the histidine at 321 position is replaced by tyrosine making the Fe^{2+} move away from the corresponding place and thus reduces the PHF2 de-methylase capacity (Horton et al., 2011).

PHF2 is ubiquitously expressed in adult tissues, and *in situ* hybridization has shown that the majority of *PHF2* gene expression is concentrated in the mouse embryonic neural tube and root ganglia (Hasenpusch-Theil et al., 1999). The expression pattern and chromosomal localization have suggested PHF2 as a candidate gene for hereditary sensory neuropathy type 1, HSN1 (Hasenbusch-Theil et al. 1999). However, mutations in *PHF2* has been identified in different malignancies including gastric, colorectal cancer (Lee et al., 2017b) and breast cancer (Sinha et al. 2008). Furthermore deregulation of *PHF2* gene has been found to negatively correlate with the overall survival of esophageal squamous cell carcinoma patients (Sun et al., 2013) and clear cell renal cell carcinoma patients (Lee et al., 2017a). However the PHF2 function is still not yet dissected.

PHF2 as a transcriptional co-activator

PHF2 has no enzymatic activity by itself (Horton et al., 2011; Wen et al., 2010) but it is functionally activated through post-translational phosphorylation by PKA. PHF2 active is able to assembly with and demethylates ARID5B; this last modification allows PHF2-ARID5B complex to bind to the DNA and activate gene transcription through de-methylation of H3K9me2 (Baba et al., 2011). Two years after it has been demonstrated

that the PHF2-ARID5B complex is a transcriptional SOX9 co-activator and has a key role in promoting chondrocyte differentiation both *in vitro* and *in vivo* (Hata et al., 2013). PHF2 has also been shown to interact with C/EBPδ or C/EBPα and promote the transcription of *PPARG*, *CEBPA* and *FABP4* supporting the hypothesis that PHF2 controls adipogenesis and it would be a interesting candidate for diabetes treatment (Lee et al., 2014; Okuno et al., 2013).

PHF2 de-methylates non histone proteins

As described above post-translational modifications (PTMs) change the chemical nature and structure of aminoacids and lead to diversity in the localization, stability, and function of proteins. PHF2, as well as LSD1, is able to demethylate non-histone proteins. In particular, PHF2 has been described to demethylate RUNX2 on its DNA binding site enhancing its transcriptional activity on *osteocalcin (OCN)* promoter, suggesting PHF2 as a positive regulator of osteoblast differentiation (Kim et al., 2014). However, while the transcriptional activation mechanism of ARID5B-PHF2 co-activator complex is a combination between ARID5B protein demethylation that drives the complex to target promoters and H3K9me2 gene promoter demethylation (Baba et al., 2011), knocking down PHF2 in osteoblasts does not change the H3K9me2 rate at the *OCN* promoter during differentiation (Kim et al., 2014).

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. Some 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 7**) and others undergo epithelial-mesenchymal transition, delaminate and migrate to the limb buds, where they give rise to limb musculature (Chevallier et al., 1977; Dietrich et al., 1999).

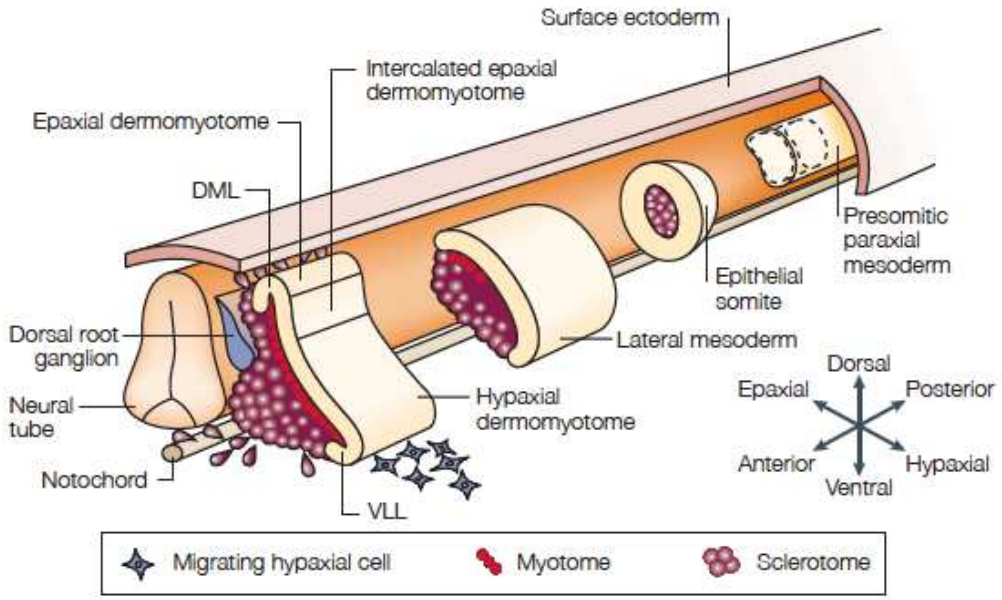


Figure 7: The embryonic origin of limb and trunk skeletal muscle (Parker et al., 2003)

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 stem cells named satellite cells are located (**Figure 8**).

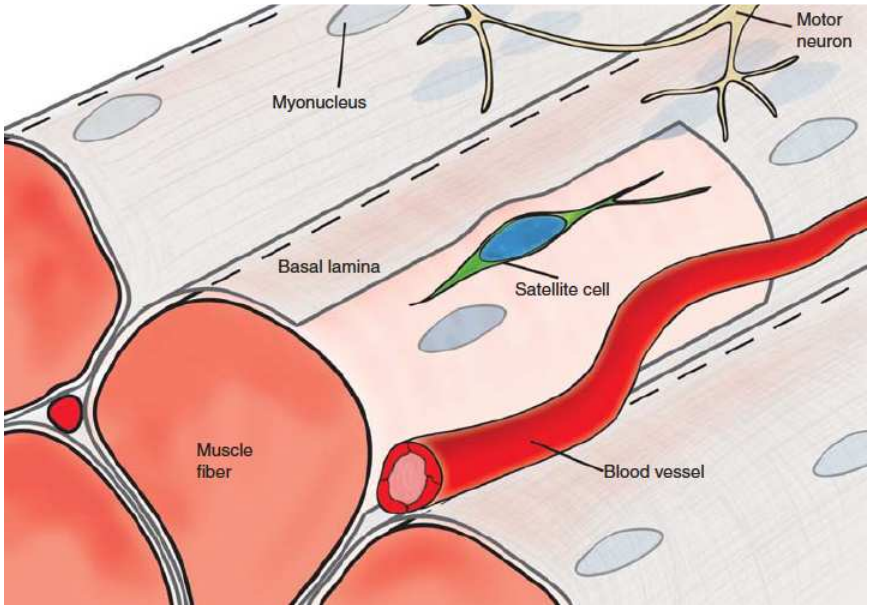


Figure 8: Representation of skeletal muscle and the satellite cell niche (Bentzinger et al., 2012).

Satellite cells normally remain quiescent in adult muscles, but they can be activated upon injury and support 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 gene expression cascade 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*-knock out (KO) mice loose all of their embryonic myofibers (Hutcheson et al., 2009), further supporting the hypothesis that *Pax3* is required for normal skeletal muscle development. In contrast, *Pax7* is dispensable for fetal myogenesis because *Pax7* KO 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* gene in mouse embryos (Hutcheson et al., 2009). *Pax7* lineage deletion resulted in the loss of fetal 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 the second 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 myoblast 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 specific 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 bHLH 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 at embryonic day (E) 8 of mouse embryonic development, and its expression starts to decrease on E11 (Buckingham and Tajbakhsh, 1993). In contrast, *MyoD* expression is initiated 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 one occurs between E9 and E11.5 and the second wave starts at E16 and persists through adulthood (Patapoutian et al., 1995). Genetic studies in mice indicate redundant and differential roles of MRFs during muscle development. Ablation of *Myf5* or *MyoD* in mice results in normal muscle development. *Myf5*-KO 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*-KO mice is fully restored by a MYOD-expressing lineage (Gensch et al., 2008; Haldar et al., 2008). On the other hand, *MyoD* deletion results in prolonged and elevated *Myf5* expression, which functionally leads to normal skeletal musculature (Rudnicki et al., 1992). However adult MYOD-KO muscle have a severe regenerative defect, supporting the idea that MYOD is required for commitment into differentiation program during adulthood (Asakura et al., 2007; Megeney et al., 1996). Interestingly, *Myf5/MyoD* double-KO mice show no skeletal myoblasts or myofibers as well as *Myogenin* expression (Rudnicki et al., 1993). These observations indicate that *Myf5* and *MyoD* have redundant roles in myogenic cell fate determination and myoblast commitment during embryonic development.

Conversely, *Myogenin*-KO 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 to fuse to form myofibers and acts downstream of MYF5 and MYOD.

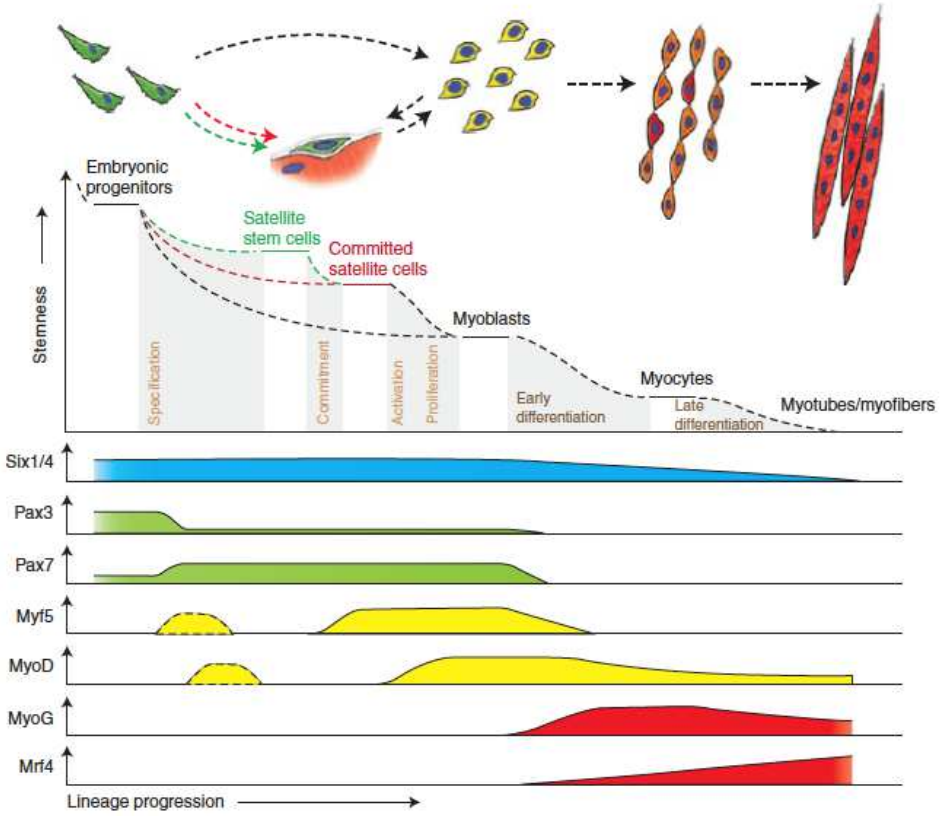


Figure 9: Hierarchy of transcription factors regulating progression through the myogenic lineage (Bentzinger et al., 2012)

MRF4-KO 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-KO results in a severe muscle deficiency that is similar to the *Myogenin*-mutant mice (Lassar et al., 1991) suggesting that MRF4 and MYOD have overlapping functions in myoblast differentiation.

All together 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 commit myoblasts, while Myogenin and MRF4 act genetically downstream to induce myocytes fusion and muscle-specific gene expression (**Figure 9**).

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 as stated above, during myogenesis, MRFs act as heterodimers together with E proteins (E12, E47 and HEB) that belong to the same transcription factor family, and bind to E boxes of 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 the onset of differentiation (Molkentin et al., 1995), allowing the formation of heterodimers MYF5/E protein or MYOD/E protein, and thus myogenic gene specific expression. Full activation of muscle-specific gene expression by MRFs requires their collaboration with myocyte enhancer factor 2 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 DNA consensus sequence for MEF2 is often close to the E-box sequences within muscle genes promoters (Johanson et al., 1999). In addition to activating muscle genes transcription, MEF2 proteins mediate myogenic bHLH gene expression in a positive feedback mechanism. Indeed upstream signals activate MYF5 and MYOD, which cooperate with MEF2 proteins to induce *Myogenin* expression (Cserjesi and Olson, 1991; Yee and Rigby, 1993). Next 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 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* ablation 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.

Interaction of MRFs with chromatin remodeling factors

In proliferating myoblast 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. Thus in order to induce the muscle specific gene expression cascade the coordinated action between MRFs but also between MRFs and chromatin-remodeling enzymes is required. Therefore, chromatin modification and remodeling are required to relax the chromatin and allow the access of transcription factors. Thus several transcriptional co-activators in muscle have been described, including p300, a functional homolog of CREB-binding protein (CBP) and p300/CBP-associated factor (PCAF), which have intrinsic histone acetyltransferase (HAT) activity; conversely, histone deacetylases (HDACs) repress transcription in skeletal muscle (McKinsey et al., 2001). MRFs and MEF2 interact with HATs and HDACs that modulate their transcriptional activity (**Figure 10**).

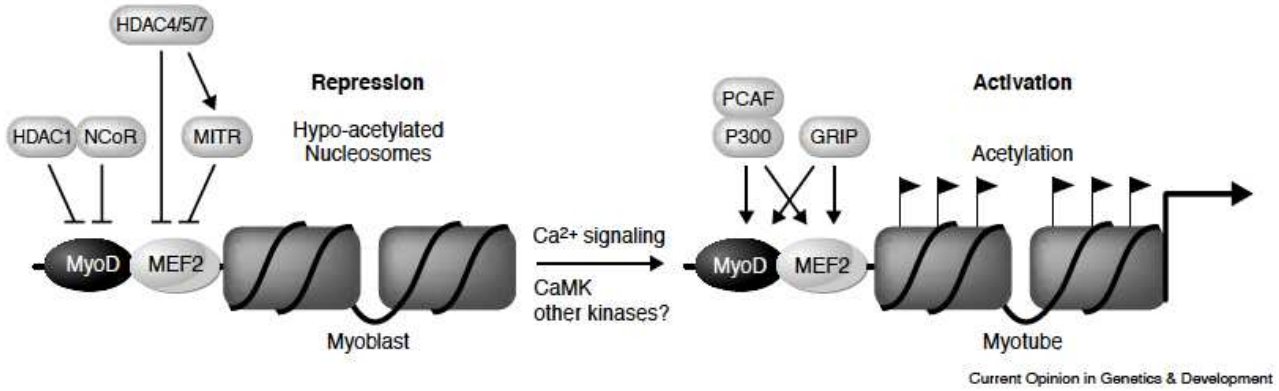


Figure 10: A model for the roles of HATs and HDACs in the control of muscle gene expression (McKinsey et al., 2001).

The 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; Puri et al., 1997a; Puri et al., 1997b; Sartorelli et al., 1997; Sartorelli et al., 1999; Yuan et al., 1996). Moreover, p300 interacts with MEF2 and acetylating it in skeletal muscle, results 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 10**).

In addition to acetylation, chromatin remodeling is also 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). Indeed, 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 11**).

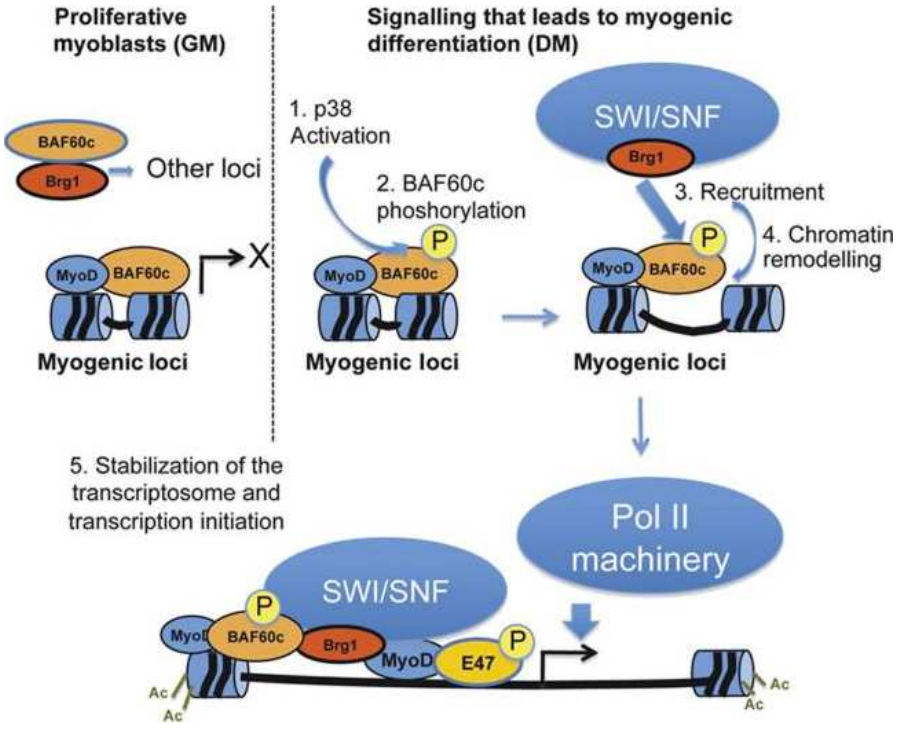


Figure 11: Cartoon of the stepwise recruitment of SWI/SNF to MYOD-target genes by pre-assembled BAF60c–MYOD complex (Forcales et al., 2012).

Until 2010, the involvement of histone methylation in the regulation of myoblast differentiation process was not clearly investigated. The first studies have been conducted

to dissect the chromatin remodeling factors that are involved in the regulation of *Myogenin* expression. Choi and co-workers have demonstrated for the first time that LSD1 demethylates MEF2D increasing its transcriptional activity (Choi et al., 2014). Moreover LSD1 in complex with MYOD/MEF2D is required to erase the H3K9me2 mark from *Myogenin* promoter (Choi et al., 2010). However, in proliferating myoblast the *Myogenin* promoter is strongly inhibited by the presence of the H3K9me3 and H3K27me3 marks, which are not LSD1 targets, thus supporting the idea of the presence of other JMJC-histone demethylases on the *Myogenin* promoter. Indeed, ΔN-KDM4A/KDM4C complex has been described at the *Myogenin* locus where it demethylates H3K9me3 to activate *Myogenin* expression (Verrier et al., 2011). Four years later another JMJC demethylase, KDM4B, has been observed to interact with MYOD and participate to the activation of *Myogenin* transcription*,* demethylating the H3K9me3 (Choi et al., 2015). Moreover, very recently the UTX/KDM6A demethylase together with MYOD and SIX4 has been identified as a key regulator of *Myogenin* expression, both *in vitro* and *in vivo,* erasing the H3K27me3 mark at *Myogenin* promoter during myoblast differentiation (Chakroun et al., 2015; Faralli et al., 2016; Seenundun et al., 2010).

All these reports have supported the idea that *Myogenin* promoter is finely controlled by a large multiproteic complex where each component might be required for the right assembly of the complex itself and/or for the substrate specificity and activity of each enzyme (Cai et al., 2014; Metzger et al., 2010; Metzger et al., 2005; Shi et al., 2005).

MYOD

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 shares two domains 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 α-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 12**).

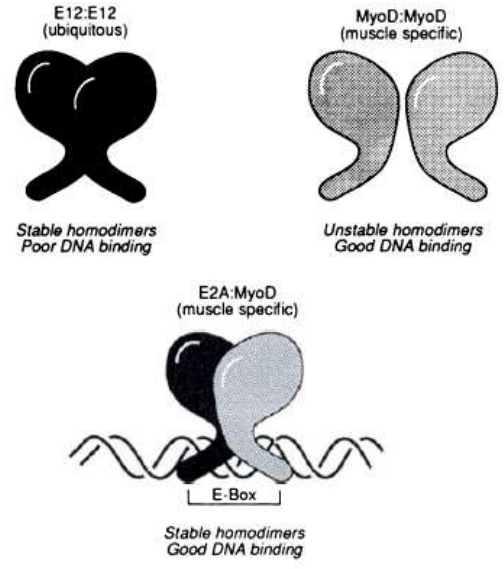


Figure 12: Model of transcriptional regulation by heterodimeric bHLH protein (Kadesch, 1993)

Once hetero-dimerized the basic regions of the two transcription factors bind specific DNA sequences. MYOD has also 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 13**).

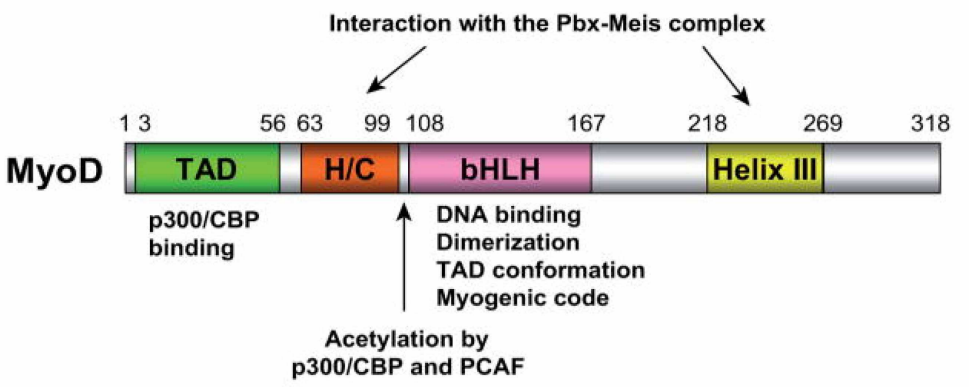


Figure 13: Structure and functional domains of MyoD.

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 gene targets via CHiP data in a fibroblast cell line containing an estrogen induced MyoD allele (Bergstrom et al., 2002). Moreover, MYOD is able to initiate the myogenic differentiation program and subsequently this program is able to regulate the activity of MYOD itself, thus MYOD is able to act 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). Post-translational modification of the MYOD protein has also been shown to affect target gene selection (Di Padova et al., 2007).

Briefly, MYOD heterodimerizes with E-proteins (E12 and E47) to activate myogenic genes through their shared HLH domains. 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 and frequently through out the mammalian genome. Thus 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; Relaix et al., 2013).

Transcriptional control of MyoD expression

Conversely to the abundant knowledge accumulated on how MYOD affects chromatin organization at its target genes promoters during muscle cell differentiation (Bergstrom et al., 2002; Berkes and Tapscott, 2005; de la Serna et al., 2005; Forcales et al., 2012; Sartorelli et al., 1997; Tapscott, 2005), the early epigenetic events involved in the activation of *MyoD* expression still lack an in depth understanding. Moreover, since it has been shown that MyoD-KO myoblasts serve as better transplant material than wild type myoblasts in mice (Asakura et al., 2007), dissect the chromatin remodeling factors, which activate regulatory regions of the powerful transcription factor MYOD, is of ultimate importance in understanding transcriptional pathway of myoblast commitment and it may be applicable in therapy to humans with muscle wasting diseases.

So far most of the 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 more 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). Indeed in *MyoD*/*Myf-5* double-KO embryos 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, is compared to a similar construct, which lacks only the CE, there is only a delay in *MyoD* expression in the brachial arc and limb buds up to E11.5, after which a normal expression profile is restored (Chen and Goldhamer, 2004; Chen et al., 2001) (**Figure 14**). These results suggest that the initial timely activation of *MyoD* is CE dependent in only a subset of early myogenic cells.

