Epigenetic control of type 2 and 3 deiodinases in myogenesis: role of Lysine-specific Demethylase enzyme and FoxO3

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ABSTRACT

The proliferation and differentiation of muscle precursor cells require myogenic regulatory factors and chromatin modifiers whose concerted action dynamically regulates access to DNA and allows reprogramming of cells towards terminal differentiation. Type 2 deiodinase (D2), the thyroid hormone (TH)-activating enzyme, is sharply upregulated during myoblast differentiation, whereas type 3 deiodinase (D3), the TH-inactivating enzyme, is downregulated. The molecular determinants controlling synchronized D2 and D3 expression in muscle differentiation are completely unknown. Here, we report that the histone H3 demethylating enzyme (LSD-1) is essential for transcriptional induction of D2 and repression of D3. LSD-1 relieves the repressive marks (H3-K9me2-3) on the *Dio2* promoter and the activation marks (H3-K4me2-3) on the *Dio3* promoter. LSD-1 silencing impairs the D2 surge in skeletal muscle differentiation while inducing D3 expression thereby leading to a global decrease in intracellular TH production. Furthermore, endogenous LSD-1 interacts with FoxO3a, and abrogation of FoxO3-DNA binding compromises the ability of LSD-1 to induce D2. Our data reveal a novel epigenetic control of reciprocal deiodinases expression and provide a molecular mechanism by which LSD-1, through the opposite regulation of D2 and D3 expression, acts as a molecular switch that dynamically finely tunes the cellular needs of active TH during myogenesis.

INTRODUCTION

Histone modifications mediate changes in gene expression by dynamically remodelling the chromatin structure and converting the compact and repressed chromatin into an accessible form for active transcription or vice versa. In particular, the lysine residues of histone tails are subject to both acetylation and methylation, and the meaning of such epigenetic marks can lead to gene activation or repression.

Determination of the myogenic lineage and differentiation of skeletal muscle cells are precisely orchestrated by the concerted action of muscle-specific transcription factors (MRFs) and chromatin modifier enzymes such as nuclear histone acetyltransferases (HATs) and deacetylases (HDACs) (1–4), as well as factors regulating the methylation states of various muscle-specific promoter genes. Although histone acetylation is a common marker of transcriptionally active chromatin, histone methylation is associated with both gene activation and repression, depending on the site where it occurs. In particular, methylation of lysine 4 in histone H3 (H3-K4) correlates with gene activation (5), whereas H3-K9 and H3-K27 methylation is associated with transcriptional repression (6). Histone lysine methylation was long regarded as an irreversible process until the recent discovery of the first histone demethylating enzyme, LSD-1/KDM1A (7). Soon after, Jumonji was identified as another enzyme able to remove methyl groups from lysine residues, and, more recently, several histone lysine demethylases (KDMs) with fine substrate specificity have been implicated in diverse processes including embryonic patterning, stem cell self-renewal, differentiation, neuronal development and spermatogenesis (8). Mutations or deregulation of KDMs are often linked to human cancers and other diseases (9,10).

LSD-1 is a flavin adenine dinucleotide-dependent mono-amine oxidase that, by specifically removing mono- and di-methyl groups, but not tri-methyl groups from methylated lysines (7,11), functions as both a transcriptional coactivator and corepressor of its substrates (12,13). LSD-1 has been identified in a number of complexes that control gene transcription, and its demethylase activity has also been linked to pathological processes including tumorigenesis. LSD-1 has been described to associate with the mixed-linkage leukaemia supercomplex (14), the elongation factor RNA polymerase II (elongation complex, containing the eleven-nineteen lysine-rich leukaemia protein (ELL)) complex (15), HDAC1 and HDAC2 (16). It is a component of complexes associated with transcription repression, such as CoREST-HDAC, CtBP and NuRD (17), and can also coactivate gene expression as demonstrated for androgen and estrogen receptor genes (11,18). Recently, LSD-1 has been shown to regulate MyoD and Mef2 expression during myogenesis and muscle regeneration by relieving repressive epigenetic marks during myoblast differentiation (19).