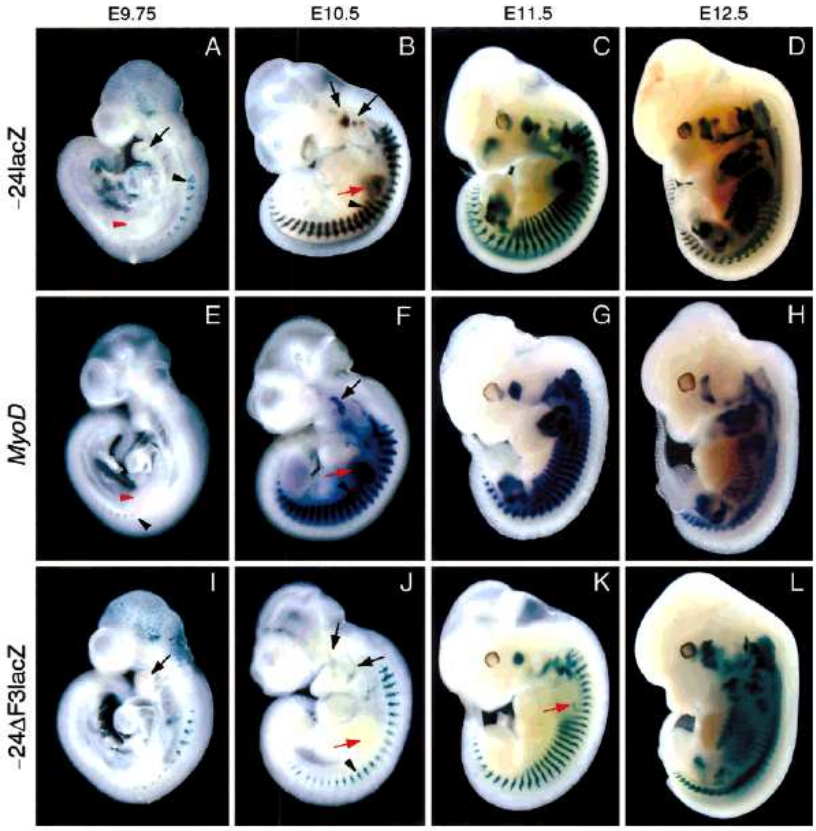


Figure 14: Representative whole mount embryos from E9.75 to E12.5 comparing expression of lacZ in transgenic lines (Chen et al., 2001).

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 MEF2 sites (Chen et al., 2001). The sequence similarities between the CE of humans and mice are 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; L'Honore et al., 2003). 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* promoter in myoblast cultures. FOX03 is further implicated as a direct activator of *MyoD* through *Fox03* KO 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; Zhang et al., 2012). 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). Moreover *in vitro* cell culture analysis shows that the histone variant H3.3, associated with transcriptionally active genes, is required on 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.

Enhancer RNAs

The development, differentiation and growth of cells rely on the specific spatiotemporal regulation of gene expression and the activation and repression of enhancer elements are instrumental to initiate such gene transcription cascade. Indeed disrupting this finely controlled process may lead to abnormal tissue growth and disease.

Until 2010 enhancer regions have been defined as *cis- or trans-acting* DNA sequence bound by specific activator proteins that interacting with mediator complex, were able to recruit RNA polymerase II (RNAPII) on gene promoters thus enhancing the transcription of the target gene. Enhancers may be located several hundred thousand base pairs upstream or downstream of the gene promoter, as well as on another chromosome, since their orientation does not affect its function.

The development of next generation sequencing (NGS) techniques has increased the understanding of the complexity of the human transcriptome. Indeed through a genome-wide analysis many extragenic regions, such as enhancers, have been found enriched by transcription factors and RNA polymerase II (RNApolII), suggesting that enhancers themselves are transcribed (Carroll et al., 2006; Kim et al., 2010; Koch et al., 2011; Nielsen et al., 2008). This new family of non-coding RNAs, named enhancer RNAs (eRNAs), has made the gene transcriptional regulation mechanism more complex.

Enhancer RNAs are non-coding RNA of 50-2000 nucleotide in size transcribed from the DNA sequence of enhancer region. Conversely from a messenger RNA (mRNA) transcription, eRNAs are generally transcribed bi-directionally. However, there is also a group of enhancers that are induced uni-directionally (Hah et al., 2013; Li et al., 2013; Schaukowitch et al., 2014). Moreover while most of eRNAs consisted of one exon and they are not polyadenylated, there have been cases where eRNAs are spliced and polyadenylated (Kim et al., 2010; Koch et al., 2011). However, it is still not clear 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. Thus understanding how eRNAs contribute to the gene regulation mechanism has become an area of active interest since their key role in the cell fate. Indeed it has been demonstrated that super-enhancers, which regulate the transcription of master genes, involved in the establishment of cell identity, transcribed for eRNAs in a tissue and time specific fashion.

Chromatin immunoprecipitation sequencing (ChIP-seq), and chromatin-conformation-capture studies have defined the chromatin signature of transcribed enhancer regions. Indeed such regions are characterized by robust enrichment of H3K4me1 and H3K27ac marks (Creyghton et al., 2010; Heintzman et al., 2007) and are associated with HAT p300 and CREB Binding Protein (CBP) (Shen et al., 2012; Visel et al., 2009). eRNA-expressing enhancers are also enriched with the transcriptional initiation complex and serine 5 phosphorylated RNA PolII, characteristics of coding gene promoter regions (Koch et al., 2011).

Transcription and function of eRNA

To date the exact requirements for eRNAs in activation of their target genes are not completely understood. So far some reports have provided evidence linking the eRNAs to transcriptional activation. Using knockdown approaches, it has been demonstrated that eRNAs positively activate the transcription of master genes and thus have a key role in many biological processes (Lai et al., 2013; Mousavi et al., 2013; Orom et al., 2010).

While these reports have revealed for the first time a role for long non-coding RNAs in the activation of neighboring protein-coding genes expression, they did not assess their specific pathway of activation.

Genome-wide studies have revealed that P53 is associated with a larger number of binding sites than expected (Melo et al., 2013). In particular two distinct extragenic P53 sites transcribed for eRNAs that were stimulated following treatment of cells with Nutlin-3, an inducer of P53. Indeed their knocked down abolished the Nutlin-3 induction of transcription of the P53-target genes (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 that transcribed for eRNAs. These eRNAs are transcribed bi-directionally and their abolishment diminished the E2-induced activation of their target genes. Indeed they demonstrated that in the case of the FOXC1 enhancer, involved in the activation of *FOXC1* gene, only the sense strand of the eRNA has the capacity to enhance the transcription of the gene (Li et al., 2013).

Furthermore genome-binding site and RNA-seq studies during myoblast differentiation have also demonstrated that the master genes, *MyoD* and *Myogenin*, also bind to

extragenic sites inducing the expression of many eRNAs. In particular the knocking down of eRNAs transcribed from the two super-enhancers of *MyoD* gene, the Core enhancer region (CE, CEeRNA) and the distal regulatory region (DRR, DRReRNA), results in the perturbation of the myoblast differentiation, affecting *MyoD* or *Myogenin* expression respectively (Mousavi et al., 2013). However, while these eRNAs are transcribed bi-directionally only one strand is able to activate the target genes expression (Mueller et al., 2015; Scionti et al., 2017).

Conversely the nuclear receptor Rev-Erbs is also binding to enhancers and results 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.

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

Mechanism of eRNAs action

Until now many evidences have suggested that eRNA transcripts *per se* can 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, which are responsible of looping with promoters of protein-coding genes, possess higher expression of eRNAs (Lin et al., 2012; Sanyal et al., 2012). Such studies have suggested a potential role of eRNAs in the process of proper formation of chromosomal looping between enhancers and gene promoter. 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; Kim et al., 2015; 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 15**).

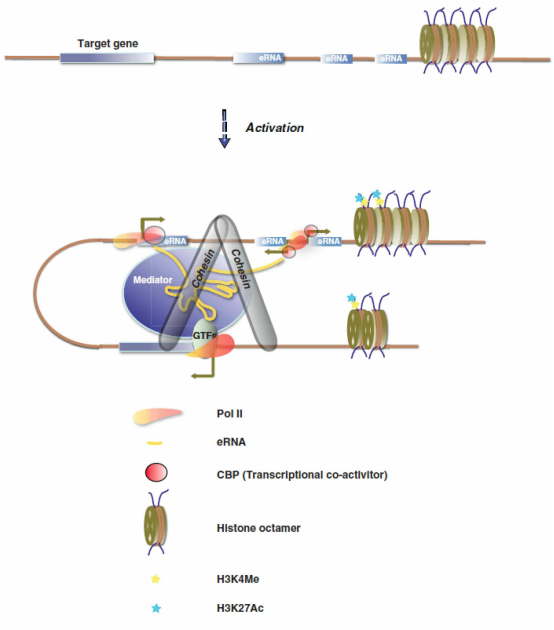


Figure 15: Cartoon of eRNA functional activity *in cis* (Lai and Shiekhattar, 2014).

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 the *CEeRNA* has been demonstrated to act *in cis* improving the RNA PolII recruitment on the *MyoD* promoter at the onset of myoblast differentiation (Mousavi et al., 2013; Scionti et al., 2017). Thus, absence of *CEeRNA* decreased RNA PolII recruitment at the promoter and gene body of MyoD. Furthermore it has also demonstrated that *Arc* eRNA facilitates the dissociation of NELF complex from paused RNA PolII which can be phosphorylated and thus able to transcribe (Schaukowitch et al., 2014)(**Figure 16**).

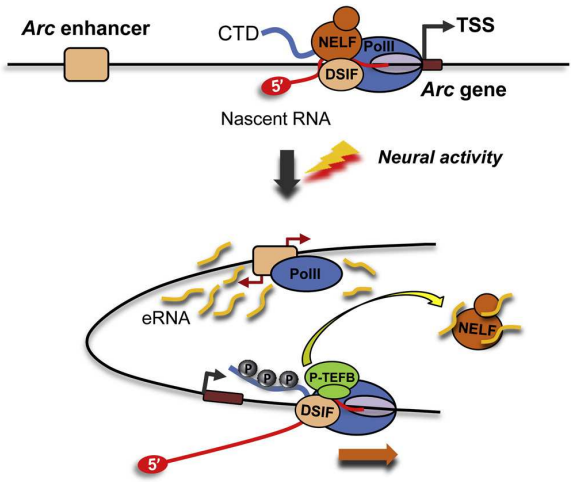


Figure 16: Schematic model of *Arc* eRNA action upon neural activity (Schaukowitch et al., 2014)

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 that the intergenic region Dlx-5/6 transcribes for an eRNA *Evf-2* which increases the transcriptional activity of the DLX2 protein, which in turn enhance the activity of the Dlx-5/6 enhancer region (Feng et al., 2006). Moreover very recently knocking down the *DRReRNAs* 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 17**).

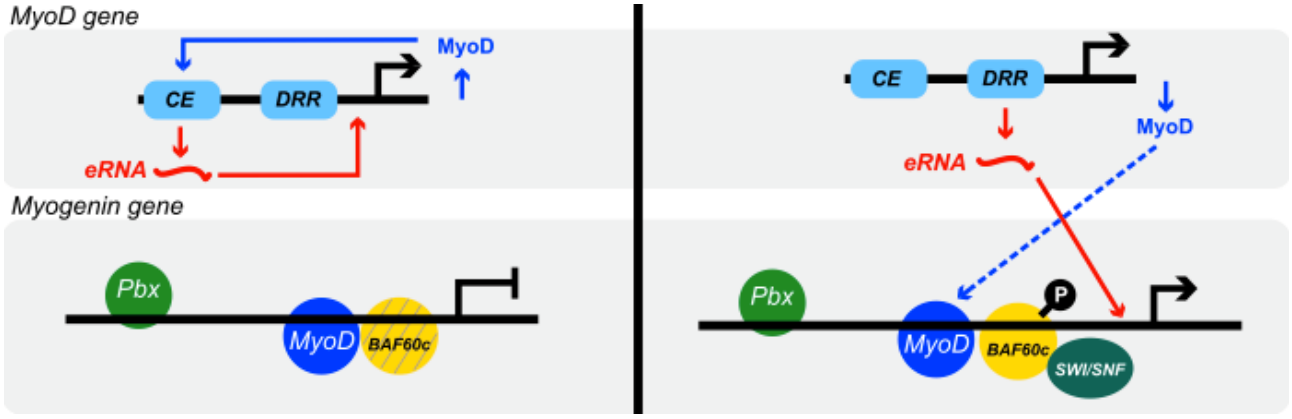


Figure 17: Schematic model of eRNAs mechanism of action for the transcriptional regulation of *MyoD* and *Myogenin* genes (adjusted from (Buckingham and Rigby, 2014)).

Results

LSD1 and PHF2 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, RT-PCR approach has been used to compare the expression of histone demethylases in innervated and denervated muscle and the expression of *Lsd1* and *Phf2* came out increased in denervated muscle (**Figure 18A**). This result was confirmed at protein level by western blot analysis (**Figure 18B**).

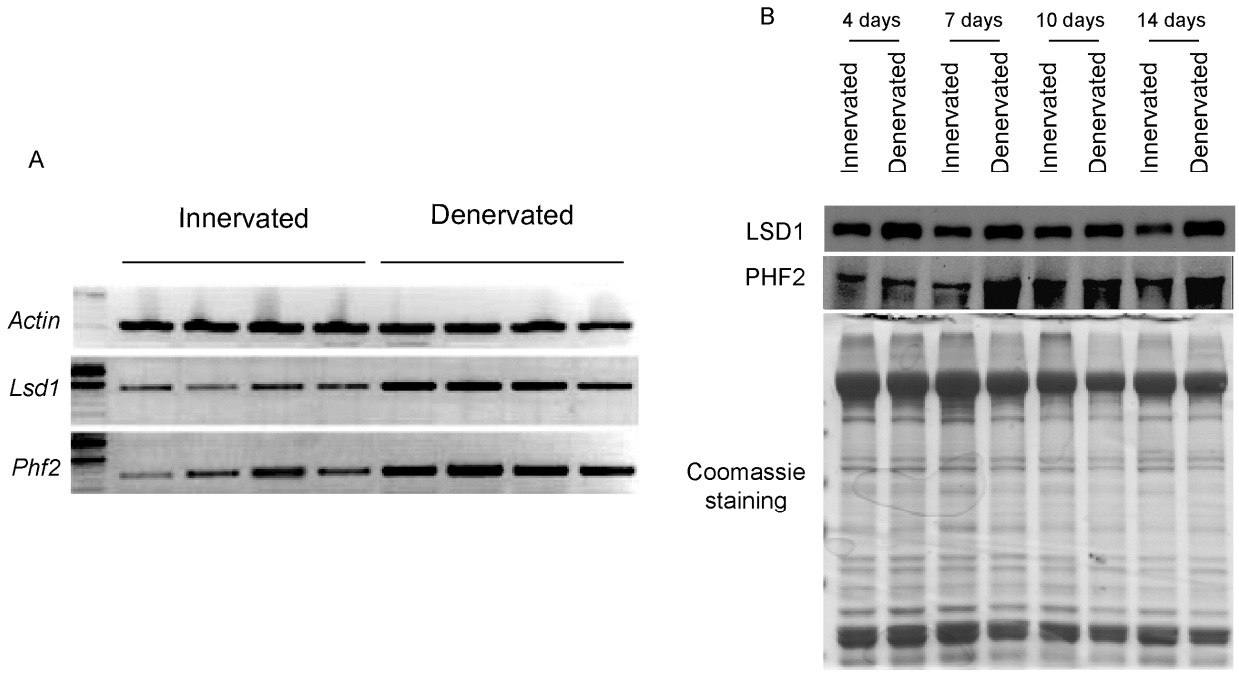


Figure 18: A) *Lsd1* and *Phf2* expression evaluated by RT-PCR on 4 innervated and denervated (48h) tibialis anterior muscles. **B)** Kinetics of total LSD1 and PHF2 protein expression after denervation at tibialis anterior muscle at different time-point.

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 and PHF2 in skeletal muscle differentiation.

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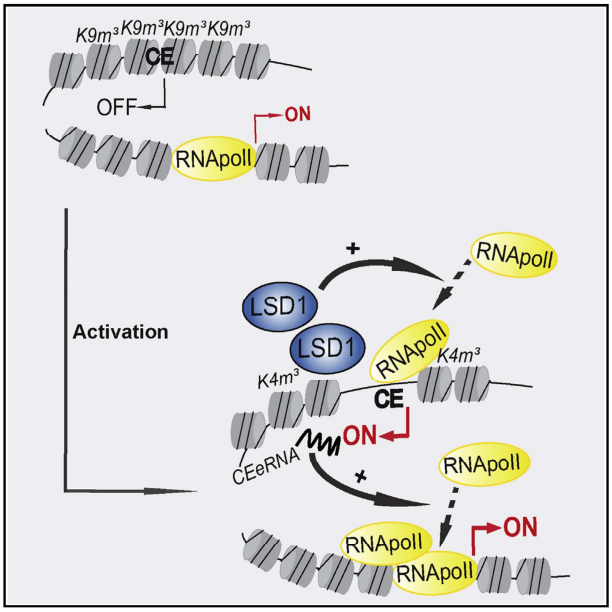
LSD1 Controls Timely MyoD Expression via MyoD Core Enhancer Transcription.

Cell Reports

Article

LSD1 Controls Timely *MyoD* Expression via MyoD Core Enhancer Transcription

Graphical Abstract



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In Brief

Scionti et al. show that LSD1 is recruited on the MyoD core enhancer, where it promotes the transcription of an enhancer RNA that controls the timing of MyoD expression during myoblast commitment. This provides the first evidence that LSD1 is required for the transcription of enhancer RNAs from a pro-differentiation enhancer.

Highlights

- LSD1 participates in enhancer function by promoting eRNA transcription
- LSD1 contributes to activate *MyoD* during commitment of muscle cells
- LSD1 is recruited on the MyoD core enhancer (CE) during muscle differentiation
- LSD1 activates the transcription of the *MyoD* core enhancer eRNA

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LSD1 controls the timely *MyoD* expression via MyoD Core Enhancer transcription

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Summary

MyoD is a master regulator of myogenesis. Chromatin modifications required to trigger *MyoD* expression are still poorly described. Here we demonstrate that the histone demethylase LSD1/KDM1a is recruited on the *MyoD* core enhancer upon muscle differentiation. Depletion of *Lsd1* in myoblasts precludes the removal of H3K9 methylation and the recruitment of RNA polymerase II on the core enhancer, thereby preventing transcription of the non-coding enhancer RNA required for *MyoD* expression (CEeRNA). Consistently, *Lsd1* conditional inactivation in muscle progenitor cells during embryogenesis prevented transcription of the CEeRNA and delayed *MyoD* expression. Our results demonstrate that LSD1 is required for the timely expression of *MyoD* in limb buds and identify a new biological function for LSD1 by showing that it can activate RNA polymerase II-dependent transcription of enhancers.

Keywords: *MyoD*, LSD1, Enhancer RNA, Chromatin modifying enzyme.

Introduction

During development, somatic progenitor cells engage into differentiation to form organs. In adult tissues, stem cells, which have self-renewal capacities, differentiate to maintain tissue homeostasis or to repair damages. The balance between self-renewal and differentiation has to be tightly controlled to allow adequate development and prevent aberrant growth of tissues. The switch between self-renewal and differentiation states is associated with profound changes in gene expression and global genomic rearrangements. Activation and repression of enhancer elements embedded in chromatin are instrumental to orchestrate these changes. Extensive studies on the role of chromatin modifications in the regulation of cell stemness and differentiation have demonstrated the importance of histone modifications and enzymes involved in the control of lysine methylation have particularly emerged as key regulators of cell fate (Agger et al., 2007; Amente et al., 2013; Pereira et al., 2010; Rajasekhar and Begemann, 2007; Zylicz et al., 2015).

Lysine specific demethylase 1 (LSD1, AOF2, KDM1A) is a monoamine oxidase that can de-methylate mono- and di-methylated lysine 4 and 9 residues of the N-terminal of histone H3 (H3K4Me1, H3K4Me2 and H3K9Me1, H3K9Me2), thus promoting either transcriptional repression or activation (Metzger et al., 2005; Mulligan et al., 2011; Shi et al., 2004; Yang et al., 2006). Whole genome distribution studies have shown that in stem cells, LSD1 preferentially localizes at enhancers, where it represses the enhancers involved in stemness maintenance at the onset of differentiation (Whyte et al., 2012). Functional approaches using *Lsd1* inactivation in mouse have also demonstrated the involvement of LSD1 in the engagement of progenitor cells into differentiation (Wang et al., 2007). The requirement of LSD1 for differentiation of progenitor cells can be explained by the need to decommission stemness enhancers to allow differentiation (Whyte et al., 2012). One of the best-characterized examples of how progenitor cells multiply and differentiate to form functional organs is myogenesis. The complex signaling and transcriptional cascades that control the specific timing expression of muscle-specific regulatory genes have been extensively studied. Among these factors, MYOD is a key regulator of the engagement into differentiation of muscle progenitor cells (Conerly et al., 2016; Tapscott et al., 1988). Conversely to the abundant knowledge accumulated on how MYOD affects chromatin organization to promote muscle cell differentiation (Bergstrom et al., 2002; Berkes and Tapscott, 2005; de la Serna et al., 2005; Forcales et al., 2012; Sartorelli et al., 1997; Tapscott, 2005), the chromatin changes on the *MyoD* promoter that trigger *MyoD*

expression, still lack an in depth understanding. Among the regulatory regions of *MyoD*, the Core Enhancer region (CE), located about 25 kb upstream the MyoD promoter, has been demonstrated to control the initiation of *MyoD* expression during myoblast commitment (Asakura et al., 1995; Chen and Goldhamer, 2004; Chen et al., 2001; Goldhamer et al., 1995). Recent findings regarding the transcriptional initiation of *MyoD* gene have shown that many different factors bind the CE (Andrews et al., 2010; L'Honore et al., 2010; Relaix et al., 2013). Moreover, involvement of epigenetic remodeling in the activation of the CE have been demonstrated by *in vitro* studies that showed the requirement of histone variant H3.3 deposition on the CE for proper expression of *MyoD* in differentiating myoblasts (Yang et al., 2011). Consistent with the association of H3.3 with transcriptionally active regions, it has been discovered that the CE region was transcribed to produce a non-coding RNA enhancer (CEeRNA) playing a key role in *MyoD* expression during early differentiation steps (Mousavi et al., 2013).

On this basis, we decided to investigate the possibility that LSD1 could regulate positively or negatively the core enhancer of MyoD and therefore the initiation of *MyoD* expression in muscle precursor cells. LSD1 inhibition in myoblasts drastically decreased *MyoD* up-regulation indicating that LSD1 might be involved MyoD expression control. Further functional and ChIP experiments revealed that upon induction of differentiation, LSD1 was recruited on the *MyoD* core enhancer where it promoted the expression of the CEeRNA, which consequently controlled the timely transcription of *MyoD*. Finally, the involvement of LSD1 in the regulation of CEeRNA expression during myogenesis was provided by conditional inactivation of *Lsd1* in muscle precursor cells using a Pax3-cre knock-in mouse strain (Engleka et al., 2005; Li et al., 2000). LSD1 conditional inactivation in PAX3 positive cells recapitulated the effect of the deletion of the MyoD core enhancer (Chen and Goldhamer, 2004; Chen et al., 2001). The expression of the CEeRNA and *MyoD* in the forelimbs at embryonic day E10.5 was drastically reduced. Altogether, our results indicate that during muscle cell commitment, LSD1 is necessary for *MyoD* core enhancer expression. LSD1 is required to prevent H3 lysine 9 (H3K9) tri-methylation and recruit RNA polymerase II for the transcription of an essential non-coding RNA enhancer.

Results

LSD1 inhibition in cultured myoblasts prevents differentiation by affecting the timely increase of *MyoD* expression

During C2C12 myoblast differentiation, an increase in LSD1 protein level was observed and coincided with that of MYOD protein and mRNA levels (Figure S1). Thus, we asked if LSD1, by modulating *MyoD* expression, could play a role in the entry of muscle cells into the differentiation process.

To test our hypothesis, LSD1 activity was inhibited in cultured myoblasts with the two LSD1 inhibitors Pargyline and OG-L002 (Figure S2A and B) (Choi et al., 2010; Liang et al., 2013; Metzger et al., 2005). After 72 hours in differentiation medium (DM), C2C12 myoblasts treated with Pargyline or OG-L002 showed a dose-dependent decrease of *MyoD* expression (Figure S2C), indicating that LSD1 de-methylase activity was required for the increase of *MyoD* expression. To further investigate the mechanism of action of LSD1 on *MyoD* transcription, C2C12 cells were stably transduced with a lentivirus expressing either an shRNA directed against LSD1 (named shLSD1) or a control shRNA (named shSCRA) (Figure S3A). Consistent with previous reports (Choi et al., 2010; Munehira et al., 2016), while shSCRA and shLSD1 cells had identical growth rates (Figure S3B) and reached the same density after 72 hours in DM (Figure S3C), shLSD1 cells showed a marked reduction in their ability to fuse and form myotubes (Figure S3D). Only 3% of shLSD1 cells underwent fusion, with the majority of myotubes containing only 2 to 5 nuclei, whereas 63% of shSCRA myoblasts formed myotubes, most of them containing more than 10 nuclei (Figure S3E and F). As previously reported, this lack of differentiation was paralleled by a reduction of both Myogenin protein (Figure S3G), and mRNA levels (Figure S3H) (Cheng et al., 2014; Choi et al., 2010). In addition, shLSD1 cells as well as primary fetal satellite cells (FSC) transiently infected with LSD1 shRNA, showed a dramatic decrease of *MyoD* mRNA level (Figure 1A and B), strongly suggesting that LSD1 and its catalytic activity are required at early stages of differentiation to up-regulate *MyoD* expression.

LSD1 is recruited on the *MyoD* Core Enhancer during differentiation

So far three regulatory regions have been identified to independently control *MyoD* expression: the proximal promoter, the distal regulatory region (DRR) and the core enhancer (CE) (Asakura et al., 1995; Goldhamer et al., 1995). While the DRR region is

required to maintain *MyoD* expression in differentiating muscle cells, the CE region controls the initiation of *MyoD* expression in newly determined myoblasts, (Asakura et al., 1995; Chen et al., 2001; Goldhamer et al., 1995). To further examine the regulatory role of LSD1 on the *MyoD* transcription we performed ChIP experiments on shSCRA myoblasts. In our *in vitro* model, 72 hours after switching cells to DM, *MyoD* expression reaches its maximal and myoblasts are committed to differentiate as evidenced by *Myogenin* expression (Figure 1A, Figure S1A-B and figure S3C). At that time, LSD1 was strongly enriched at the *MyoD* core enhancer (Figure 2A and Figure S4).

Furthermore, the presence of LSD1 on the CE coincided with a reduction of the H3K9me2 and H3K9me3 repressive marks, along with a reduction of H3K4me1. Similar ChIP experiments performed on shLSD1 myoblasts placed 72 hours in DM showed that conversely to what we observed in shSCRA cells the H3K9me3 repressive mark did not only fail to decrease, but strongly increased in shLSD1 cells (Figure 2B).

Previous ChIP-seq studies have suggested that transcriptional enhancers are associated with high level of H3K4me1 (Heintzman et al., 2009). Pekowska and colleagues further demonstrated that H3K4me1 is not indicative of enhancer activity but that there is a functional link between enhancer activity and H3K4me3 enrichment (Pekowska et al., 2011). Interestingly the presence of LSD1 positively correlates with a strong increase of the activation mark H3K4me3 (Figure 2B) in that region after 72 hours in DM. Consistently, by analyzing two published ChIP-seq data (Asp et al., 2011; Mousavi et al., 2012), we observed an enrichment of H3K4me3 in the CE region during myoblast differentiation (data not shown). Altogether, these results pointed to a central role of LSD1 in the activation of the CE region.

LSD1 participates to the activation of the CEeRNA transcription

Activation of the CE region was recently shown to trigger the transcription of the CEeRNA that improves the recruitment of RNApolII on the *MyoD* proximal promoter and thus participates to the timely increase of *MyoD* expression and myoblast differentiation (Mousavi et al., 2013). A possible role of LSD1 in the transcription of the CEeRNA was investigated. Seventy-two hours in DM induced a significant increase of the of CEeRNA level in shSCRA cells (Figure 3A) whereas it remained unchanged in shLSD1 cells as well as in myoblasts treated with Pargyline or OG-L002 (Figure 3B). Accordingly, FSC transduced with LSD1 shRNA (Figure 3C) failed to activate CEeRNA expression during differentiation. Consistently, RNApolII was less enriched on the CE and near the *MyoD*

transcription start site (TSS) in shLSD1 cells than in shSCRA myoblasts after 72 hours in DM (Figure 3D).

To ensure that the inhibition of CEeRNA expression was due to the knockdown of LSD1, rescue experiments were performed by expressing either a human *wild-type* LSD1 (hLSD1) or a catalytically inactive LSD1 mutant (hLSD1 K661A; (Lee et al., 2006)) that are not targeted by the mouse LSD1 shRNA (Figure 3E). Expression of hLSD1 efficiently restored the expression of the CEeRNA after 72 hours in DM in shLSD1 cells. Conversely, the hLSD1 K661A mutant failed to rescue the CEeRNA expression (Figure 3F). These results demonstrate the requirement of LSD1 and of its de-methylase activity for the activation of the CEeRNA expression.

To determine if allowing transcription of the CEeRNA is the main function of LSD1 in the activation of MyoD expression, the CEeRNA was overexpressed in shLSD1 cells and their ability to differentiate was explored. ShLSD1 myoblasts were transfected with either an empty vector, or CEeRNA expression vectors (Figure 4A and Figure S5A). After 72 hours in DM, examination of *MyoD* mRNA levels revealed that neither the empty vector, nor the vector containing the CEeRNA cloned in the + orientation rescued *MyoD* expression in shLSD1 cells (Figure 4B). Conversely, in shLSD1 cells transfected with the vector expressing the CEeRNA (- strand), *MyoD* expression was restored to the same level than in shSCRA cells (Figure 4B). Consistently, MYOD protein levels were also restored in these cells (Figure 4C and Figure S5B-C). Moreover, expression of the CEeRNA (- strand) in shLSD1 cells allowed a 10-fold improvement of their ability to form myotubes (Figure 5A-B). Indeed, 30% of the cells fused to form myotubes with an average of 6 to 10 nuclei per myotube, whereas only 3% of the shLSD1 cells transfected with the empty or CEeRNA (+ strand) vectors underwent fusion (Figures 5B and C). In conclusion, our data demonstrate that LSD1 controls *MyoD* expression during myoblast differentiation via the activation of the CEeRNA transcription.

Lsd1 **inactivation in muscle precursor cells prevents the timely expression of** ***MyoD***

The CE region upstream of the *MyoD* locus has long been known to control the spatiotemporal pattern of expression of *MyoD* during embryogenesis (Chen and Goldhamer, 2004; Chen et al., 2001). *In vivo*, removing the core enhancer from the *MyoD* regulatory regions induces a temporary inhibition of *MyoD* expression. At embryonic day (E) 11.5, a mild reduction of *MyoD* expression in the somites and a major impairment of *MyoD* expression in the forelimbs can be observed, indicating that in the forelimb region

MyoD expression is Core-enhancer dependent (Chen and Goldhamer, 2004). One day later, *MyoD* expression is back to normal (Chen and Goldhamer, 2004; Chen et al., 2001). LSD1 immunofluorescence on E11.5 control embryo transverse sections showed that LSD1 was more expressed in muscle progenitors (PAX3 positive cells) in the forelimb than in the somite region (Figure 6A). To evaluate the requirement of LSD1 for CE dependent-*MyoD* expression *in vivo*, we conditionally ablated *Lsd1* in muscle progenitors (*LSD1 cKO,* Figure S6A) by crossing $Lsd1^{tm1Schüle}$ mice carrying a new conditional allele for *Lsd1* deletion engineered in the Schüle group (Zhu et al., 2014) and $Pax3^{Cre/+}$ mice (Engleka et al., 2005; Li et al., 2000). *In situ* hybridization on E11.5 *LSD1 cKO* embryos showed that LSD1 inactivation in muscle progenitor cells resulted in a mild and strong temporary impairment of *MyoD* expression in the somites and in the forelimbs respectively (Figure 6B). Indeed at E12.5, *MyoD* expression was restored to the same levels observed in control embryos (Figure 6B). *In vivo* ablation of LSD1 fully mimics that of the core enhancer. Of note, other PAX3-expressing cells, such as the neural crest derived lineage, were not affected as seen with *Sox10* expression (Figure S6B). To confirm *MyoD* down-regulation, western blot experiments were performed on E11.5 *LSD1 cKO* and control embryo total protein extracts. MYOD protein level was reduced in the absence of LSD1 (Figure S6C). No alteration of PAX7 and MYF5 protein levels were observed (Figure S6C), supporting the idea that at early stages of muscle progenitor differentiation LSD1 specifically controls *MyoD* expression but does not affect the expression of other early myogenic determination factors. This would explain why in the absence of MYOD (Conerly et al., 2016; Rawls et al., 1998) and LSD1 myogenesis is delayed but ultimately proceeds. To evaluate the impact of LSD1 inactivation on the proportion of progenitors that turned on *MyoD* expression, MYOD and PAX3 positive cells in the forelimb of E11.5 embryos were visualized by immunofluorescence. Counting PAX3 and MYOD positive cells revealed that the percentage of MYOD positive cells in the forelimb at E11.5 was significantly lower in *LSD1 cKO* compared to control (Figure 6C). Consistent with the delay in *MyoD* expression and with the previously reported role of LSD1 on *Myogenin* activation (Cheng et al., 2014; Choi et al., 2010), a strong reduction in *Myogenin* expression was observed in LSD1 cKO forelimbs at E11.5 (Figure S6C-D).

Lsd1 **inactivation in muscle precursor cells prevents CEeRNA expression**

In vitro results indicated that the control of MyoD expression by LSD1 was mediated by the expression of the CEeRNA. CEeRNA expression was therefore evaluated in E10.5 control

and LSD1 cKO embryos, both by *in situ* hybridization and by RT-qPCR on dissected forelimbs. Both approaches showed that in LSD1 cKO embryo forelimbs, CEeRNA and *MyoD* mRNA levels were dramatically reduced (Figures 7A-B and Figure S7B). Consistent with our *in vitro* results, only the CEeRNA (- strand) was significantly expressed in the forelimb region (Figure S7A). These results demonstrate that *in vivo* LSD1 is essential for the MyoD core enhancer transcription in muscle cells commitment.

Discussion

Although the action of MYOD on chromatin remodeling during muscle differentiation has been extensively studied, still little is known about the chromatin remodeling events associated with the increase of *MyoD* expression. The core enhancer of *MyoD* is required for the initiation of *MyoD* expression in newly determined myoblasts (Asakura et al., 1995; Chen and Goldhamer, 2004; Chen et al., 2001; Goldhamer et al., 1995). In this work, we have demonstrated that LSD1 is required for the transcription of the CEeRNA from the Core Enhancer region. So far, LSD1 is the first chromatin-modifying enzyme identified to regulate the activity of the core enhancer of MyoD.

The inhibition of myoblast differentiation and of CEeRNA expression by two different LSD1 pharmacological inhibitors (Pargyline and OG-L002) or a catalytically inactive LSD1 mutant shows that LSD1 enzymatic activity is required to increase *MyoD* expression. However, the loss of H3K9 tri-methylation cannot be directly attributed to LSD1 enzymatic activity, suggesting that LSD1 might work together with other histone de-methylases to prevent H3K9 tri-methylation upon differentiation. Consistently, the absence of LSD1 in differentiating myoblasts induced a strong increase in H3K9 tri-methylation (Figure 2B). Increased H3K9me3 in the absence of LSD1 could be due to the fact that LSD1 prevents H3K9 tri-methylation by removing mono- and di-methylation, or/and that LSD1 prevents the recruitment/activity of a methyl transferase. This possibility would fit with the idea that LSD1 belongs to large multiproteic complexes and could affect the composition of the complexes recruited on the core enhancer.

Indeed, the function of histone de-methylases is not only defined by their active site. Both interactions with the histone substrates and with protein partners can profoundly affect substrate specificity and activity (Cai et al., 2014; Metzger et al., 2010; Metzger et al., 2005; Shi et al., 2005). In addition, LSD1 could also de-methylate non-histone substrates, such as components of co-activator complexes. Regarding H3K4 methylation, as part of

co-activator complexes LSD1 could favor RNA polymerase II recruitment, which comes along with the COMPASS complex that catalyzes H3K4 tri-methylation (Dehe and Geli, 2006; Terzi et al., 2011). In the absence of LSD1, RNA polymerase II recruitment on the *MyoD* Core enhancer is reduced and the level of H3K4 tri-methylation is strongly impaired indicating that LSD1 could be required for RNA polymerase II recruitment on the Core enhancer of *MyoD*.

In 2013, Mousavi et al., have shown that transcription of the core enhancer by RNA polymerase II generated a non-coding enhancer RNA that promoted the recruitment of RNA polymerase II on the proximal promoter of MyoD (Mousavi et al., 2013). However, which strand of the CEeRNA had to be transcribed to regulate *MyoD* transcription remained unknown. Our results show that only the transcription of the minus strand of the CEeRNA promotes *MyoD* transcription and that in forelimbs, only this strand is expressed. Whether this is due to unidirectional transcription of the core enhancer or to different stabilities of the RNA transcribed from the plus and minus strands remains an open question.

Recently, LSD1 was shown to bind and activate enhancers stimulated by androgen receptors (AR-stimulated enhancer) (Cai et al., 2014). However, the mechanism described in that case was different from the one we report here. While activating the transcription of AR-dependent genes, LSD1 still catalyzed H3K4 de-methylation on AR-stimulated enhancers. Our study shows that LSD1 can have a different enhancer-activating activity, which involves H3K4 methylation via RNA polymerase II recruitment on transcribed enhancer.

Several observations argue in favor of the idea that the main function of LSD1 during muscle cells engagement is the timely control of *MyoD* expression *via* the activation of the CEeRNA: i) LSD1 inactivation effect can be efficiently rescued by the expression of the CEeRNA (- strand), ii) LSD1 inactivation in mouse muscle progenitors inhibits the expression of the CEeRNA and mimics *MyoD* core enhancer deletion phenotype, iii) LSD1 inactivation does not interfere with the alternative mechanisms that allow delayed muscle differentiation in the absence of *MyoD*, indeed the expression of other muscle determination factors such as PAX7 and MYF5 is not affected by the inactivation of LSD1.

The specific action of LSD1 in the early steps of differentiation does not exclude the possibility that LSD1 may also be involved in later stages of muscle differentiation. Indeed, LSD1 has been shown to directly regulate *Myogenin* expression in cultured myoblasts (Cheng et al., 2014; Choi et al., 2010). This could explain why in the rescue experiments

with the CEeRNA, *Myogenin* expression is only partially rescued (figure S5B). This would also explain why although expression of the CEeRNA efficiently restored myoblast fusion in the absence of LSD1, myotubes remained thinner and incorporated less nuclei than control cells (figure 5).

In conclusion, our data show that LSD1 is required for the timely expression of *MyoD* via the activation of the *MyoD* core enhancer. More generally, our results indicate that in addition to repress stemness enhancers, LSD1 can participate to cell engagement into differentiation by activating pro-differentiation enhancers. This raises the question of the mechanisms that drive LSD1 to selectively silence stemness enhancers and/or activate pro-differentiation enhancers upon progenitor cell commitment.

Experimental Procedures

Cell lines, culture conditions, infection and transfection

C2C12 mouse myoblasts were maintained as myoblasts in growth medium (GM): Dulbecco modified Eagle medium, supplemented with 15% fetal calf serum and antibiotics. Primary fetal satellite cells (FSC) were maintained on Matrigel coated dishes in growth medium (GM): Dulbecco modified Eagle medium F12, supplemented with 20% fetal calf serum, 5 ng/ml of FGF and antibiotics. C2C12 cells and FSC cells were differentiated into myotubes by replacing GM with media containing 2% horse serum with antibiotics (differentiation medium, DM). For stable knockdown of *Lsd1* in C2C12 cells, lentiviral vector containing the mouse Lsd1-targeting sequence pLKO.1-sh-LSD1 (TRCN0000071377, ShLSD1), purchased from Open Biosystem, was used. As a scrambled control (shSCRA), the pLKO.1 vector SHC016V purchased from Sigma-Aldrich was used. Twenty-four hours after lentiviral infection, C2C12 were selected with puromycin (1µg/ml) for fourteen days. To avoid problems with clonal variation, all the clones (50–100 per transfection) were pooled and then used for experiments.

Primary fetal satellite cells were infected with the pLKO.1-sh-LSD1 (FSC shLSD1) and with the pLKO.1 vector SHC016V (FSC shSCRA). Twenty-four hours after lentiviral infection, FSC were induced to differentiate.

Pargyline (1mM) and OG-L002 (5µM, 7µM or 10 µM) were added to C2C12 cells concomitantly to DM and again 48 hours after.

Cell transfections with pRNAT vector (pRNAT-CMV3.1/Neo by GenScript), CEeRNA vectors were performed as follows: 300,000 shSCRA and shLSD1 cells were seeded in 35

mm petri dishes. Three hours later, shSCRA cells were transfected with pRNAT empty vector, shLSD1 cells were transfected with pRNAT empty vector or CEeRNA (+ strand) or CEeRNA (- strand) with jetPRIME® (polyplus transfection) according to manufacturer's instructions. Twenty-four hours after transfection, cells were seeded (150,000 cells per 35 mm petri dishes) in DM for 72 hours for RNA or protein analysis and 120 hours for nuclei counting. Cell transfections with hLSD1 and hLSD1 K661A plasmids were performed as previously described. Twenty-four hours after transfection, cells were seeded (150,000 cells per 35 mm petri dishes) in DM for 72 hours for RNA or protein analysis.

Cloning

CEeRNA constructs were generated with the Phusion Green High-Fidelity DNA Polymerase (Thermo Scientific) and confirmed by DNA sequencing. The full length CEeRNA was cloned in the pRNAT vector (pRNAT-CMV3.1/Neo by GenScript) in the sense [CEeRNA (+ strand)] and antisense [CEeRNA (- strand)] orientations under the control of the strong H1 promoter, using the BAMHI site. For oligonucleotides details, see also supplemental experimental procedures**.**

Real-Time PCR (RT-qPCR)

Total RNA was isolated from cultured cells grown in 100-mm dishes using Trireagent (Sigma). RNA was analyzed by real-time PCR using QuantiFast SYBR® Green PCR Kit (Qiagen). Relative gene expression was determined using the ΔCt method. Total RNA from dissected forelimbs and heads of control and LSD1cKO embryos at E10.5 was isolated using the RNeasy Micro Kit (Qiagen) according to the manufacturer's instructions. For oligonucleotides details, see also supplemental experimental procedures.

Immunoblotting

Proteins were extracted from total embryos and cells and quantified using the DC™ Protein assay (Bio-Rad). Total proteins were separated by 10% SDS-PAGE electrophoresis and transferred onto PVDF Immobilon®-P membranes (Millipore™). Immunoblots were performed with the ECL PLUS reagent (Amersham or GE Healthcare) according to the manufacturer's instructions. For antibodies details, see also supplemental experimental procedures.

ChIP- Chromatin ImmunoPrecipitation

1 x 10^7 C2C12 cells were incubated in 1% formaldehyde on a rotating wheel for 10 minutes at room temperature. Reactions were stopped by adding glycine at a final concentration of 0,125 M and incubated on a rotating wheel for 10 minutes at room temperature. After PBS wash, pellet was dissolved in ice-cold cell lysis buffer (5mM PIPES, 85mM KCl, 0,5% NP40) and incubated on ice for 10-20 minutes. Nuclei were centrifuged at 3000 rpm for 5 minutes at 4°C, dissolved in ice-cold RIPA (150mM NaCl, 0,5% NaDoc, 1% NP40, 0,1% SDS, 50mM TrisHCl) buffer and incubated on ice for 10-20 minutes. Nuclei were sonicated with a Bioruptor® PLUS combined with the Bioruptor® Water cooler (Diagenode). The size of chromatin fragments was checked. Chromatin was then pre-cleared by incubation with Protein A-Sepharose® 4B fast flow (Sigma) for 15 minutes at 4°C with constant rotation. After centrifugation, specific antibodies were added and rotated overnight at 4°C. Protein A-Sepharose® 4B fast flow (Sigma) was added and incubated with constant rotation for 30 minutes at room temperature. Beads were then washed and chromatin IP was de-cross-linked with Proteinase K at 65°C for 6 hrs. Chromatin IP and INPUT were extracted and dissolved in 10 mM TrisHCl pH 8. Three sites: Core enhancer, Negative and Transcriptional start site regions (CE, NEG and TSS respectively) were tested for RT-qPCR amplification. RT-qPCR data analysis for LSD1 and RNApolII IPs has been performed calculating the percentage of input for each genomic region and then data are shown as the relative enrichment to the control genomic region (NEG region) that does not interact with the protein of interest. RT-qPCR data analysis for H3, H3K9me2, H3K9me3, H3K4me1 and H3K4me3 IPs has been performed as previously described. Data were also normalized to the occupancy of H3 in each genomic region and shown as the relative enrichment to the control genomic region (NEG region). For oligonucleotides details, see also supplemental experimental procedures.

Nuclei counting and percentage of fusion

The nuclei counting of myotubes was performed as follows. 300,000 shSCRA and shLSD1 cells were seeded in 35 mm petri dishes. Three hours later, shSCRA cells were transfected with pRNAt empty vector, shLSD1 cells were transfected with pRNAt empty vector or CEeRNA (+ strand) or CEeRNA (- strand). 24 hours after transfection, cells were seeded (150,000 cells per 35 mm petri dishes) in DM for 120 hours. Cells were then fixed for 20 min in 4% PFA in PBS and washed 3 times in PBS-0,1% triton-X100 to permeabilize membranes. Cells were then incubated for 20 minutes with DAPI to stain nuclei and

washed 3 times in PBS. Cells were mounted with Vectashield and observed with a fluorescent microscope (AxioImager).

Mouse breeding and embryo harvesting

Lsd1^{tm1Schüle} and *Pax3$^{Cre/+}$* mice were previously described (Engleka et al., 2005; Li et al., 2000; Zhu et al., 2014). All mouse handling, breeding, and sacrificing were done in accordance with European legislations on animal experimentation. Experimental mice (*LSD1 cKO*) were generated by crossing *Pax3$^{Cre/+}$*:Lsd1$^{tm1Schüle\ /+}$ males with Lsd1^{tm1Schüle} females. The uterus was removed and placed into dishes filled with PBS. Individual embryos were collected and placed into 4% paraformaldehyde (PFA) in PBS overnight at 4°C on a shaker for whole mount *in situ* hybridization and immunofluorescence or frozen in liquid nitrogen for protein extraction.

Whole mount *in situ* hybridization

Gentle rocking of embryos occurred during all following incubations. Embryos were fixed in 4% PFA 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. Embryos were digested with proteinase-K/PBS-T and then fixed in 0.1% glutaraldehyde/4% PFA/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. Digoxygenin labeled RNA probe (Sassoon et al., 1989) was then added and incubated at 68°C overnight. Embryos were washed in 68°C pre-warmed hybridization mix at 68°C. Embryos were then incubated for 10 minutes as 68°C in a 1:1 mix of hybridization mix and MAB-T buffer. Embryos were then washed in MAB-T at room temperature and then incubated in 2% boehringer blocking reagent (bbr) in MAB-T for 1 hour at room temperature. Anti-digoxygenin-ap fab fragment (roche #11093274910) antibody was then added to a 1:2000 dilution and incubated 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. After replacing NTMT with BM purple AP substrate (Sigma-Aldrich, cat #11442074001) color is developed to appropriate level, usually 6-8 hours. After color development level is reached, embryos were re-fixed in 4% PFA and stored at 4°C. The *MyoD*, *Myogenin* and *Sox10* riboprobes

were synthesized as described previously (Hayashi et al., 2011; Sassoon et al., 1989). CEeRNA probe were generated by PCR amplification from genomic DNA using the following primers: Forward 5'-GGAGCACCCCACAACATGAGC-3' and Reverse 5'-AGTCTGTGCGGGTGAGGCAG-3'. The resulting 516 bp fragment was subcloned in pGEMT-easy (Promega). Antisense and sense riboprobes were synthesized using the DIG RNA labeling kit (SP6/T7) (Sigma).