Thyroid hormone (TH) is a pleiotropic agent that has long been known to affect muscle development and maturation through direct regulation of several muscle-specific genes (20,21). It influences fibre-type composition and is the main determinant of the resting metabolic rate of muscle fibres (20). A large body of evidence indicates that TH is required for the correct execution of the myogenic programme, and alterations in muscle physiology are common clinical features of hyper- and hypo-thyroid patients. Moreover, TH fluctuations have been demonstrated to exacerbate myopathies such as myasthenia gravis and myotonic dystrophy (22).

TH action starts with the monodeiodination of the prohormone T4 that produces the active hormone T3. The three iodothyronine deiodinases (D1, D2 and D3, encoded by the *Dio1, 2* and *3* genes) are involved in the peripheral activation and inactivation of TH in space and time through their tissue-specific expression patterns. D1 and D2 catalyse the conversion of the prohormone thyroxine (T4), to the active hormone, $3,5',3'$-triiodo-thyronine (T3). D3 triggers the major inactivating pathway by terminating the action of T3 and preventing activation of T4.

D2 is expressed in the pituitary gland, the central nervous system, thyroid, bone, brown adipose tissue and skeletal muscle (23). D2 expression in muscle is under the control of FoxO3a and is an essential requirement for skeletal muscle differentiation and muscle regeneration. Notably, the regeneration process after an injury is significantly delayed in D2KO mice (24), which implies that an increase in D2-generated intracellular T3 is required for complete muscle repair.

Here, we show that acetylation and methylation of histone tails are fundamental components of the transcriptional regulation of the *Dio2* gene. LSD-1 activates *Dio2* gene expression in differentiating myoblasts by relieving the repressing histone marks (H3-K9). At the opposite, LSD-1 negatively affects D3 expression during muscle cell differentiation. Moreover, LSD-1 physically interacts with FoxO3a on the *Dio2* promoter, and an integral

FoxO3a–LSD-1 complex is essential to ensure that the level of D2 is increased during muscle cell differentiation. siRNA-mediated knockdown of LSD-1, its chemical inhibition or over-expression of dominant negative LSD-1 mutants impair D2 expression and D2-mediated TH production. Our experiments therefore establish that, by dual opposite but functionally convergent mechanisms on D2 and D3 expression, LSD-1 plays a central role in regulating TH signalling in muscle cell differentiation. Our data indicate that regulators of the structure and function of chromatin, such as enzymes modulating the turnover of histone methylation and histone acetylation, represent an attractive novel tool to finely modulate TH action in various systems, including muscle.

MATERIALS AND METHODS

Cell cultures, reagents and transfections

Primary muscle cultures (mpcs) were isolated as described previously (24). C2C12 and HEK293 cells were obtained from ATCC. Proliferating cells were cultured in 20% fetal bovine serum Dulbecco's modified Eagle's medium. To induce differentiation, cells at 70% confluence were switched from 20% fetal bovine serum to 2% horse serum Dulbecco's modified Eagle's medium supplemented with insulin, and transferrin. Transient transfections were performed using Lipofectamine 2000 (Life Technologies Ltd., Paisley, Scotland) according to the manufacturer's instructions.

Anti-MyoD (sc-304), myogenin (sc-12732) and tubulin (sc-8035) antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Anti Ha antibody (12CA5) was from Roche and anti-Flag from Sigma (St Louis, MO, USA). Anti-LSD-1 (ab16632), anti H3-Ac (06-599), anti-H3K4me1 (ab8895), me2 (ab7766) and me3 (ab8580), anti-H3K9me1 (07-450), me2 (07-441) and me3 (ab8898) and anti-H3K27me3 (07-449) were all obtained from Upstate Biotechnology (Charlottesville, VA, USA). Cells were treated for 24–48 h with the following reagents: Pargilin 3 mM, Sirtinol 10 nM, Trichostatin A (TSA) 30 nM, sodium butyrate (NaB) 1 mM and XLI 30 nM all obtained from Sigma.