Immunofluorescence

Embryos and cells were fixed with 4% PFA at 4°C 2 hours with rotation and at room temperature 20 minute respectively. The embryos and cells were washed with cold PBS. The fixed embryos were 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 tissues were cryosectioned at 12 microns, washed and then permeabilized with 100% methanol for 6 min at -20°C. Slides and cells 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 for 1 hour at room temperature with secondary antibody diluted in PBS-B-T. After three washes, slides and cells were counterstained with DAPI and mounted. Fluorescent images were acquired on a confocal microscope (Leica TCS SP5) and processed with Photoshop CS4 (Adobe system). For antibodies details, see also supplemental experimental procedures.

Statistical analysis

Statistical significance was determined by Bonferroni test after one-way ANOVA analysis using the software Graph-Pad Prism version 5.00 for windows, Graph-Pad Software, San Diego, California, USA, **www.graphpad.com**. A p value of <0.05 was considered significant.

Author Contributions

L.S., F.R., I.S. and E.G. conceived the research. I.S. performed all cell biology, molecular cloning, ChIP and RT-qPCR experiments and analysis, H.S. carried out the immunofluorescence and *in situ hybridization* on E11.5 and E12.5 embryos, S.M made mouse breeding and embryo harvesting, western blot on mouse embryos, S.M and K.A.

performed C2C12 myoblast differentiation experiments with LSD1 inhibitors, E.G. performed immunofluorescence on C2C12 cells, J.E.L. performed the *in situ hybridization* of CEeRNA on E10.5 embryos, V.M. dissected forelimbs and head from E10.5 embryos, T.S. has analyzed the GSE25308 and GSE25549 ChIP-seq data, P.M. and M.W. have isolated fetal satellite cells from E18.5 *wild-type* embryos, E.M., R.S. generated $Lsd1^{tm1Schüle}$ mice and hLSD1 and hLSD1 K661A constructs, L.S. I.S. and E.G. wrote the manuscript.

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Figure Legends

Figure 1: Inhibition of Lsd1 in cultured myoblasts drastically reduces MyoD expression

MyoD mRNA levels A) in shSCRA and shLSD1 cells and B) Primary fetal satellite cells infected with an shRNA scrambled or an shRNA against LSD1 (respectively FSC shSCRA and FSC shLSD1) during differentiation. RT-qPCR values were normalized to the *Ppib* mRNA. mRNA levels are shown as the fold variation compared to shSCRA or FCS shSCRA cells at DM0. Data are represented as mean ± SEM of at least three experiments. **p < 0.01, ***p <0,001 (Bonferroni test after one way-ANOVA). See also Figure S1-S3.

Figure 2: LSD1 recruitment on the *MyoD* Core enhancer region correlates with its activation.

A) Localization of LSD1 at the Core Enhancer (CE) region of *MyoD* gene locus after 72 hours in DM. ChIP analysis was performed on shSCRA cells with an anti-LSD1 antibody. Enrichment values were normalized to input. B) ChIP analysis of the CE region on shSCRA and shLSD1 cells at DM0 and after 72 hours in DM, using antibodies against H3K9me2, H3K9me3, H3K4me1 and H3K4me3. Enrichment values were normalized to input and to the occupancy of the core H3. Two sites: Core enhancer and Negative regions (CE and NEG respectively) were tested for RT-qPCR amplification. Data are shown as fold difference relative to the NEG region and represented as mean ± SEM of at least three experiments. **p < 0.01, ***p < 0.0005 (Bonferroni test after one way-ANOVA). See also Figure S4.

Figure 3: Demethylase activity of LSD1 is required to promote CEeRNA transcription.

CEeRNA expression in A) shSCRA and shLSD1 cells, B) control C2C12 cells treated with pargyline or OG-L002 and C) FSC shSCRA and FSC shLSD1 . RT–qPCR values were normalized to the *Ppib* mRNA levels, and are shown as the fold difference with DM0. D) Localization of RNApolII at the *MyoD* gene locus. ChIP analysis was performed on

shSCRA and shLSD1 cells after 72 hours in DM with an anti-RNA polII antibody. Three sites; Core enhancer, Negative and Transcriptional start site regions (CE, NEG and TSS respectively) were tested for RT-qPCR amplification. Enrichment values were normalized to input, and are shown as the fold difference relative to NEG region. E) Western blot analysis of LSD1 protein levels after 72 hours in DM in shSCRA, shLSD1, and shLSD1 cells expressing wild type or hLSD1 K661A hLSD1. F) CEeRNA expression after 72 hours in DM in shSCRA, shLSD1 and shLSD1 cells expressing wild type or hLSD1 K661A hLSD1. RT–qPCR values were normalized to the *Ppib* mRNA levels, and are shown as the fold difference with shSCRA at DM0. Data are represented as mean ± SEM of at least three experiments. **p < 0.01, ***p < 0.0005 (Bonferroni test after one way ANOVA).

Figure 4: LSD1 driven CEeRNA expression is required for *MyoD* expression.

A) Schematic representation of pRNAT constructs expressing the CEeRNA used in rescue experiment. B) *MyoD* mRNA levels in shSCRA transiently transfected with empty pRNAT vector, and in shLSD1 cells transiently transfected with empty pRNAT vector, CEeRNA (- strand) or (+ strand) vectors after 72 hours in DM. RT–qPCR values were normalized to the *Ppib* mRNA levels, and are shown as the fold difference with shSCRA at DM0. Data are represented as mean ± SEM of at least three experiments. *p < 0.05 (Bonferroni test after one way ANOVA) C) Confocal pictures showing MYOD immunostainings in shSCRA myoblasts transiently transfected with pRNAT empty vector, and in shLSD1 cells transiently transfected with pRNAT empty, CEeRNA (- strand) or CEeRNA (+ strand) vectors after 72 hours in DM. Scale bar: 20 µm. Data are representative of at least 3 independent experiments. See also Figure S5.

Figure 5: CEeRNA (- strand) overexpression rescues myotube formation in absence of LSD1.

A) Representative images of shSCRA transiently transfected with empty pRNAT vector, shLSD1 transiently transfected with empty pRNAT vector, CEeRNA (+ strand) or CEeRNA (- strand) cells after 120 hours in DM. Scale bars represent 50 µm. B) Percentage of fused cells was calculated as the proportion of GFP positive cells containing two or more nuclei. C) Nuclei were counted in shLSD1 cells transfected with pRNAT empty, CEeRNA (-strand) or CEeRNA (+strand) (180, 132 and 102 cells, respectively) vectors, and in 110 shSCRA cells transfected with pRNAT empty vector. Graphs represent three different experiments.

Figure 6: LSD1 depletion spatio-temporally impairs *MyoD* expression during embryogenesis

A) LSD1 and PAX3 immunostainings of transverse sections of E11.5 control embryos in forelimb and somite regions. Scale bars represent 100 µm. B) Whole-mount *in situ* hybridization for *MyoD* mRNA in Control and *LSD1 cKO* embryos at E11.5 and E12.5. Insets are higher magnification of the forelimb C) PAX3 and MYOD immunostainings in the forelimbs of E11.5 control and LSD1 cKO embryos. Scale bar: 50 µm. Right panel shows quantification of the relative proportion of PAX3 and MYOD-positive cells in control and *LSD1 cKO* forelimb shown in left panel and data are expressed as percentage over total immunostained cell population. Histogram data are means ± SEM. ***p < 0.01 (n = 3 embryos for each condition) (Bonferroni test after one way ANOVA). See also Figure S6.

Figure 7: LSD1 depletion impairs CEeRNA expression *in vivo* at E10.5

A) Whole-mount *in situ* hybridization for CEeRNA using a sense probe in control and *LSD1 cKO* embryos at E10.5. Insets are higher magnification of the forelimb region B) CEeRNA level in dissected forelimb and head from control and LSD1 cKO embryos at E10.5. RT–qPCR values were normalized to the *Ppib* mRNA levels, and are shown as the fold difference with control head. ***p<0,0001 (6 control and 4 LSD1 cKO embryos) (Bonferroni test after one way ANOVA). See also Figure S7.

Figure 1

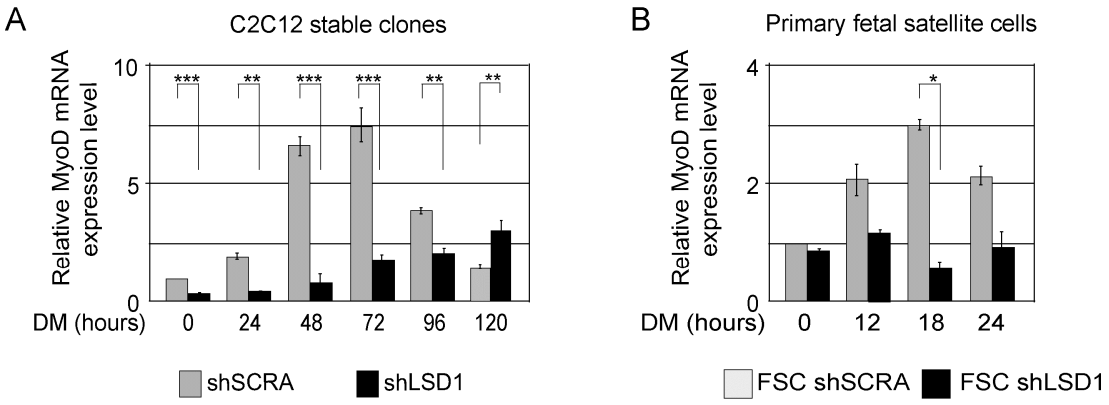

A
C2C12 stable clones
Relative MyoD mRNA expression level
10
5
0
***
**
***
***
**
**
DM (hours)
0
24
48
72
96
120
shSCRA
shLSD1
B
Primary fetal satellite cells
Relative MyoD mRNA expression level
4
2
0
*
DM (hours)
0
12
18
24
FSC shSCRA
FSC shLSD1

Figure 2:

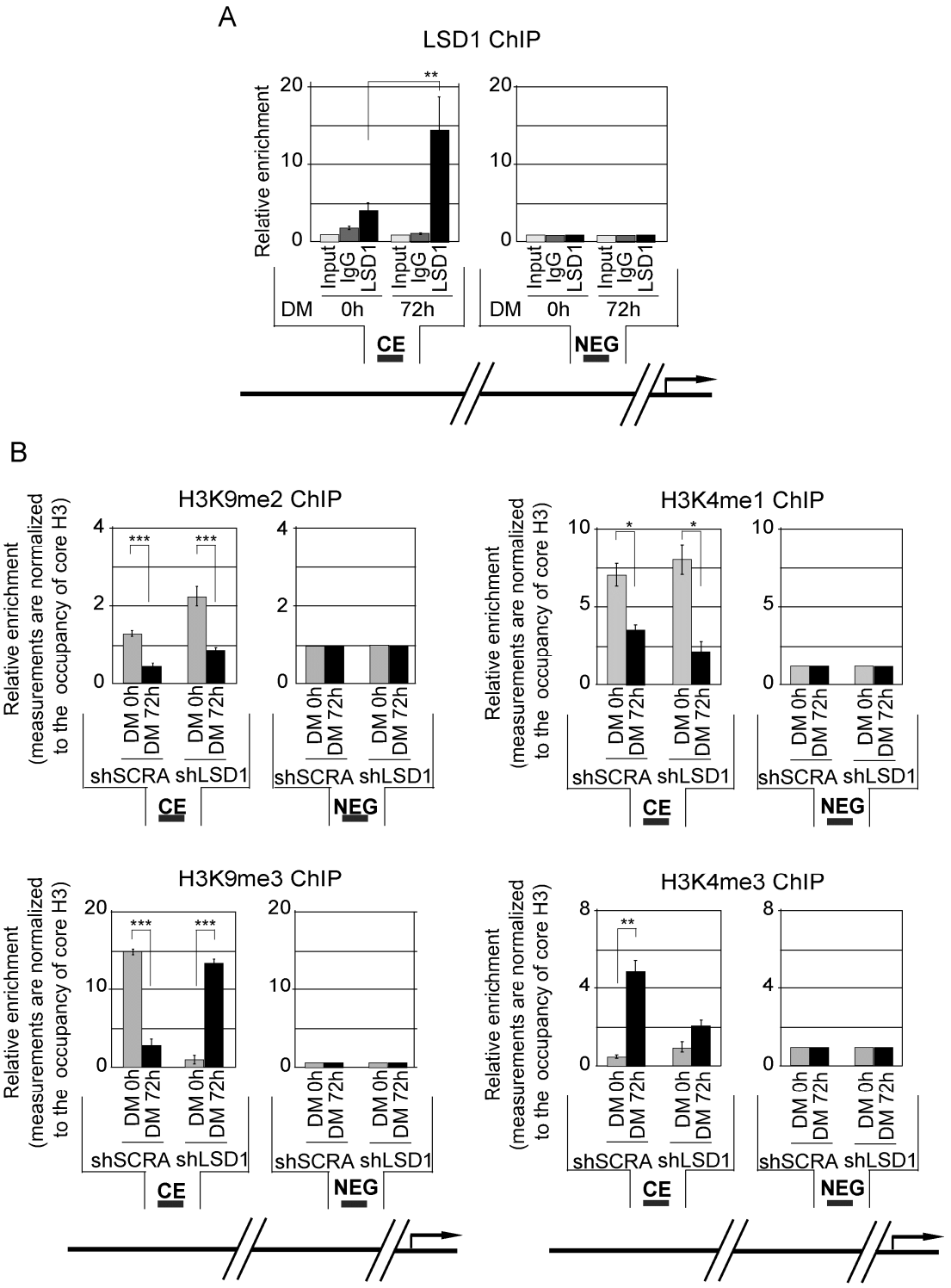

A
LSD1 ChIP
Relative enrichment
20
10
0
**
Input
IgG
LSD1
DM
0h
72h
CE
NEG
B
H3K9me2 ChIP
H3K4me1 ChIP
H3K9me3 ChIP
H3K4me3 ChIP
Relative enrichment (measurements are normalized to the occupancy of core H3)
***
*
**
DM 0h
DM 72h
shSCRA shLSD1
CE
NEG

Figure 3:

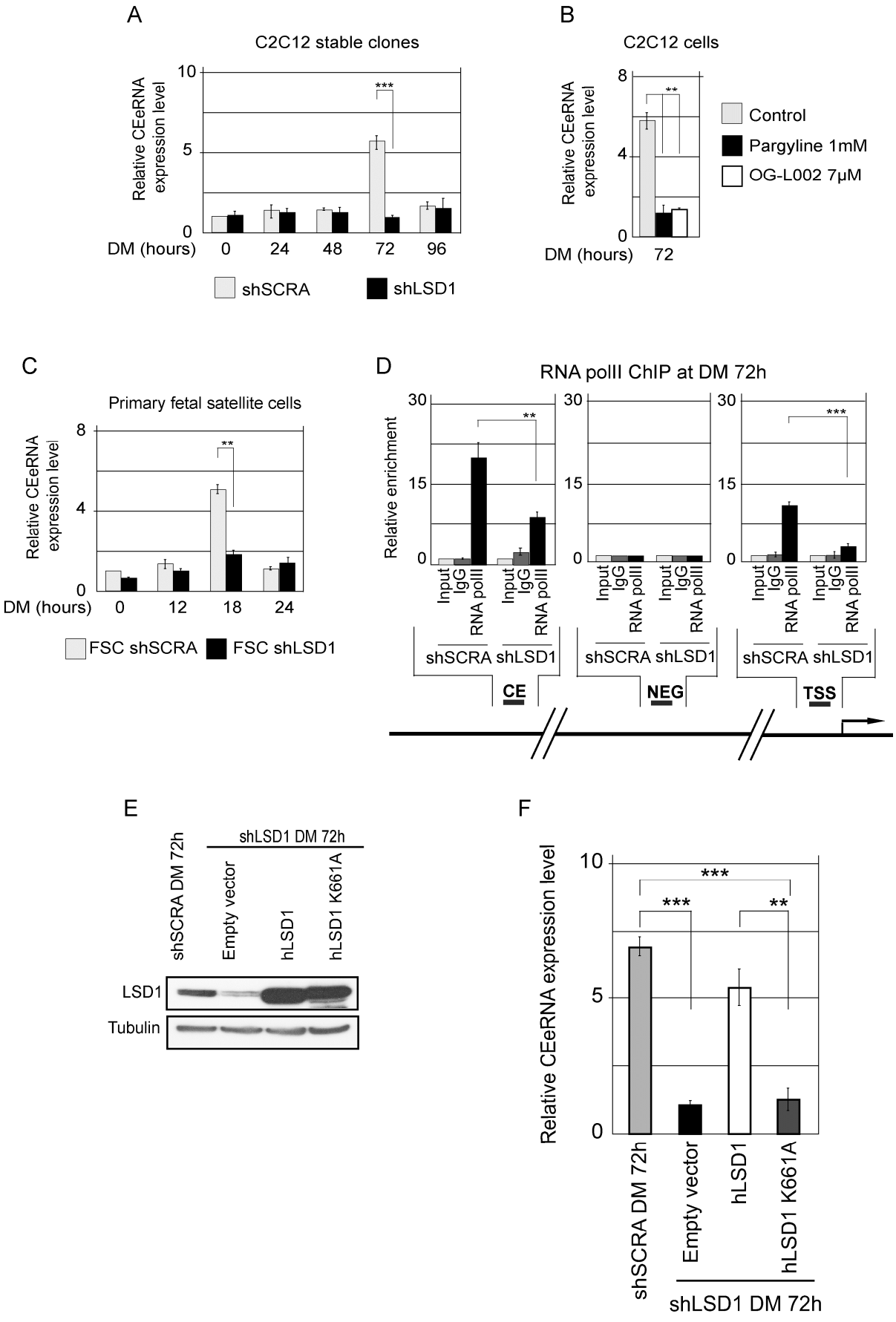

A
C2C12 stable clones
Relative CEeRNA expression level
10
5
0
***
DM (hours) 0 24 48 72 96
shSCRA
shLSD1
B
C2C12 cells
Relative CEeRNA expression level
8
4
0
**
DM (hours) 72
Control
Pargyline 1mM
OG-L002 7μM
C
Primary fetal satellite cells
Relative CEeRNA expression level
8
4
0
**
DM (hours) 0 12 18 24
FSC shSCRA
FSC shLSD1
D
RNA polII ChIP at DM 72h
Relative enrichment
30
15
0
**
***
Input
IgG
RNA polII
shSCRA shLSD1
CE
NEG
TSS
E
shLSD1 DM 72h
shSCRA DM 72h
Empty vector
hLSD1
hLSD1 K661A
LSD1
Tubulin
F
Relative CEeRNA expression level
10
5
0
***
***
**
shSCRA DM 72h
Empty vector
hLSD1
hLSD1 K661A
shLSD1 DM 72h

Figure 4:

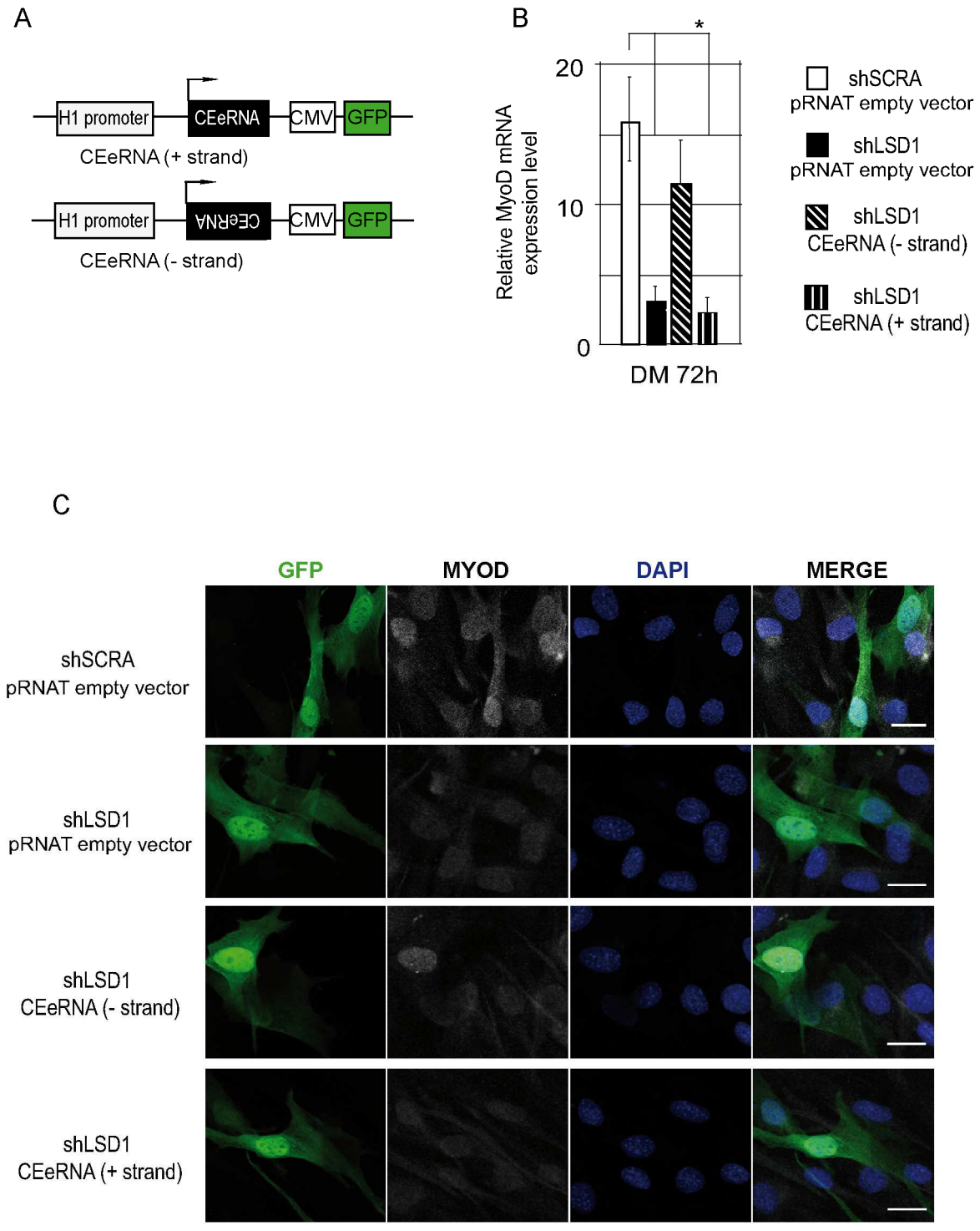

A
H1 promoter
CEeRNA
CMV
GFP
CEeRNA (+ strand)
H1 promoter
CMV
GFP
CEeRNA (- strand)
B
*
20
10
0
Relative MyoD mRNA expression level
DM 72h
shSCRA
pRNAT empty vector
shLSD1
pRNAT empty vector
shLSD1
CEeRNA (- strand)
shLSD1
CEeRNA (+ strand)
C
GFP
MYOD
DAPI
MERGE
shSCRA
pRNAT empty vector
shLSD1
pRNAT empty vector
shLSD1
CEeRNA (- strand)
shLSD1
CEeRNA (+ strand)

Figure 5:

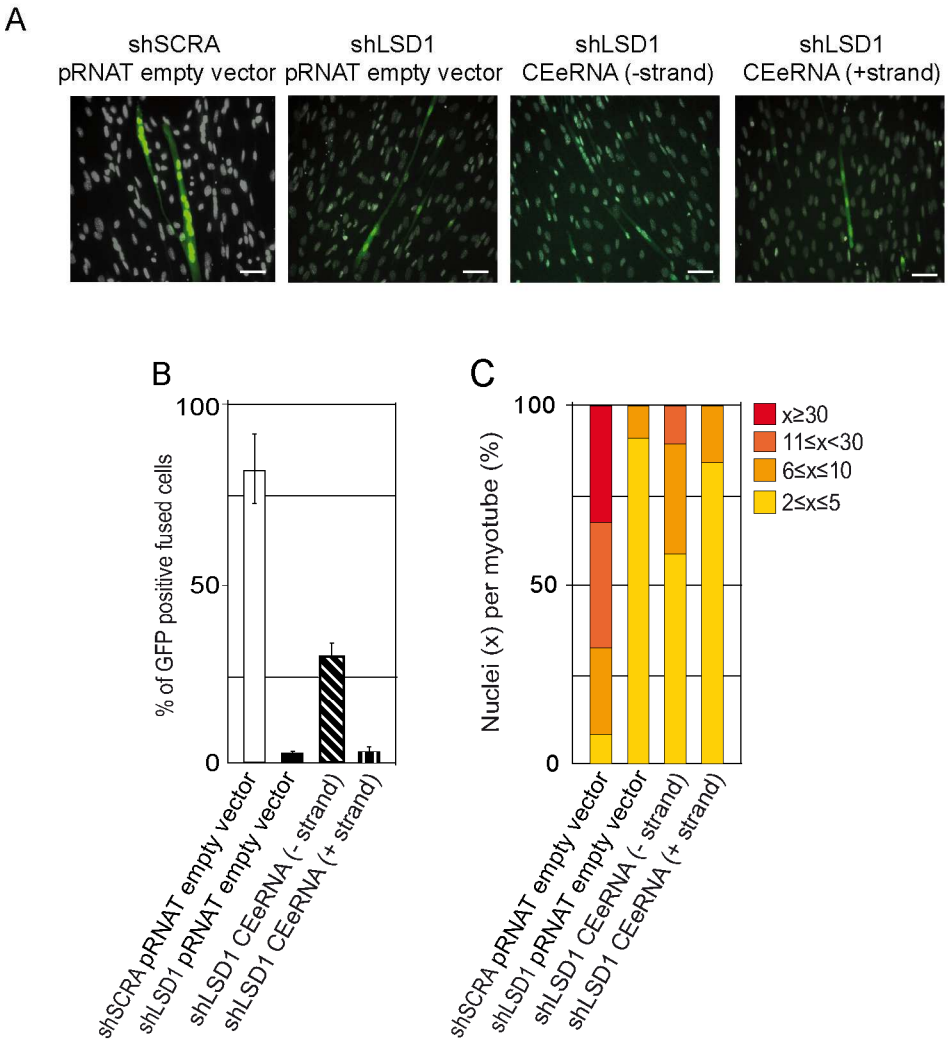

A
shSCRA pRNAT empty vector
shLSD1 pRNAT empty vector
shLSD1 CEeRNA (-strand)
shLSD1 CEeRNA (+strand)
B
% of GFP positive fused cells
100
50
0
shSCRA pRNAT empty vector
shLSD1 pRNAT empty vector
shLSD1 CEeRNA (- strand)
shLSD1 CEeRNA (+ strand)
C
Nuclei (x) per myotube (%)
100
50
0
x≥30
11≤x<30
6≤x≤10
2≤x≤5
shSCRA pRNAT empty vector
shLSD1 pRNAT empty vector
shLSD1 CEeRNA (- strand)
shLSD1 CEeRNA (+ strand)

Figure 6:

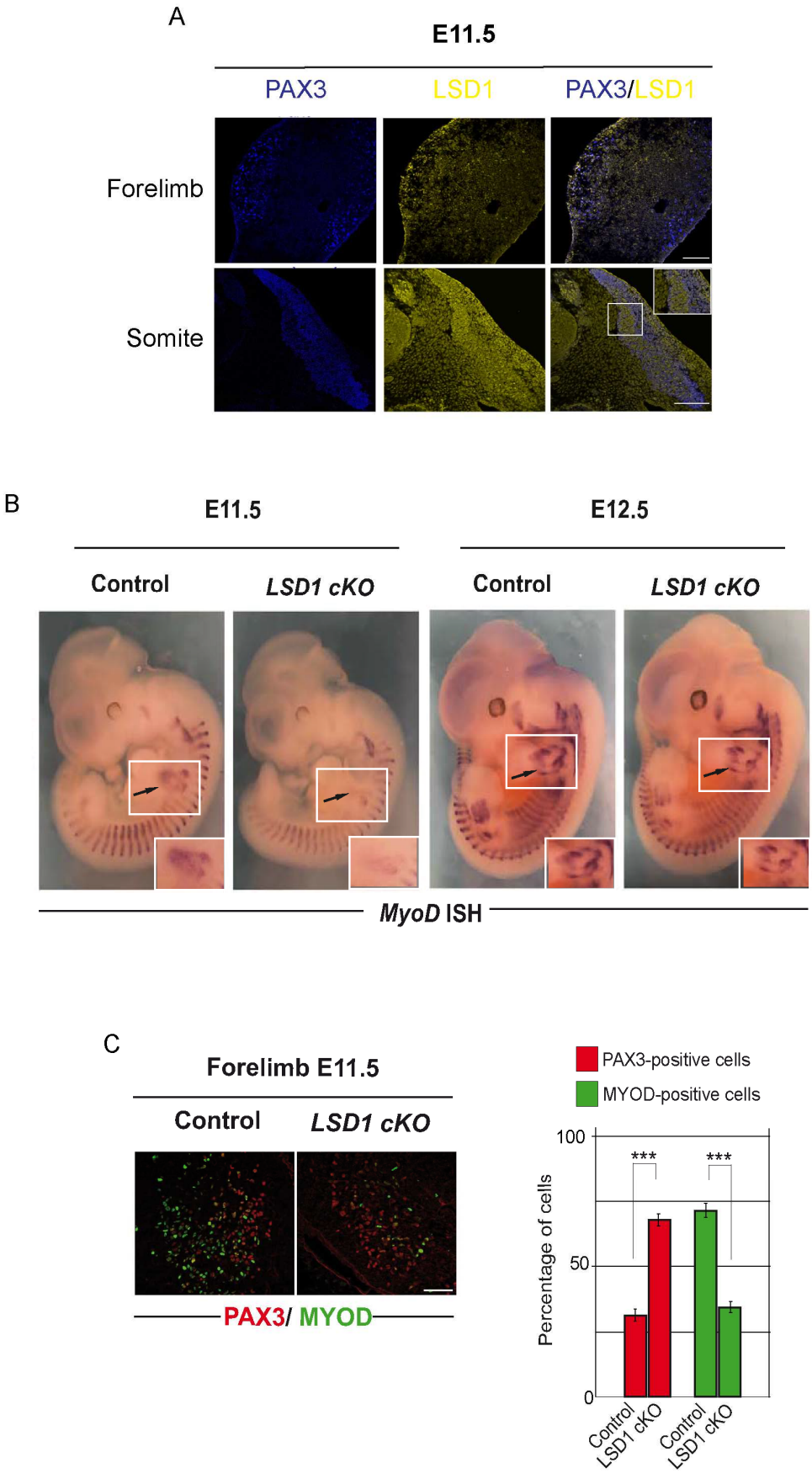
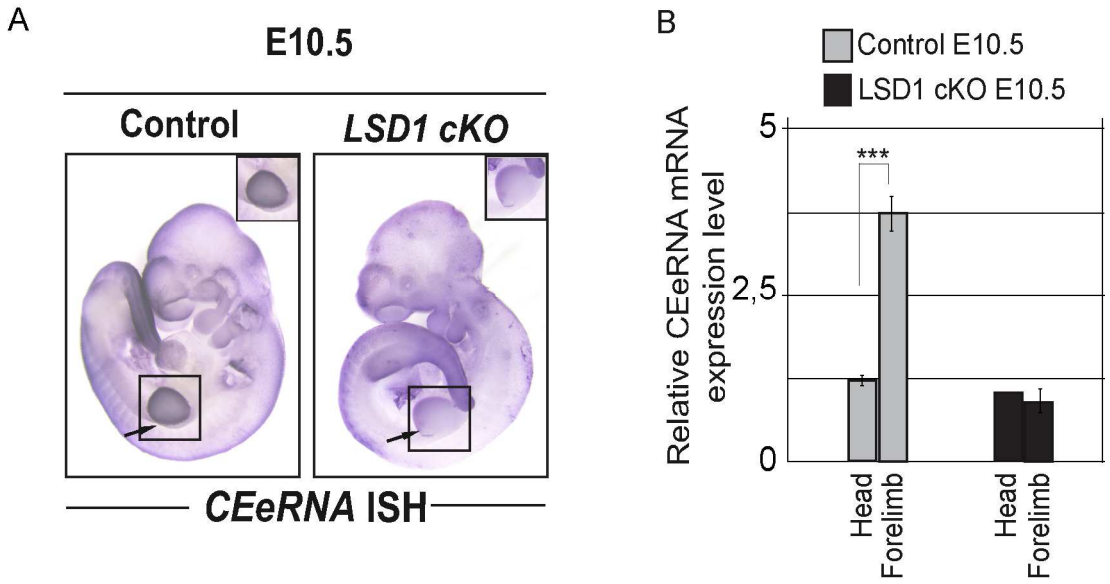

A
E11.5
PAX3
LSD1
PAX3/LSD1
Forelimb
Somite
B
E11.5
E12.5
Control
LSD1 cKO
Control
LSD1 cKO
MyoD ISH
C
Forelimb E11.5
Control
LSD1 cKO
PAX3/ MYOD
PAX3-positive cells
MYOD-positive cells
Percentage of cells
100
50
0
***
***
Control
LSD1 cKO
Control
LSD1 cKO

Figure 7:


A
E10.5
Control
LSD1 cKO
CEeRNA ISH
B
Control E10.5
LSD1 cKO E10.5
Relative CEeRNA mRNA expression level
5
2,5
0
***
Head
Forelimb
Head
Forelimb

Cell Reports, Volume *18*

Supplemental Information

LSD1 Controls Timely *MyoD* Expression via MyoD Core Enhancer Transcription

Isabella Scionti, Shinichiro Hayashi, Sandrine Mouradian, Emmanuelle Girard, Joana Esteves de Lima, Véronique Morel, Thomas Simonet, Maud Wurmser, Pascal Maire, Katia Ancelin, Eric Metzger, Roland Schüle, Evelyne Goillot, Frederic Relaix, and Laurent Schaeffer

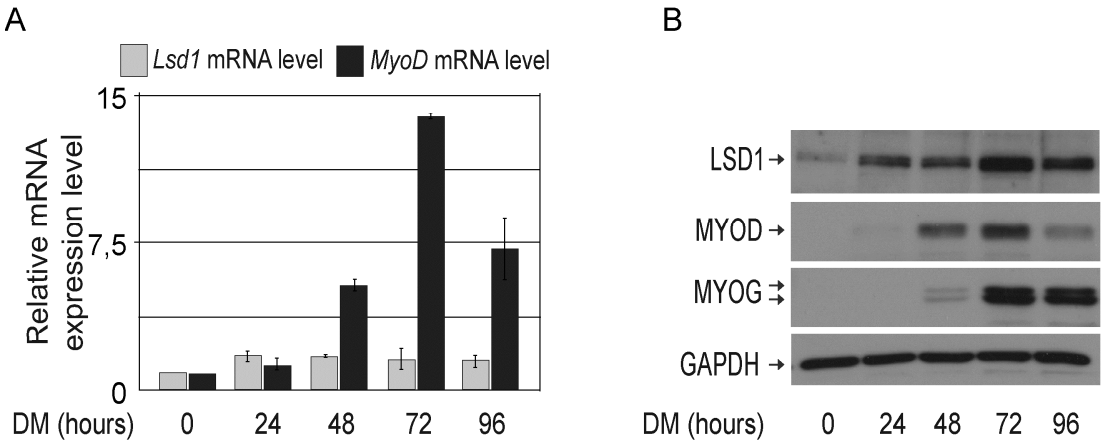


Figure S1. [LSD1 and MyoD expression during C2C12 myoblast differentiation], Related to Figure 1.

A) *Lsd1* and *MyoD* mRNA levels in C2C12 cells during differentiation. RT–qPCR values were normalized to the *Ppib* mRNA levels. mRNA levels are shown as the fold variation compared to C2C12 cells at DM0, i.e., in proliferation conditions. Data are represented as mean ± SEM of at least three experiments. B) LSD1, MYOD and MYOG immunoblots on C2C12 cell extracts during differentiation. GAPDH was used as a loading control.

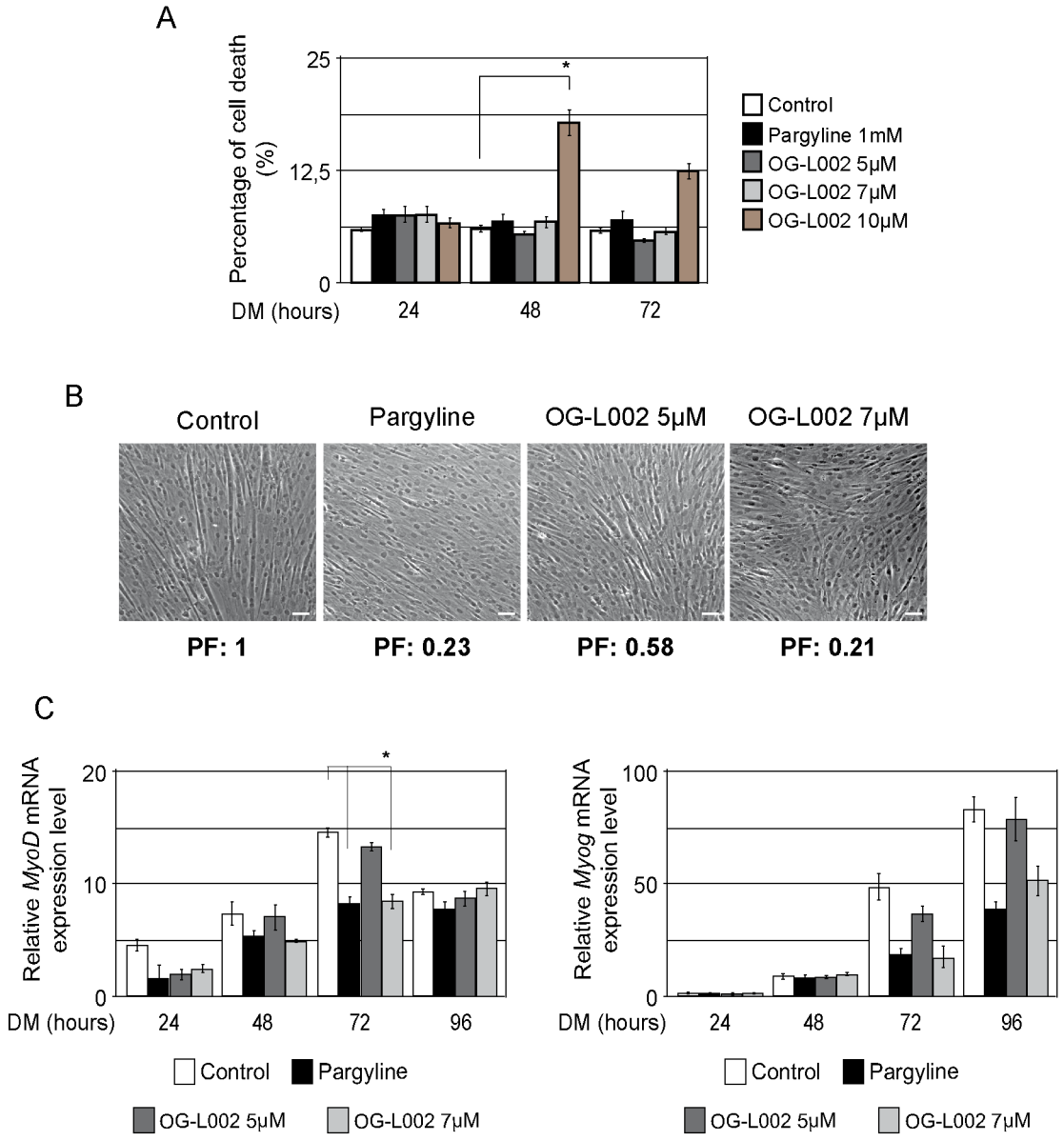


Figure S2. [LSD1 demethylase activity is required to induce myoblast differentiation], Related to Figure 1.

A) Percentage of C2C12 cell death at 24, 48 and 72 hours of differentiation after treatment with Pargyline 1mM and OG-L002 at three different concentrations (5µM, 7µM and 10µM). Measurements were made by cytometry analysis after cell suspension staining with propidium iodide. Data are represented as mean ± SEM of at least three experiments. B) Phase contrast images of Pargyline 1mM and OG-L002 (5µM and 7µM) treated C2C12 cells after 120 hours in DM. Percentage of fusion (PF), calculated as the proportion of cells containing two or more nuclei, are shown below the pictures. Scale bar: 50 µm. C) *MyoD* and *Myog* mRNA levels in C2C12 cells treated with Pargyline 1mM and OG-L002 5µM and 7µM during differentiation. RT–qPCR values were normalized to *Ppib* mRNA levels, and are shown as the fold difference with C2C12 at DM0. Data are represented as mean ± SEM of at least three experiments. *p <0,01 (Bonferroni test after one way ANOVA).

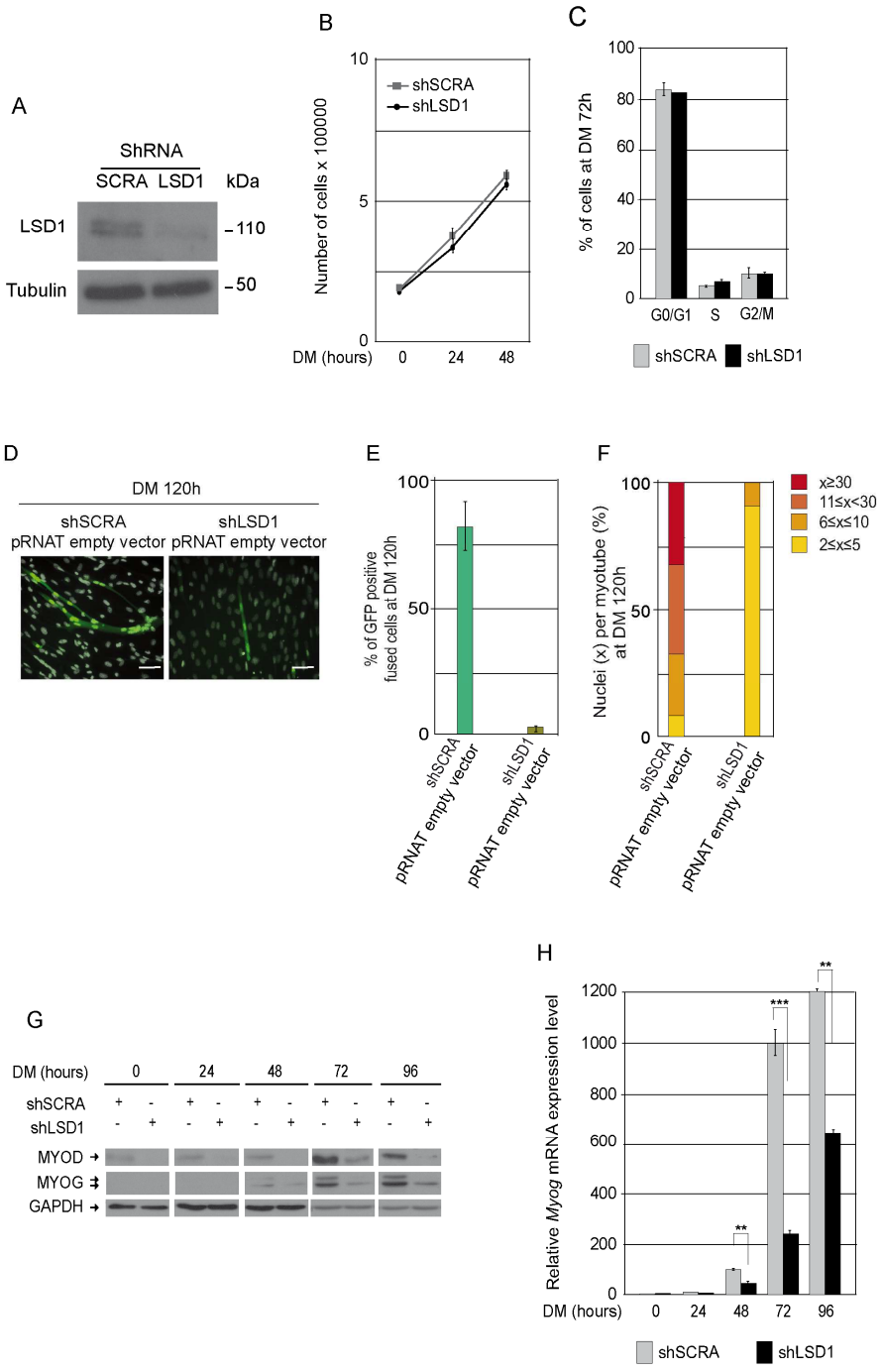


Figure S3. [Absence of LSD1 does not affect myoblast proliferation but impairs their differentiation], Related to Figure 1.

A) Immunoblot for LSD1 on shSCRA and shLSD1 cell extracts showing the efficiency of the shRNA targeting LSD1. Tubulin was used as a loading control. B) shLSD1 and shSCRA cell numbers at DM0, DM 24hours and DM 48 hours. C) shSCRA and shLSD1 cell cycle analysis by cytometry after 72 hours in DM. D) pRNAT vector expressing GFP was transfected in shLSD1 and shSCRA myoblasts to help distinguish cell contours. Cells were allowed to differentiate in DM for 120 hours and were stained with DAPI to visualize nuclei. Transfected cells, identified by green fluorescence, were observed by epifluorescence microscopy. Representative images of GFP positive shLSD1 and shSCRA cells are shown. DAPI was changed to grey to allow better visualization. Scale bars represent 50 µm. E) The percentage of fused

cells was calculated as the proportion of GFP positive cells containing two or more nuclei. F) The number of nuclei in 100 shLSD1- and 110 shSCRA- GFP positive cells was counted. G) MYOD and MYOG immunoblots on shSCRA and shLSD1 cell extracts. GAPDH was used as a loading control. H) *Myog* mRNA levels in shSCRA and shLSD1 cells during differentiation. RT–qPCR values were normalized to the *Ppib* mRNA levels, and are shown as the fold difference with shSCRA at DM0. Data are represented as mean ± SEM. **p < 0.01, ***p <0,001 (Bonferroni test after one way ANOVA).

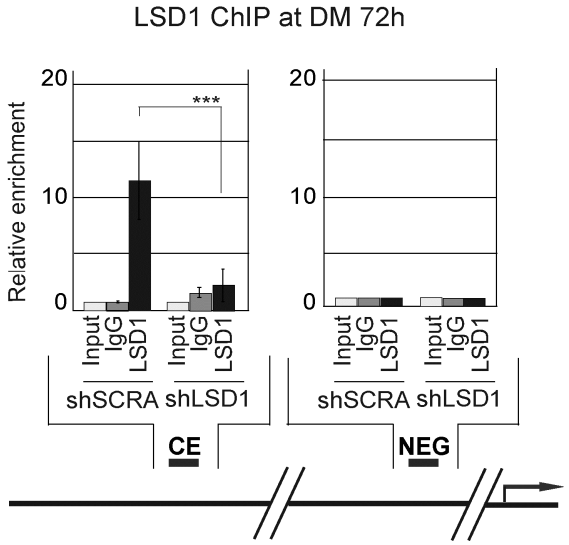


Figure S4. [Validation of LSD1 antibody], Related to Figure 2.

Localization of LSD1 at the Core Enhancer (CE) region of MyoD gene locus after 72 hours in DM. ChIP analysis was performed on shSCRA and shLSD1 cells with an anti-LSD1 antibody. Ct values were normalized to input. Two sites Core enhancer (CE) and Negative regions (NEG) were tested for RT-qPCR amplification. Data are shown as relative enrichment to the NEG region. Data are represented as mean ± SEM of at least three experiments. ***p < 0.0005 (Bonferroni test after one way-ANOVA).