Plasmids and expression constructs

D2-Luc and TRE3TK-Luc plasmids are described elsewhere (24). Dominant negative (dn) form of FoxO3a and FoxO plasmids are described elsewere (24). To knockdown LSD-1 protein, siRNA targeting the sequence 5′-CG GACAAGCTGTTCCTAAA-3′ was purchased from Santa Cruz (sc-60971) and transiently transfected into mpcs using the Lipofectamine 2000 (Invitrogen).

Western blot and co-immunoprecipitation analysis

Protein extracts from C2C12 or mpcs were resolved by sodium dodecyl sulfate (SDS)-12% polyacrylamide gel electrophoresis. For co-immunoprecipitation (Co-IP) assays, total cell lysates of transiently transfected HEK-293 cells were prepared in RIPA lysis buffer (50 mM Tris (pH 7.5), 150 mM NaCl, 1% Nonidet-P40,

0.1% SDS, 0.5% Sodium Deoxycholate, 0.5 mM phenyl-methylsulfonyl fluoride, 1 mM sodium vanadate, 50 mM sodium floride). Total cell extracts (0.8 mg) were immuno-precipitated overnight with the indicated antibodies and conjugated to agarose beads for 2 h. Immunocomplexes were washed five times with RIPA buffer, resuspended in sample buffer and heated at 99°C for 5 min. The super-natant was transferred to fresh tubes containing 5% b-mercaptoethanol. Proteins were resolved by SDS–poly-acrylamide gel electrophoresis and blotted onto an Immobilon P (Millipore, Bedford, MA, USA) membrane for 12 h at a constant current of 150 mA. LSD-1 and FoxO3 were immunodetected with a monoclonal anti-Flag antibody (M2) and anti-Ha, respectively.

Chromatin immunoprecipitation and Sequential Chromatin Immunoprecipitation (Re-ChIP) assays

For Chromatin Immunoprecipitation (ChIP) assays, proliferating or differentiated mpcs were fixed with 1% formaldehyde and subjected to ChIP assay as previously described by using the indicated antibodies (25). DNA fragments were purified after immunoprecipitation and used for real-time polymerase chain reaction (RT-PCR) analysis with oligonucleotide pairs to specifically amplify the *Dio2* promoter region containing the FoxO3- and MyoD-binding elements within the 5′-flankin region (Figure 2A). As negative control, PCR was carried out by using unrelated oligonucleotides, and the presence of equivalent amounts of chromatin in each sample was con-firmed by PCR without prior immunoprecipitation (input).

In Re-ChIP experiments, after IP with anti-FoxO3 antibody and washes, complexes were eluted by incuba-tion for 30 min in Re-ChIP buffer [2 mM DL-Dithiothreitol (DTT), 1% Triton X-100, 2 mM ethylenediaminetetraacetic acid, 150 mM NaCl, 20 mM Tris–HCl (pH 8)], diluted 50 times with diluition buffer and subjected again to the ChIP procedure using the anti-LSD-1 antibody. Quantitative PCR were performed on a iQ5 Multicolor Real Time Detector System (BioRad, Hercules, CA, USA) with the fluorescent double-stranded DNA-binding dye SYBR Green (BioRad).

RT-PC

The mRNAs were extracted with Trizol reagent (Life Technologies, Ltd). The cDNAs were prepared with Superscript III (Life Technologies, Ltd) as indicated by the manufacturer. The cDNAs were amplified by PCR in an iQ5 Multicolor Real Time Detector System (BioRad, Hercules, CA, USA) with the fluorescent double-stranded DNA-binding dye SYBR Green (BioRad). Specific primers for each gene were designed to work under the same cycling conditions (95°C for 10 min followed by 40 cycles at 95°C for 15 s and 60°C for 1 min), generating products of comparable sizes (∼200 bp for each amplification). Primer combinations were positioned whenever possible to span an exon–exon junction, and the RNA was digested with DNAse to avoid genomic DNA interference. For each reaction, standard curves for reference genes were constructed based on six 4-fold serial dilutions of cDNA. All samples were run in

triplicate. The template concentration was calculated from the cycle number when the amount of PCR product passed a threshold established in the exponential phase of the PCR. The relative amounts of gene expres-sion were calculated with ß-actin or Abelson expression as an internal standard (calibrator). The results, expressed as N-fold differences in target gene expression, were determined as follows: $N *target = 2^{(Ct\ sample\ Ct\ calibrator)}$.