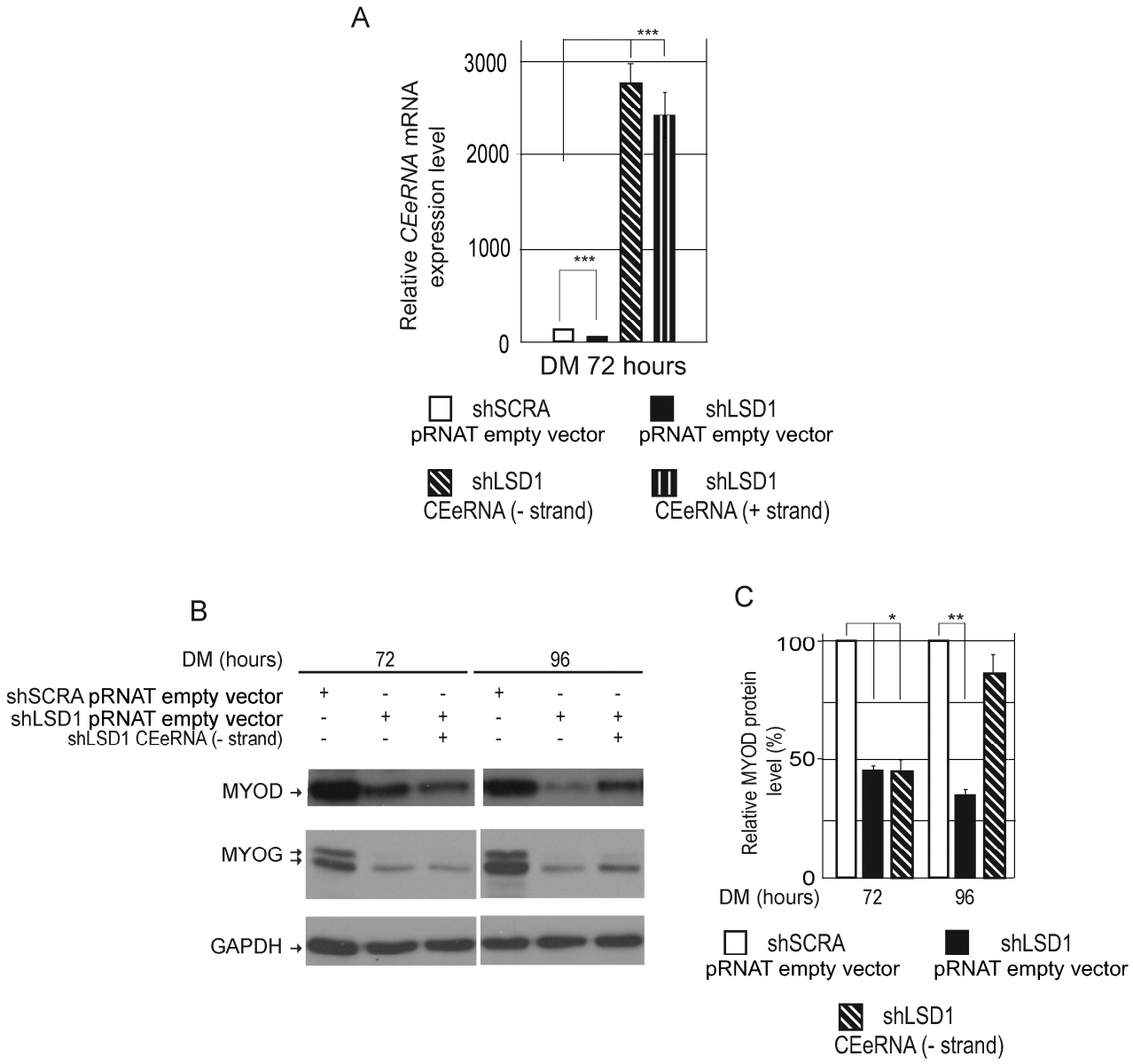


Figure S5. [CEeRNA expression is required for MyoD expression], Related to Figure 4.

A) CEeRNA mRNA levels in shSCRA transiently transfected with pRNAT empty vector, and in shLSD1 cells transiently transfected with empty pRNAT, CEeRNA (- strand) or CEeRNA (+ strand) vectors after 72 hours in DM. RT–qPCR values were normalized to *Ppib* mRNA levels and are shown as the fold difference with shSCRA at DM0. B) MYOD and MYOG immunoblots on extracts of shSCRA cells transiently transfected with empty pRNAT vector and shLSD1 cells transiently transfected with empty pRNAT or CEeRNA (- strand) vectors after 72 and 96 hours in DM. GAPDH was used as loading control. C) Relative MYOD protein levels were quantified using Image J software and compared to MYOD in shSCRA control cells. Data are represented as mean ± SEM of at least three experiments. *$p < 0.05$, **$p < 0.01$. ***$p < 0.005$ (Bonferroni test after one way ANOVA).

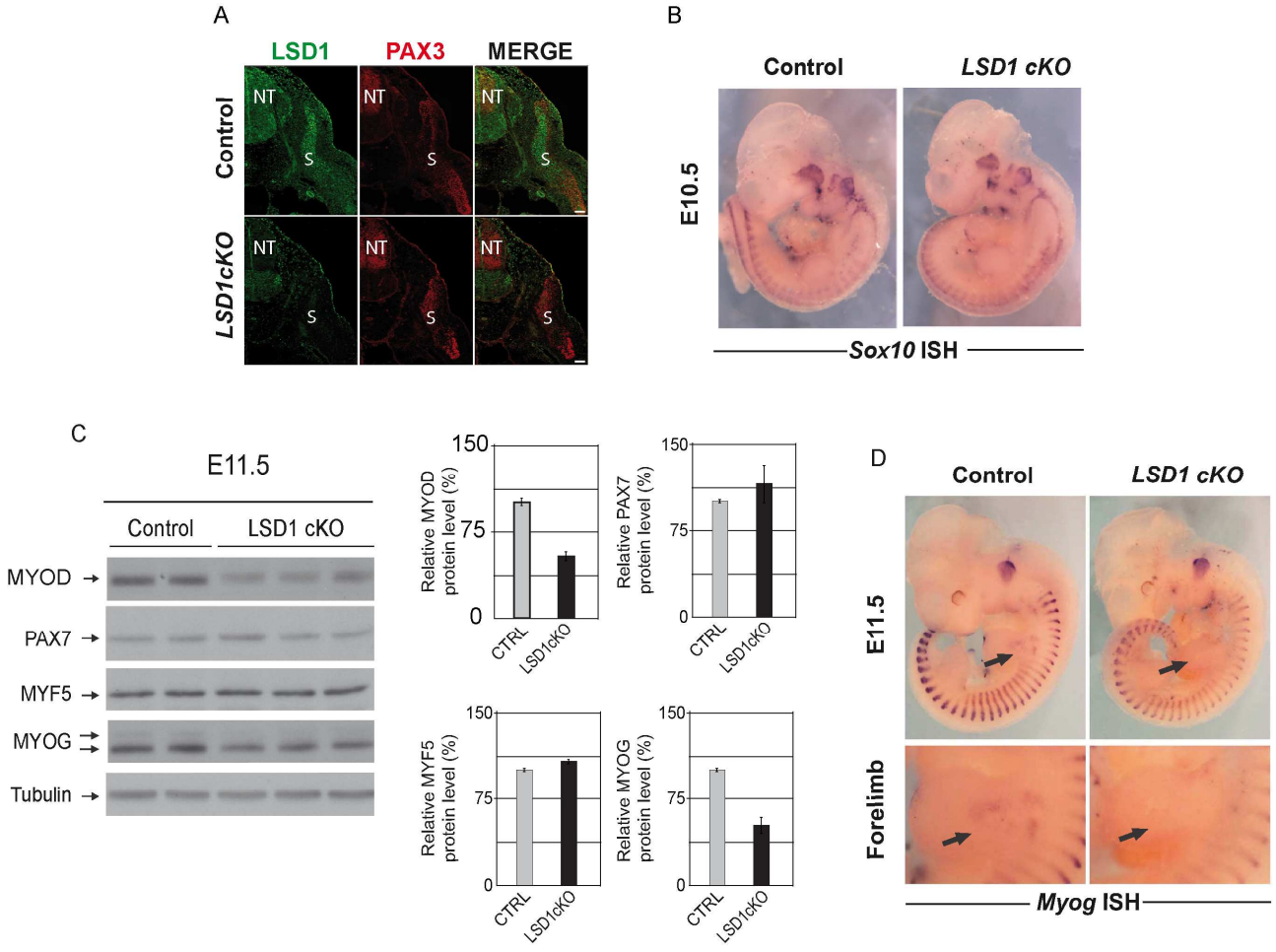


Figure S6. [LSD1 deficiency does not affect peripheral nervous system development but delayed myogenesis in vivo], Related to Figure 6.

A) LSD1 and PAX3 immunostainings of transverse sections of E11.5 control and LSD1cKO embryos in the neural tube (NT) and the somites (S). Scale bars represent 50 µm. B) Whole-mount *in situ* hybridization with a *Sox10* RNA probe in control and LSD1 cKO embryos at E10.5. C) MYOD, PAX7, MYF5 and MYOG protein levels were analyzed by immunoblotting E11.5 control (n=2) and LSD1cKO (n=3) total embryo protein extracts. Relative protein levels were quantified using Image J software and compared to levels in control embryos. D) Whole-mount *in situ* hybridization with *Myog* probe in control and LSD1 cKO embryos at E11.5. Arrowheads show forelimbs. Close-up of the forelimb region (lower panels).

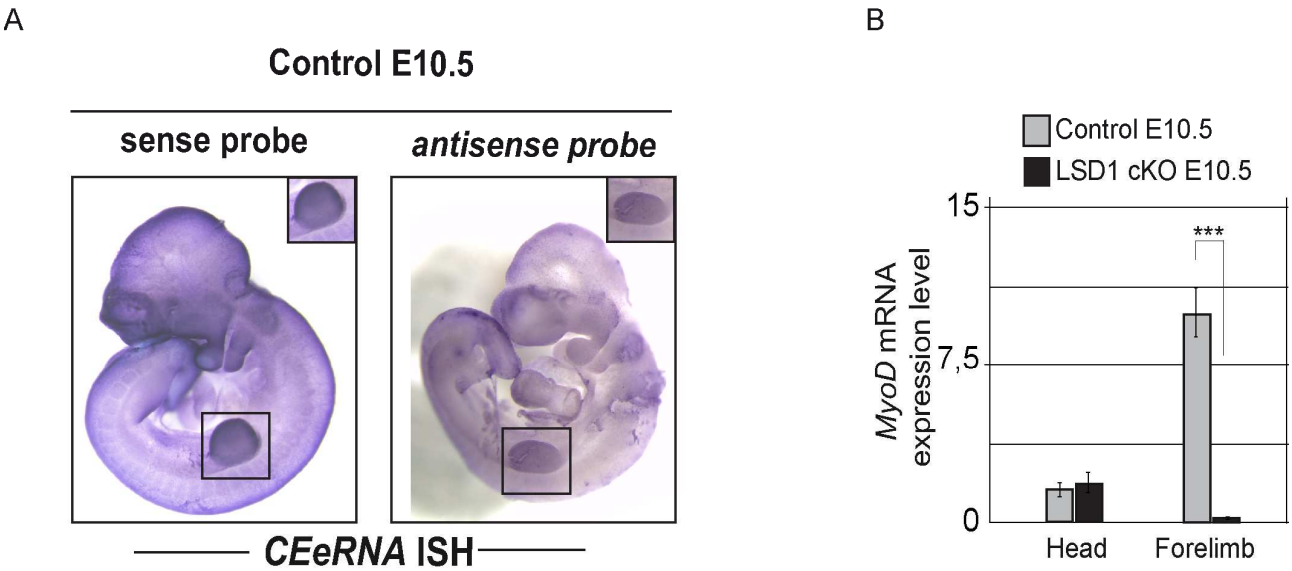


Figure S7. [CEeRNA (– strand) expression in control and LSD1cKO E10.5 embryos], Related to Figure 7.

A)Whole-mount *in situ* hybridization with two CEeRNA RNA probes in E10.5 control embryos. Antisense probe hybridizes the CEeRNA (+ strand) while the sense probe binds the CEeRNA (– strand). Insets are higher magnification of the forelimb region. B) *MyoD* mRNA levels in dissected forelimbs and heads from control (n=6) and LSD1cKO (n=4) embryos at E10.5. RT–qPCR values were normalized to the *Ppib* mRNA levels. Data are represented as mean ± SEM. ***$p < 0.005$ (Bonferroni test after one way ANOVA).

Supplemental Experimental procedures

List of oligonucleotides

Gene or region	Application	Sense primer	Antisense primer
MyoD	RT-qPCR	AGCACTACAGTGGCGACTCA	GCTCCACTATGCTGGACAGG
Ppib	RT-qPCR	GATGGCACAGGAGGAAAGAG	AACTTTGCCGAAAACCACAT
CEeRNA	RT-qPCR	GCCAAGTATCCTCCTCCAGC	AAGCTGAGCACTCTGGGAGA
Myog	RT-qPCR	CAATGCACTGGAGTTCGGTC	ACAATCTCAGTTGGGCATGG
MyoD TSS	ChIP	AGATAGCCAAGTGCTACCGC	CCAGGGTAGCCTAAAAGCCC
MyoD NEG	ChIP	CCCTTCATCCAGGGCACTAC	TTGGGAACCCAGCAGTAAGC
MyoD CE	ChIP	CTAAACACCAGGCATGAGAGG	ACTCACTTTCTCCCAGAGTTGC
CEeRNA	Cloning	CACGTGATGAAAAGTGAGGACA	TGACGTCACCAACAACGGTA
CEeRNA	ISH	GGAGCACCCCACAACATGAGC	AGTCTGTGCGGGTGAGGCAG

List of antibodies

Name	Application	Compagny
Anti-LSD1	ChIP 5µg/IP IF 1:100	Abcam
Anti-LSD1	Western blotting 1:1000	Active motif®
Anti-MYOD	Western blotting 1:500 IF 1:200	Santa-cruz Biotecnology®
Anti-MYOG	Western blotting 1:200	Santa-cruz Biotecnology®
Anti-GAPDH	Western blotting 1:10000	Cell signaling technology®
Anti-H3K4me1	ChIP 5µg/IP	MilliporeTM
Anti-H3K4me3	ChIP 5µg/IP	MilliporeTM
Anti-H3K9me2	ChIP 5µg/IP	Active motif®
Anti-H3K9me3	ChIP 5µg/IP	MilliporeTM
Anti-H3	ChIP 5µg/IP	Active motif®
Anti-MYF5	Western blotting 1:500	Santa-cruz Biotecnology®
Anti-PAX3	IF 1:100	DSHB
Anti-PAX7	Western blotting 1:200	Santa-cruz Biotecnology®
Anti-RNApol II	ChIP 5µg/IP	Abcam
Anti-α Tubulin	Western blotting 1:20000	Sigma

PHF2 and skeletal muscle differentiation in vitro

Phf2 inhibition in cultured myoblasts prevents MyoD expression and myoblasts differentiation

So far, no evidences have been shown to assess if PHF2 may be directly involved in skeletal muscle differentiation. During myoblasts differentiation, an increase in PHF2 protein level was observed one day before the increase of the LSD1 and MYOD proteins level (**Figure 19**). Thus, we asked if PHF2 could play a role in the commitment of myoblast cells into differentiation process, modulating *MyoD* expression in muscle precursor cells.

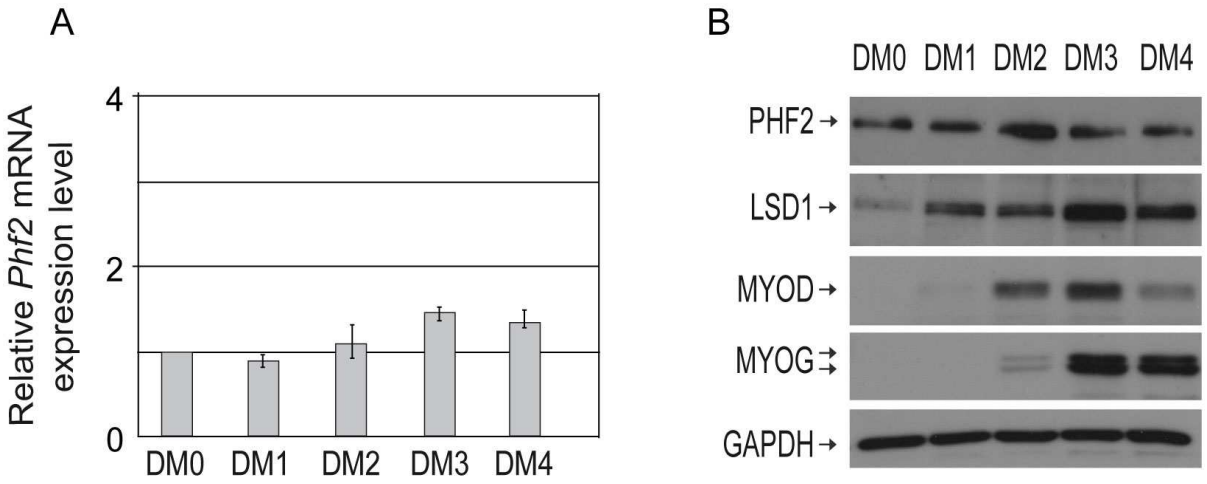


Figure 19: A) *Phf2* mRNA levels in C2C12 cells during differentiation. RT–qPCR values were normalized to the *Ppib* mRNA levels. mRNA level is shown as the fold variation compared to C2C12 cells at DM0. Data are represented as mean ± s.d. **B)** PHF2, LSD1, MYOD and MYOG western blots on C2C12 cells during differentiation.

For this purpose, I have tested 5 different shRNAs stably infecting C2C12 myoblasts with lentiviral vectors containing the mouse Phf2-targeting sequences (purchased from Open biosystem). As a control, the pLKO.1 vector SHC016V purchased from Sigma was used (**Figure 20**). These cell lines will be named shPHF2#0-4 and shSCRA respectively.

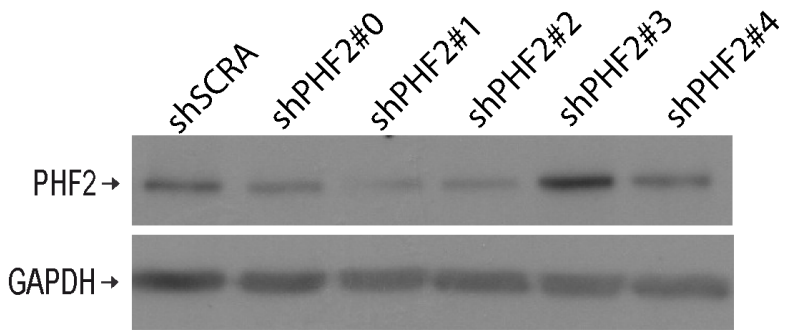


Figure 20: Specific shRNA-mediated knockdown of PHF2 in C2C12 cells.

The shPHF2#1 and #2 cells are characterized by the lowest PHF2 protein expression compared to shSCRA cells, and have been used for all the myoblast differentiation experiments. Immunofluorescence experiments have also confirmed that PHF2 is mostly localized in the nucleus of cells and is efficiently ablated in shPHF2# 1and #2 cells (**Figure 21**).

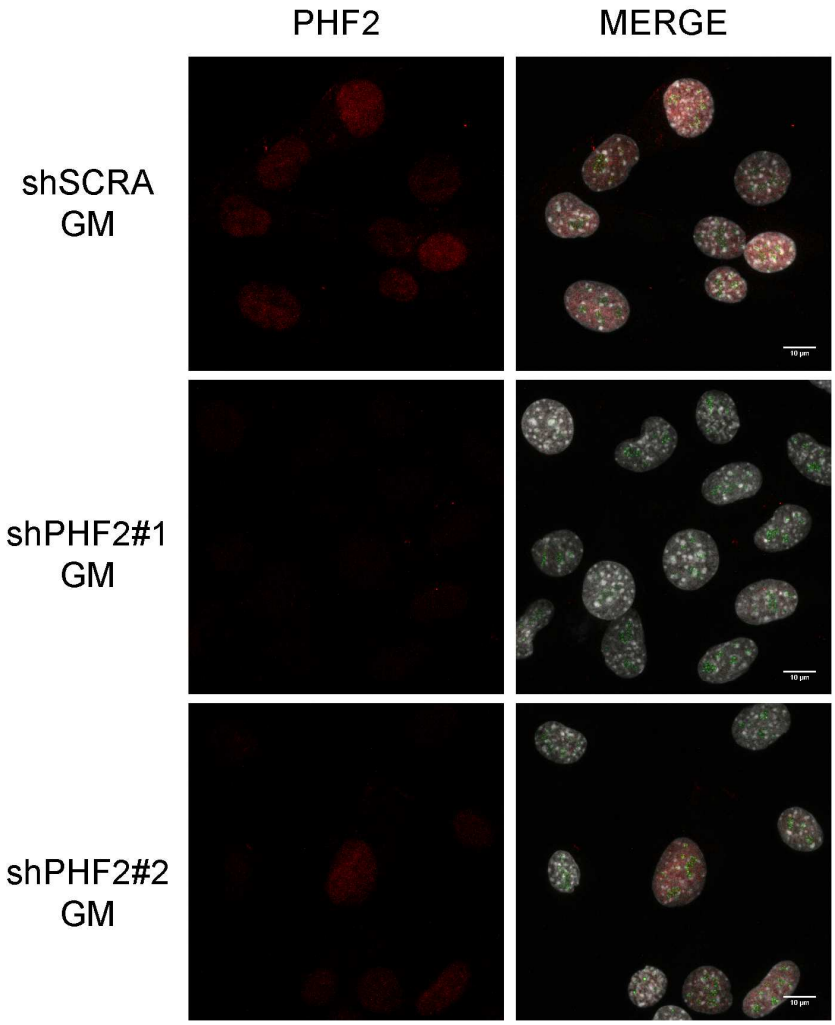


Figure 21: Nuclear localization of PHF2 in C2C12 cells. Immunofluorescence analysis. shSCRA, shPHF2#1 and #2 cells were immunostained with a rabbit polyclonal PHF2 antibody (red) and their nuclei were stained with DAPI (grey). Scale bar: 10um.

Then I asked whether *Phf2* down-regulation could be involved in myoblast differentiation affecting myotube formation. Indeed, after 5 days shPHF2#1 and #2 cells showed reduced ability to fuse and form myotubes (**Figure 22A**).

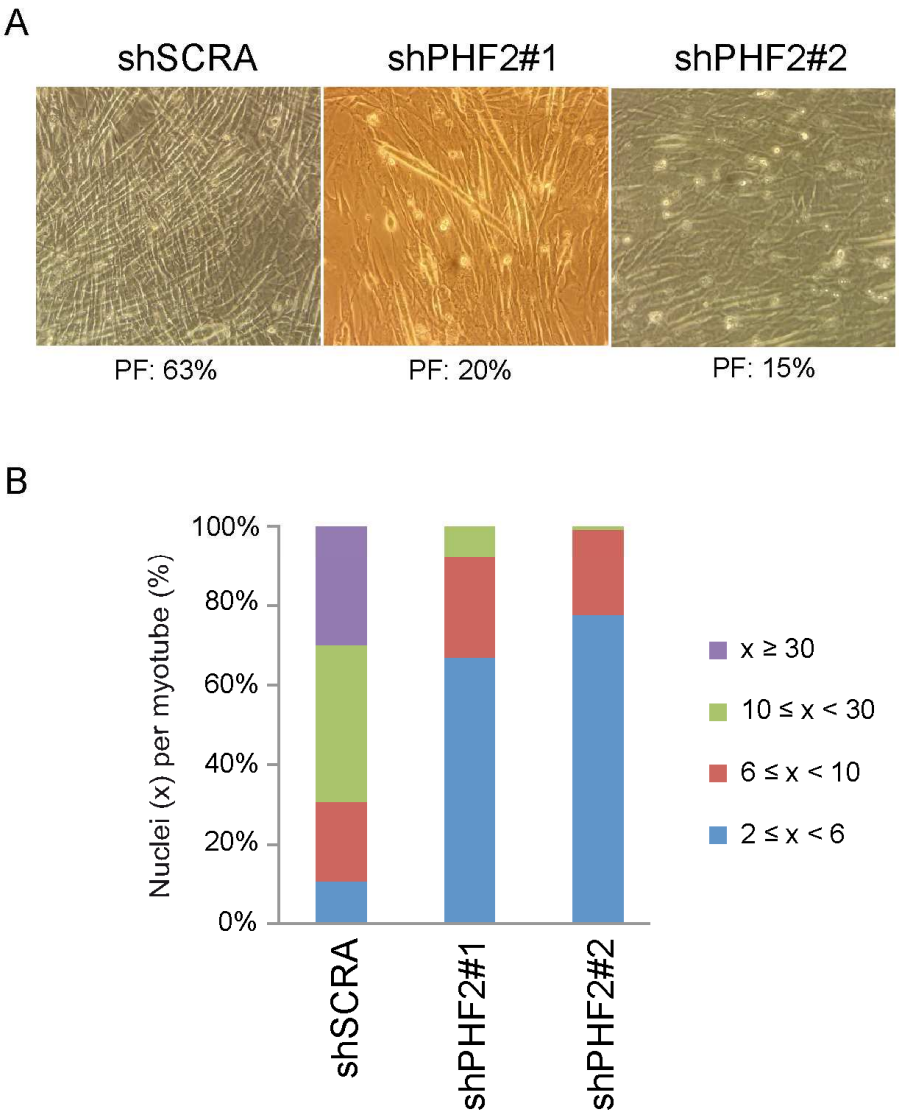


Figure 22: A) Phase contrast images of shSCRA, shPHF2#1 and shPHF2#2 cells after 5 days in differentiation medium. The percentage of fusion (PF) is the proportion of cells containing two or more nuclei. **B)** shSCRA, shPHF2#1 and shPHF2#2 cells were transfected with pRNAt vector grown in GM for 24 hours 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 140 pRNAt-transfected shPHF2#1 cells, 120 pRNAt-transfected shPHF2#2 and 110 pRNAt-transfected shSCRA.

Actually only about 20% of shPHF2#1 and #2 cells underwent fusion with the majority of myotubes (**Figure 22A**) with 2-5 nuclei (**Figure 22B**) whereas 63% of shSCRA cells formed myotubes containing more than 10 nuclei (**Figure 22**). These results are reminiscent of shLSD1 cells phenotype (Scionti et al., 2017) and are consistent with the increased *Phf2* expression after *in vivo* denervation, supporting the hypothesis that PHF2 has a key role during myoblast differentiation.

As previously described during muscle differentiation there is a hierarchical relationship between MRFs whereby MYOD is implicated in commitment of myoblasts, while Myogenin acts to induce myocytes fusion into myotubes.

Considering the effect of PHF2 on cell fusion and myotube formation I wondered whether PHF2 could affect the expression of *MyoD*. Thus shPHF2#1, #2 and shSCRA cells were induced to differentiate and mRNA and protein expression level of MYOD and Myogenin (MYOG) were monitored during 4 days after DM addition (**Figure 23**).

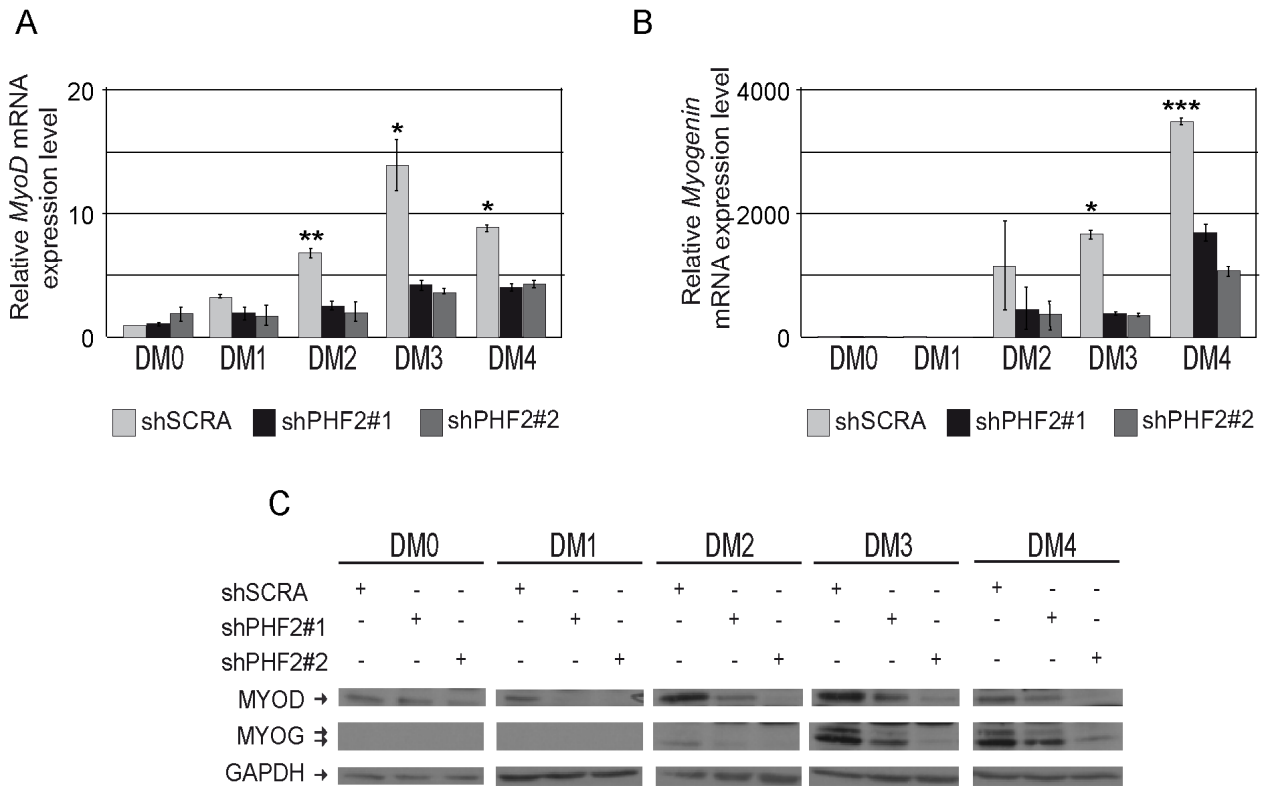


Figure 23: A) mRNA expression levels of *MyoD* gene under shSCRA, shPHF2#1 and #2 during differentiation. RT–qPCR values were normalized to the expression levels of the *Ppib* gene, and are shown as the fold difference against shSCRA DM0. **B)** mRNA expression levels of *Myogenin* gene under shSCRA, shPHF2#1 and #2 during differentiation. RT–qPCR values were normalized to the expression levels of the

Ppib gene, and are shown as the fold difference against shSCRA DM0. All histogram data are means ± s.d. of triplicate results. *$p < 0.01$ **$p < 0.002$, ***p <0,0001 (Bonferroni test after ANOVA). **C)** Total MYOD and MYOG protein levels were determined by western blot during differentiation in shSCRA, shPHF2#1 and #2.

As shown in Figure 23 during myoblast differentiation *MyoD* mRNA and protein level in shPHF2#1 and #2 cells is significantly lower compared to shSCRA cells. As expected *Myogenin* mRNA and protein level in shPHF2#1 and #2 cells does not reach the same level of shSCRA (**Figure 23 B-C**).
These results clearly show that PHF2 depletion perturbs *MyoD* expression and muscle differentiation process.

PHF2 is recruited on the MyoD core enhancer during myoblast differentiation

Given the inhibition of *MyoD* expression I observed *in vitro* with the inactivation of *Phf2* during myoblasts differentiation, I investigated a possible direct involvement of PHF2 in the *MyoD expression*. ChIP experiments were performed with an anti PHF2 antibody to detect the presence of PHF2 on the core enhancer (CE), which is directly involved in the increase of *MyoD* expression (Mousavi et al., 2013).
After 3 days in differentiation medium, PHF2 was strongly enriched on CE region compared to negative one (NEG) (**Figure 24A**). Interestingly, similarly to LSD1, the enzymatic activity of PHF2 cannot be responsible for the histone modification at the CE at the onset of differentiation (Scionti et al., 2017). However, the *CEeRNA* expression in shPHF2#1 and #2 myocytes after 3 days in DM is not increased compared to the level reached in the shSCRA cells and such difference is statistically different (**Figure 24B**).
Taken together these results strongly support the idea that PHF2, as LSD1, controls *MyoD* expression through the activation of the CE region.

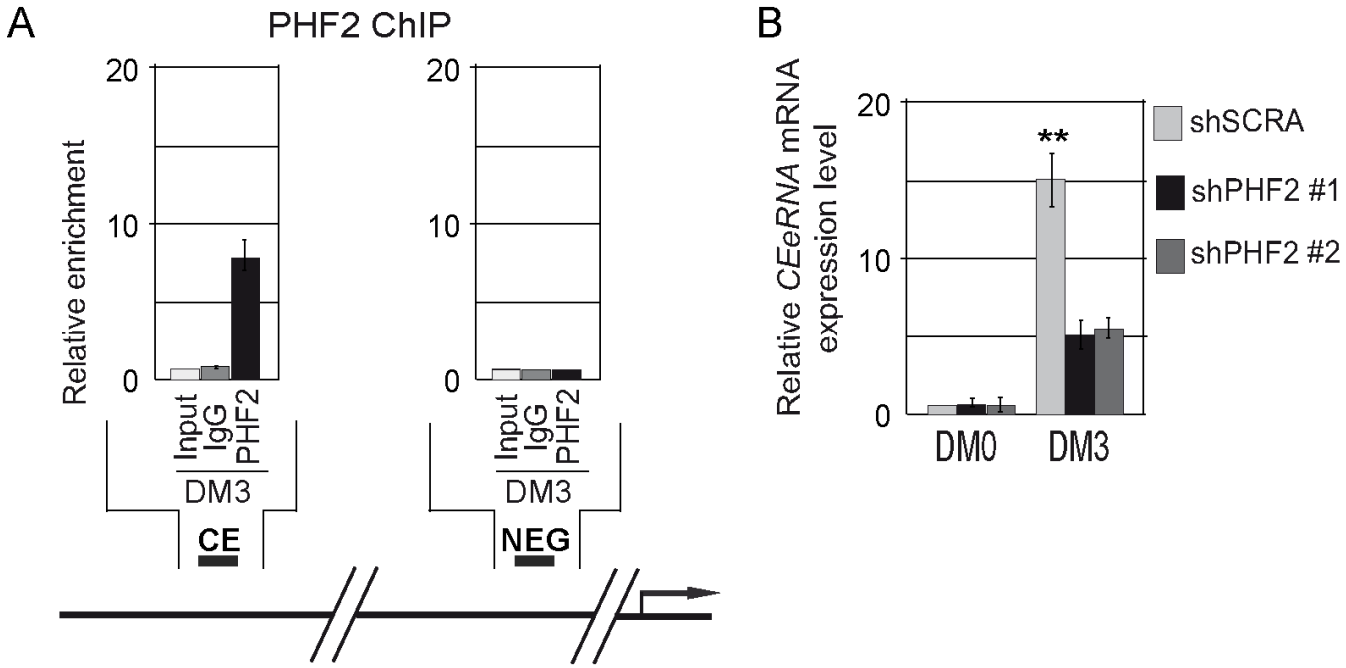


Figure 24: A) Localization of PHF2 at the *Myod* Core enhancer region. ChIP analyses were performed in C2C12 cells with an anti-PHF21 antibody. Two sites (CE; NEG) were tested for RT-qPCR amplification. Enrichment values were normalized to input, and shown as the fold difference relative to region NEG. Input (white bars) control IgG (light grey bars), anti-PHF2 antibody (dark grey bars). **B)** *CEeRNA* expression in shSCRA, shPHF2#1 and shPHF2#2 cells after 3 days of differentiation. RT–qPCR values were normalized to the expression levels of the *Ppib* gene, and are shown as the fold difference against shSCRA DM0.
All histogram data are means ± s.d. of at least triplicate results. $^{**}p < 0.01$. (Bonferroni test after ANOVA).

PHF2 ablation affects LSD1 protein stability

Since ablation of PHF2 phenocopies the one observed down-regulating LSD1 (Scionti et al., 2017) I tested the hypothesis that PHF2 affects *CEeRNA* expression indirectly regulating *Lsd1* gene expression. Thus shPHF2#1, #2 and shSCRA cells were induced to differentiate and *Lsd1* mRNA level was monitored during 4 days after DM addition. As shown in Figure 24, during myoblast differentiation the expression of *Lsd1* is not affected by the ablation of PHF2 (**Figure 25**).

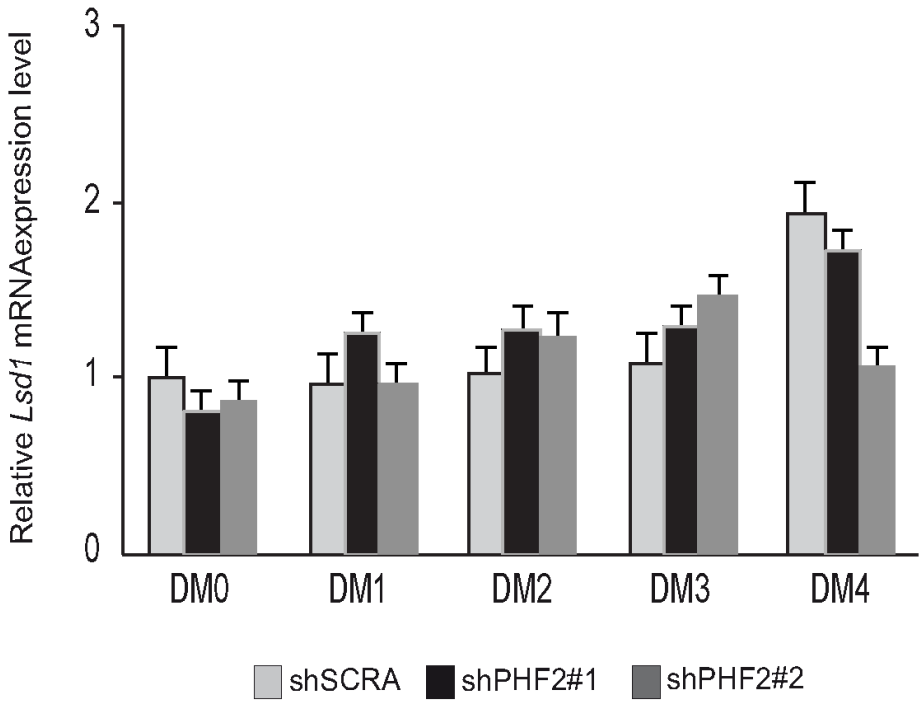


Figure 25: mRNA expression levels of *Lsd1* gene under shSCRA, shPHF2#1 and #2 during differentiation. RT–qPCR values were normalized to the expression levels of the *Ppib* gene, and are shown as the fold difference against shSCRA DM0. All histogram data are means ± s.d. of triplicate results.

Surprisingly, the LSD1 protein level at DM3 is strongly reduced in the shPHF2#1 and #2 cells and is comparable to the level of LSD1 protein in the shLSD1 cells after 3 days in DM (**Figure 26 A-B**).

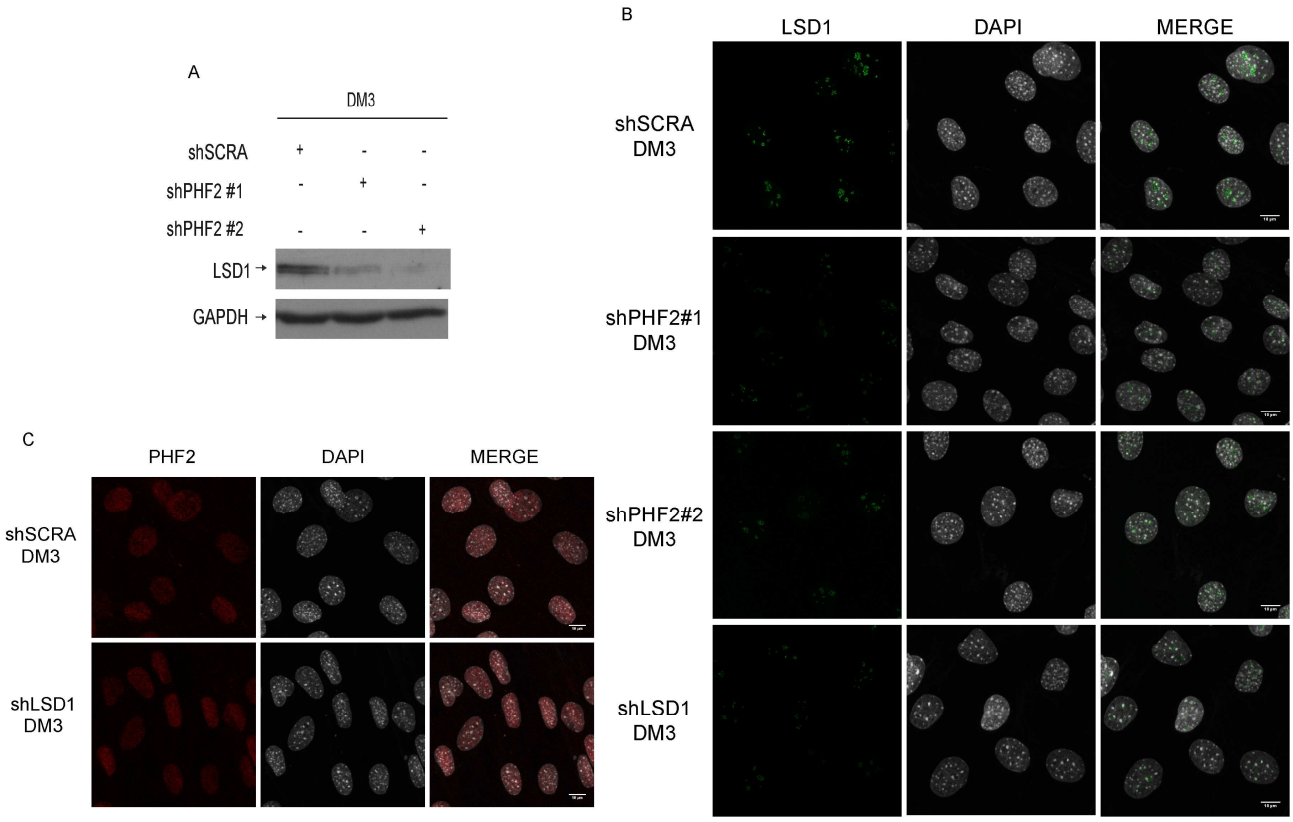


Figure 26: A) Total LSD1 protein levels were determined by western blot at 3 days of differentiation in shSCRA, shPHF2#1 and #2. **B)** Immunofluorescence analysis, shSCRA, shPHF2#1, #2 and shLSD1 cells were immunostained with a mouse monoclonal LSD1 antibody (green) and their nuclei were stained with DAPI (grey). C) Immunofluorescence analysis, shSCRA and shLSD1 cells were immunostained with a rabbit polyclonal PHF2 antibody (red) and their nuclei were stained with DAPI (grey). Scale bar: 10um.

Moreover the level of PHF2 in shLSD1 cells after 3 days in DM does not differ from the shSCRA cells (**Figure 26C**) suggesting that PHF2 regulates the turnover of LSD1 protein. Thus I performed a time course treatment with cyclohexamide, which blocks the protein synthesis, on shSCRA, shPHF2#1 and #2 at DM3. While nuclear LSD1 protein in shSCRA cells remain stable even after 4 hours of CHX treatment, in shPHF2#1 and #2 the 50% of LSD1 is already degraded at the same time point of treatment, supporting the hypothesis that PHF2 is involved in the stabilization of LSD1 (**Figure 27**).

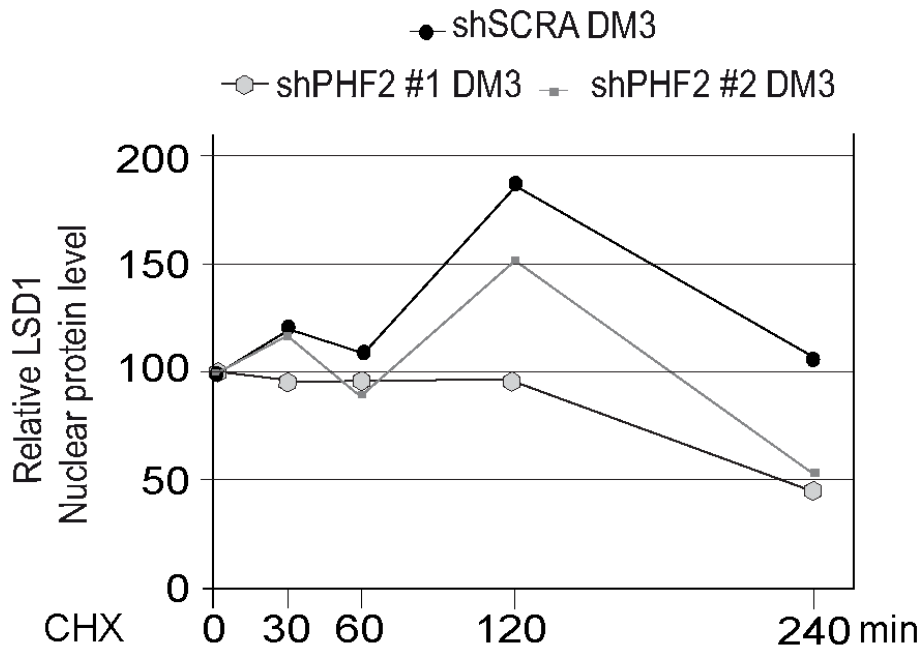


Figure 27: Time course Cyclohexamide (CHX) treatment on shSCRA, shPHF2#1, #2 at 3 days of differentiation. Quantification of the relative levels of LSD1 protein after the CHX treatment. The amount of nuclear LSD1 protein was normalized to the histone 3 levels.

Since ablation of PHF2 reduced the stability of LSD1 protein and such event might be responsible for the phenotype observed in shPHF2#1 and #2 cells, I checked if LSD1 is still recruited on the CE region. ChIP experiments performed with an anti LSD1 antibody in shPHF2#1 and #2 cells after 3 days in DM have demonstrated that LSD1 is still but much less enriched on the CE region compared to shSCRA cells. These data suggested that while PHF2 is not necessary for LSD1 recruitment on the CE, it is responsible of stabilizing LSD1 and the transcriptional complex and thus allowing the expression of *CEeRNA* (**Figure 28**).