Statistical analysis

Differences between samples were assessed by Student's two-tailed *t*-test for independent samples. *P*-values <0.05 were considered significant. Relative mRNA levels (in which the first sample was arbitrarily set as one) are reported as the results of RT-PCR in which the expres-sion of cyclophilin A was used as housekeeping gene. All experiments were repeated and analysed three to five times.

RESULTS

Histone deacetylase inhibitors upregulate D2 expression and increase thyroid hormone signalling in muscle cells

We previously demonstrated that D2 expression is induced during muscle cell differentiation (24). To investigate the possible epigenetic regulation of D2 expres-sion by histone acetylation, we first measured D2 mRNA levels in C2C12 cells treated with various histone deacetylase inhibitors (HDACIs). As shown by RT-PCR (Figure 1A), D2 mRNA was significantly induced by class I and II (TSA, NaB) and class III (Sirtinol, XLI) HDACIs at concentrations that inhibit HDAC activity (millimolar-level for NaB and nanomolar-level for TSA, Sirtinol and XLI). *Dio2* promoter activity was also induced, suggesting a transcriptional control of D2 expres-sion (Figure 1B). Stimulation of D2 expression was associated with a time-dependent increase in TH sig-nalling, as indicated by increased Luciferase gene expres-sion driven by TRE3TK promoter (Figure 1C).

These data demonstrate that histone acetylation of chromatin of the *Dio2* promoter is essential for D2 expres-sion in muscle cells.

Acetylation and methylation of core histones are critical components of D2 transcriptional regulation in muscle cells