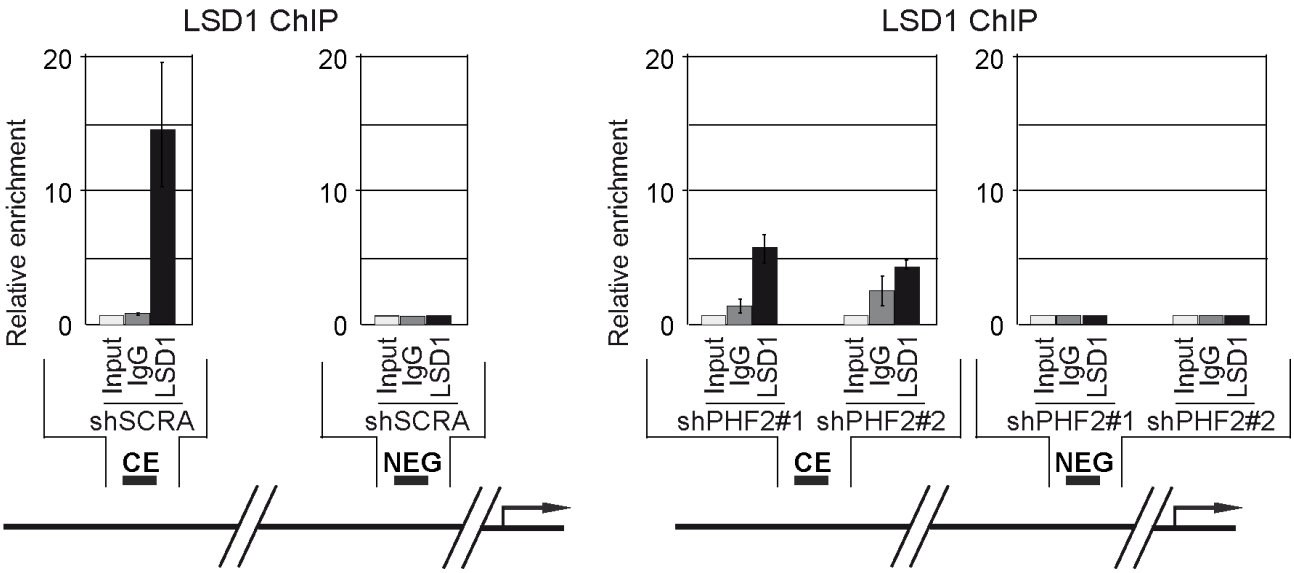


Figure 28: ChIP analyses on shSCRA (left) and shPHF2#1 and #2 cells after 3 days in DM using an antibody against LSD1. Two sites (CE; NEG) were tested for RT-qPCR amplification. Enrichment values were normalized to input, and shown as the fold difference relative to region NEG. Input (white bars) control IgG (light grey bars), anti-LSD1 antibody (dark grey bars). All histogram data are means ± s.d. of at least triplicate results.

PHF2 and LSD1 are in same complex

These results support the idea that PHF2 could be necessary for LSD1 function, thus I have tested the hypothesis that PHF2 and LSD1 interact each other and cooperate to the proper activation of the CE region.

Co-immunoprecipitation experiments in the nuclear fraction of C2C12 after 3 days in DM have been performed and as shown in Figure 29 PHF2 and LSD1 bound each other, suggesting that PHF2 might regulate the turnover of LSD1 via direct interaction.

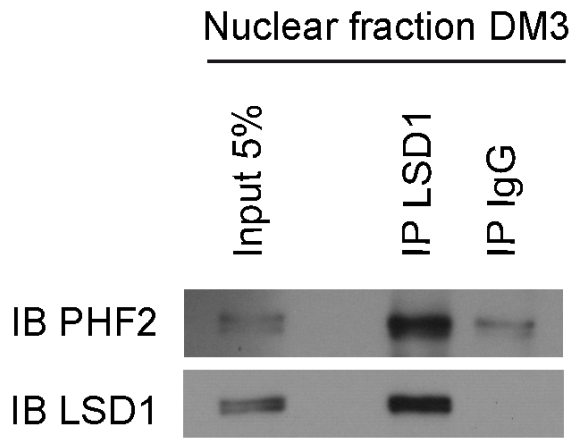


Figure 29: Nuclei of C2C12 cells were isolated and lysed under non-denaturing conditions after 3 days in DM. Nuclear LSD1 was immunoprecipitated and membrane was immunoblotted for PHF2. The blot was then stripped and re-probed for LSD1.

PHF2 and muscle development in vivo

Generation of a Pax3 conditional Knock-out mouse for PHF2

Phf2 mRNA is widely expressed at low level in all developing organs during mouse embryogenesis. However, the highest level of *Phf2* mRNA is in the neural tube and in the dorsal root ganglia (Hasenpusch-Theil et al., 1999).

Based on the *in vitro* results I have collected, to further delineate the function of PHF2 in muscle lineage determination and differentiation, PHF2 has been conditionally inactivated in muscle progenitors cells by crossing Phf2 $^{Flox/Flox}$ mice (Okuno et al., 2013) and Pax3$^{cre/+}$: Phf2 $^{Flox/+}$ transgenic mice (Li et al., 2000), hereafter named PHF2cKO.

Conversely from LSD1cKO and according to previous report (Okuno et al., 2013), knocking down *Phf2* in PAX3 positive cells does not result in a lethal phenotype, with the ratio among genotypes respected at birth.

Nevertheless, CTRL and PHF2cKo mouse embryos were collected from E 11.5. In collaboration with Dr Fredéric Relaix, in situ hybridization experiments have been performed using a specific probe on *MyoD* and *Myogenin* gene. Interestingly neither *MyoD* nor *Myogenin* mRNA levels are decreased in PHF2cKO embryos at E11.5 compared to control one (**Figure 30**). However, while these *in vivo* results revealed that PHF2 is not

involved in the regulation of *MyoD* expression during embryogenesis, the *in vitro* data suggested that PHF2 role could be carried out in adulthood during muscle regeneration.

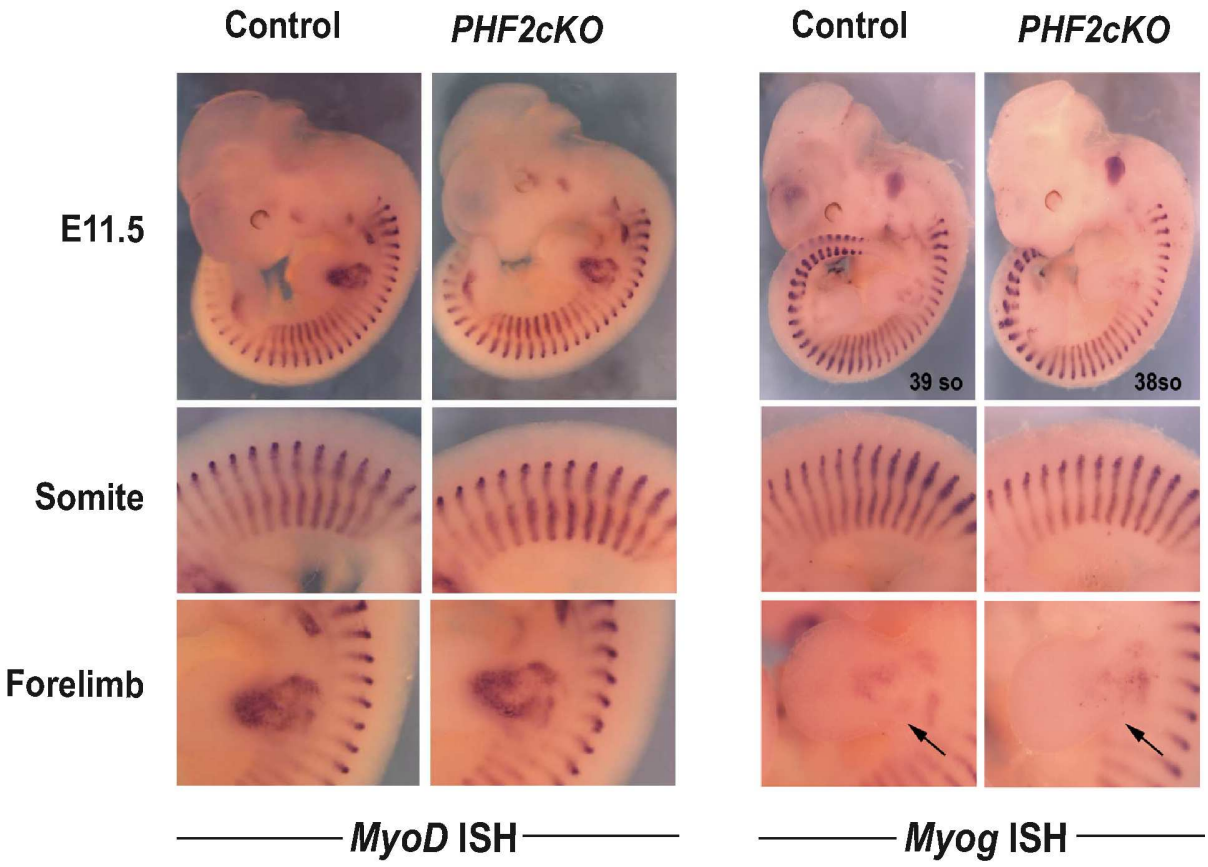


Figure 30: Control and PHF2cKO embryos at E11.5 were hybridized with *MyoD* and a *Myog*-specific probes.

Materials and Methods

Cell Lines, Culture Conditions, Infection, and Transfection

C2C12 mouse myoblasts were maintained as myoblasts in growth medium (GM): Dulbecco's modified Eagle's medium (DMEM) supplemented with 15% fetal calf serum and antibiotics. Differentiation of C2C12 cell was induced by switching the cell into low serum medium (DMEM+2% of Horse serum, DM). For stable knockdown of *Phf2* in C2C12 cells, 5 different lentiviral vectors containing the mouse Phf2-targeting sequences pLKO.1-sh-PHF2 (TRCN0000104900 ,01, 02, 03 and 04, shPHF2#0-4), purchased from Open Biosystem, were used. As an shSCRA, the pLKO.1 vector SHC016V, purchased from Sigma-Aldrich, was used. Twenty-four hours after lentiviral infection, C2C12 were selected with puromycin (1 µg/mL) for 14 days.

Cell transfections with the pRNAT vector (pRNAT-CMV3.1/Neo by GenScript), was performed as follows: 300,000 shSCRA, shPHF2#1 and #2 cells were seeded in 35-mm petri dishes. Three hours later, cells were transfected with pRNAT vector with jetPRIME (polyplus transfection) according to the manufacturer's instructions. Twenty-four hours after transfection, cells were seeded (150,000 cells/35-mm petri dishes) in DM for 5 days for nuclei counting.

For protein stability assay, cells were maintained in DM for 3 days and then treated for the indicated times by adding 50 µg/ml of cycloheximide (Sigma) to inhibit protein synthesis. Nuclear proteins were extracted as stated below and analyzed by western blotting.

Protein Cell Fractionation

Nuclear proteins were prepared from shSCRA, shPHF2#1 and #2 after 3 days of DM addition. After pelleted cells and rinsed them in PBS buffer, cells were allowed to swell for 15 min in ice-cold buffer A (20mM Tris-HCl ph8, 1mM EDTA, 5mM DTT, protease inhibitors and phosphatase inhibitors). Cells were then disrupted with 20 strokes in a dounce homogenizer using a loose-fitting pestle. Nuclei were pelleted at 1,500g for 5 min and then resuspended in buffer B (20mM Tris HCl ph8, 20% glycerol, 0,42M NaCl, 1,5M $MgCl_2$, 0,2mM EDTA, 0,5mM DTT, protease inhibitors and phosphatase inhibitors) and incubated for 30 min at 4°. Nuclei were then sonicated for 10 min (30 sec ON and 30 sec OFF). Nuclear protein extracts were collected after 30 min of centrifugation at 14,000g. Nuclear protein concentration was measured and analyzed by western blotting.

Co-immunoprecipitation

Interactions between PHF2 and LSD1 were performed by co-immunoprecipitation assay. One mg of C2C12 nuclear extracts at DM3 were diluted in IP buffer (10mM Tris-HCl ph8, 150mM NaCl, 0,1% NP40, 10% glycerol, protease inhibitors and phosphatase inhibitors) and immunoprecipitated with 30µl of Protein A-Sepharose 4B fast flow (Sigma), covalently conjugated with LSD1 antibody overnight at 4°. Immunoprecipitated nuclear proteins were loaded into 6% SDS-PAGE gel before electrophoretic transfer onto PVDF membrane. Immunoblots were performed with enhanced chemiluminescence (ECL) PLUS reagent (Amersham or GE Healthcare) according to the manufacturer's instructions.

Statistical analysis

Statistical significance was determined by Bonferroni test after one-way ANOVA using GraphPad Prism version 5.00 for Windows (Graph-Pad, http:// www.graphpad.com). $p < 0.05$ was considered significant.

Perspectives

LSD1 and PHF2 function in satellite cells upon muscle injury

In this thesis by using both molecular and genetic approaches, I provide the first evidence that LSD1 and PHF2 play a role in the early steps of skeletal myogenesis. They are both recruited on the CE region and regulate the master gene, *MyoD*, via the activation of the *CEeRNA* (**Figure 31**). Therefore, defect in the activation of the core enhancer region led to a delay in the timely increased of *MyoD* expression. Indeed, it is important to point out that myogenesis is not completely inhibited in the absence of these enzymes but it is delayed compared to control. Moreover, while during embryonic myogenesis, the myogenic regulatory factors (MRFs) play redundant roles in myogenic commitment and differentiation (Rudnicki et al., 1992), during post-natal myogenesis the absence of *MyoD* expression is not compensated by the increased expression of others MRFs, indicating that MRFs achieve different function in adulthood (Ustanina et al., 2007). Consistently, MYOD-KO muscles, upon injury, show a severe regenerative defect, while preserving their satellite cells pool (Asakura et al., 2007).

Muscle is made of very long-lived cells and thus has to adapt to physiological and environmental changes throughout life. It is therefore not surprising that muscle cells have developed unique plasticity skills to allow constant adaptation.

The plasticity of skeletal muscles relies on the presence of resident quiescent satellite cells, which confer to skeletal muscles unique regenerative capacities. Interestingly satellite cells have the same embryonic origin than embryonic muscle precursors. When activated, these cells can participate to physiological hypertrophy but their main function is to repair or replace muscle fibers when necessary, i.e. after mechanical injury, too intense muscle exercise or during aging, or in pathological situations such as muscle dystrophies. Indeed, in response to injury or disruption of the basal lamina, while a subset of the satellite cells are activated (expressing *MyoD*), proliferate and either fuse to form multinucleated myotubes, others re-establish a residual pool of quiescent satellite cells that have the capacity of supporting additional rounds of growth/regeneration. Maintaining the equilibrium between these two events is crucial for muscle homeostasis. Thus, it is of great importance unveiling how the composition and activity of the complexes that regulate muscle genes expression, such as *MyoD,* and chromatin are regulated by extracellular inputs and especially how membrane receptors, cytoskeleton, intracellular signaling pathways and chromatin modifications are linked.

Results presented here suggest that LSD1 and PHF2 might act as a molecular switch between self-renewal and differentiation of satellite cells, a cell choice that is critical to simultaneously ensure satellite cell pool maintenance and also generating differentiated cells. 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. Indeed I 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 or PHF2 activity, and thus delaying *MyoD* expression during dystrophic or aging conditions would slow down but not affect satellite cells differentiation thus preventing the premature depletion of satellite cell pool.

LSD1 and PHF2 candidate targets for regenerative medicine

One of the challenges for regenerative medicine is to improve the therapeutic stem cell transplantation. Indeed as soon as the satellite cells are activated or isolated and cultured on matrigel-coated plastic dishes they increase the expression of *MyoD* and proliferate loosing their stemness fashion, resulting in an increase of apoptotic events and failure in the replenishment of host satellite cells pool after transplantation (Asakura et al., 2007). Thus, the goal of regenerative medicine is to ameliorate the capacity of transplanted healthy satellite cells to self-renew thus reducing the number of treatment on patients. Therefore, since the LSD1 and PHF2 function is to delay and not completely suppress *MyoD* expression I would expect to observe an improvement of the self-renewal potential of transplanted satellite cells after having temporally inhibited the enzymatic activity of LSD1 or PHF2.

PHF2 regulates LSD1 protein stability

To date LSD1 enzymatic activity and its function have been extensively investigated, giving to this demethylase a key role in many biological processes. Thus, due to its critical role it is not surprising that cells have developed posttranscriptional methods of regulating its level and activity. However, LSD1 transcriptional and post- transcriptional regulation is still not well elucidated.

Few reports have demonstrated that LSD1 is subjected to post-transcriptional control mechanisms that regulate its activity and stability. In particular it has been described how the LSD1 phosphorylation by polo like kinase 1 (PLK1) inhibits its transcriptional activity promoting its release from chromatin during mitosis and allowing the cell cycle progression (Peng et al., 2017). Conversely, LSD1 phosphorylation by PKCα enhances its ability to bind to transcriptional complexes increasing their gene transcriptional activation (Feng et al., 2016; Lim et al., 2017; Nam et al., 2014). Moreover LSD1 phosphorylation by CK2 is necessary and sufficient to recruit the complex LSD1/RNF168/53BP1 at the DNA damage site allowing the DNA repair and cell survival (Peng et al., 2015). Very recently it has been published that in glioblastoma GSK3β and CK1α phosphorylate LSD1 at two different sites and such post-transcriptional modifications increase the binding to the ubiquitin specific peptidase 22 (USP22) that stabilize LSD1 protein, thus favoring tumorigenesis.
So far, there are no evidences about LSD1 protein methylation/demethylation. However, LSD1 contains more than 51 lysines and 43 arginines so it is not surprising that LSD1 transcriptional activity or stability could be also influenced by this kind of post-translational modifications. Consistent with this hypothesis, in this thesis I provided data that PHF2 and LSD1 interact each other and PHF2 is directly involved in the turnover of LSD1 protein during myoblast differentiation (**Figure 31**). Indeed, I could speculate that such stability might be achieved by demethylation of LSD1 lysines. Moreover I could hypothesize that, at the onset of myoblast differentiation PHF2 and LSD1 are in same transcriptional activator complex on the CE region. However, even though PHF2 seems not to be necessary for the recruitment of LSD1 on the CE region, its role is to stabilize LSD1 protein, which in turn would be able to assembly the activator complex on the CE region.

How do LSD1 and PHF2 activate the CEeRNA expression?

Depletion of LSD1 in myoblasts decreased the recruitment of RNA polymerase II on the core enhancer, thereby preventing the transcription of the *CEeRNA*. However, the histone modification changes occurred on the Core enhancer region cannot be directly attributed to the enzymatic activity of LSD1 or PHF2. Thus as LSD1 could also de-methylate non-histone substrates and affects their transcriptional activity and stability (Chuikov et al., 2004; Huang and Berger, 2008; Wang et al., 2009), it would worth to investigate LSD1 interactors responsible for the RNA polymerase II recruitment on the Core Enhancer

(**Figure 31**).

The activation of satellite cells is controlled by a complex array of signals including mechanical properties of the matrix, inflammatory molecules secreted by macrophages in response to muscle injury, as well as signaling molecules also involved in muscle embryogenesis such as Wnt members. Moreover, it has been extensively demonstrated that the transition between Notch signaling to Wnt/β-catenin signaling in muscle progenitors is necessary to drive the initiation of myoblats differentiation. Indeed disruption of Wnt/β-catenin signaling causes muscle developmental and regenerative defects (Hutcheson et al., 2009; Lacour et al., 2017; Parisi et al., 2015; Rudolf et al., 2016). The presence of Wnt ligands activates the Wnt/β-catenin signaling pathway through stabilization and accumulation of cytosolic β-catenin. The effector β-catenin is thus able to shuttle into the nucleus, where it directly binds the *MyoD* core enhancer region, and thus initiates the myogenic specific genes cascade (Pan et al., 2015). Since a yeast two hybrid already performed in collaboration with the laboratory of Marc Vidal (Dana Farber Center, Boston, USA) has revealed LSD1 interaction with several intermediates of the Wnt signaling pathway, I would speculate that LSD1 could participate to β-catenin action. β-catenin has no DNA binding ability thus it forms a transcriptional activator complex with TCF/LEF, p300/CBP, and other proteins functioning as a transcriptional activator following nuclear translocation. Due to its key role in cancer development many studies have been performed to characterize the different component of the transcriptional complex. More and more co-activators and regulators have been found, which influence Wnt/β-catenin transcriptional activity. Thus LSD1 could be one of them both demethylating one or more members of this complex stabilizing it and thus enhance β-catenin transcriptional activity or acting on β-catenin itself. Consistent with this last hypothesis, two reports have already linked β-catenin protein stability with post-translational lysine methylation/demethylation (Lu et al., 2015; Shen et al., 2015).

Knowledge about the factors that regulate satellite cell activity is crucial for their direct manipulation. Results presented in this thesis will contribute to better understand the molecular mechanisms that control the specification of satellite cells and shed light on their complexity. Indeed I demonstrated that PHF2 and LSD1 act at two different levels on the regulation of *MyoD* expression and thus on the satellite cells “fate”. As they are enzymes they are druggable and they could be candidate targets for stem cell therapy. Thus, a more in depth study of their role in satellite cells is needed.

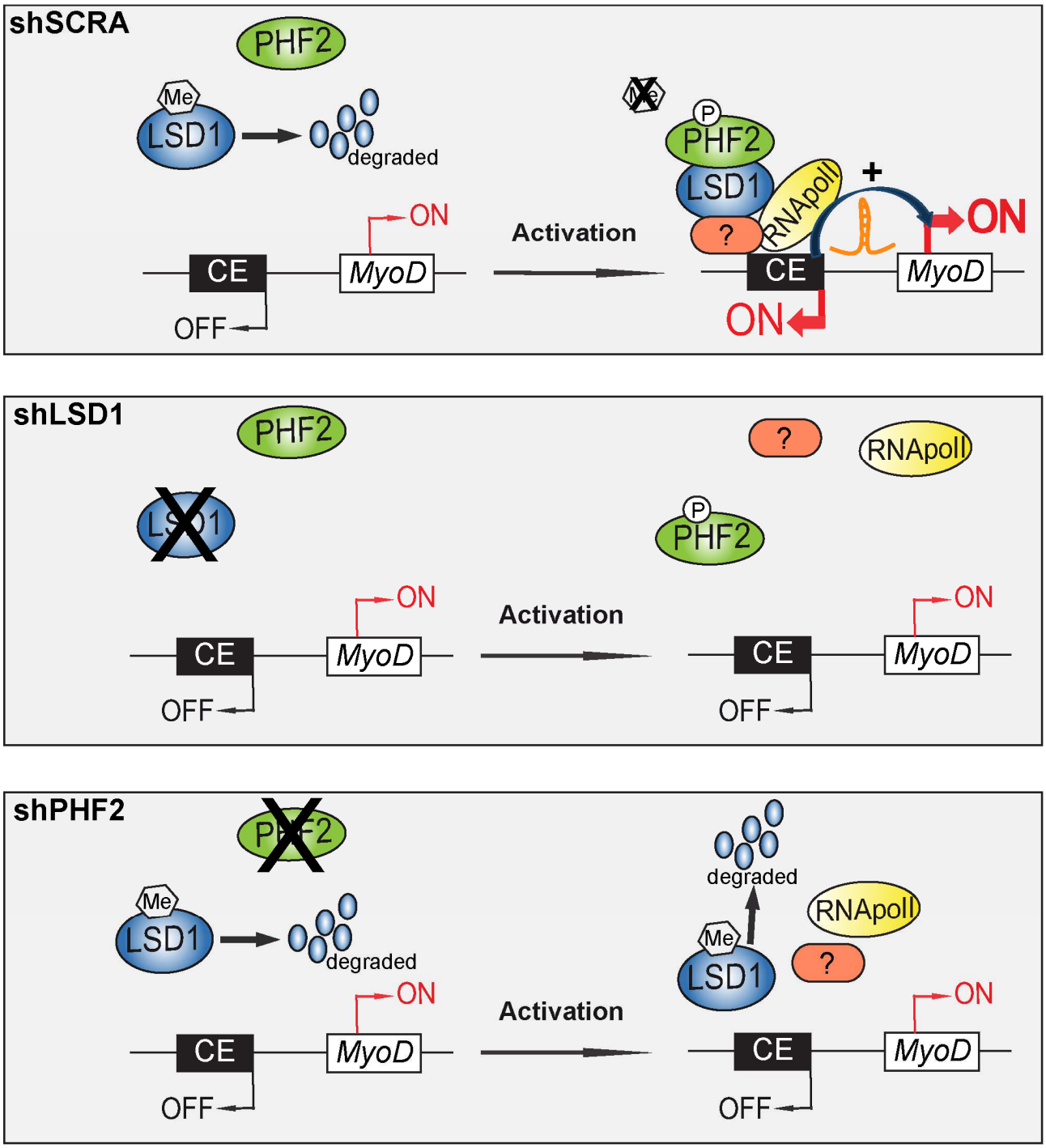


Figure 31: Schematic representation of *CEeRNA* expression regulation upon myoblast activation in shSCRA, shLSD1 and shPHF2 cells.

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