To assess the molecular signals that prompt increased acetylation of the *Dio2* promoter chromatin in differentiating muscle precursor cells, we performed ChIP assays to decipher the histone code of the promoter chromatin in proliferating and differentiated mpcs, an enriched population of mouse primary muscle stem cells. First, we analysed the acetylation status of histone H3 bound to the *Dio2* promoter. Precipitated chromatin DNA was analysed by RT-PCR using primers designed to amplify the 5′-flanking region of the promoter spanning the FoxO3- and MyoD-binding sites (Figure 2A, top). As shown in Figure 2A, acyl-histone H3 (Ac-H3 histone) physically associates with the *Dio2* promoter, and this association increased with
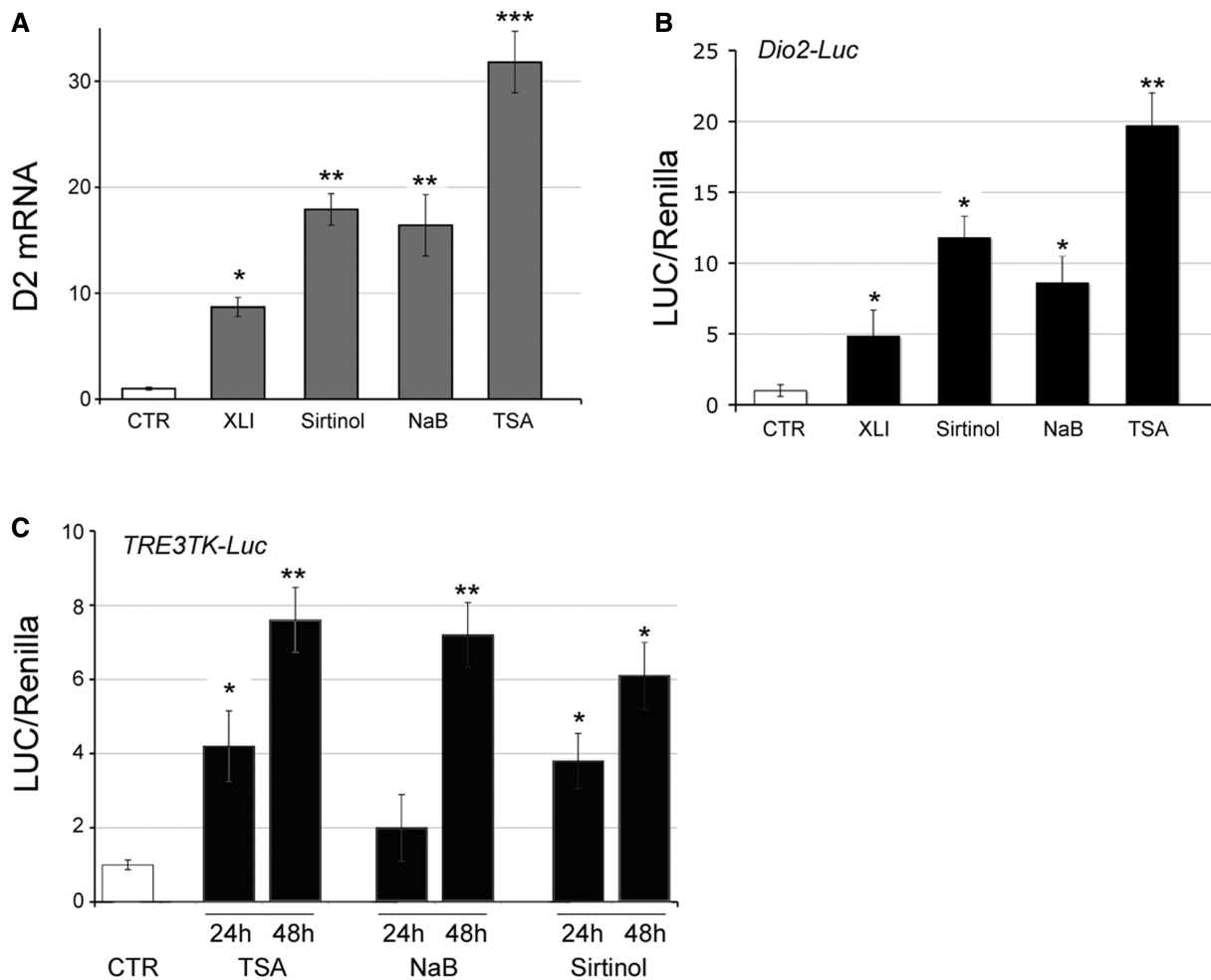
Figure 1. Deacetylase inhibitors upregulate D2 expression and thyroid hormone concentration. (**A**) Proliferating C2C12 cells were treated with different deacetylase inhibitors (see 'Materials and Methods' section) for 24 h, and harvested for RT-PCR analysis of D2 mRNA expression. Cyclophillin A was used as internal control. Normalized copies of D2 in untreated cells (CTR) were set as 1. (**B**) C2C12 cells were transfected with a 1300 bp mouse *Dio2* 5′-flanking region driving the Luciferase construct (*Dio2*-Luc) and CMV-Renilla as internal control and treated with different deacetylase inhibitors for 24 h. The results are shown as means ± SD of the LUC/Renilla ratios from at least three separate experiments, performed in duplicate. (**C**) C2C12 cells were transfected with TRE3TK-Luc construct and CMV-Renilla as internal control and treated with different deacetylase inhibitors for 24 and 48 h. The results are shown as means ± SD of the LUC/Renilla ratios from at least three separate experiments, performed in duplicate. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

differentiation, in agreement with the robust D2 expression in differentiating myoblasts. To investigate whether histone acetylation was associated with methylation at lysine residues, we analysed the methylation profile of lysine 4 (K4) or 9 (K9) on histone3 (H3) on *Dio2* promoter chromatin. Indeed, methylated H3-K4 (H3-K4me1-2-3), which mark transcriptionally active chromatin, were at their lowest levels in proliferating myoblasts, but enriched in myotubes (Figure 2B). Conversely, methylation levels of repressive histone marks H3 at lysine 9 (H3-K9me1-2-3) were high in proliferating cells and greatly reduced in differentiated cells (Figure 2C). H3-K27 residues (H3-K27me1-2-3) were not significantly modified on the chromatin of the *Dio2* promoter in either proliferating or differentiated cells (data not shown).

Taken together, these data demonstrate that the levels of methylation of histone H3 at lysine 4, which usually correlate with active gene transcription, are enhanced on

the *Dio2* promoter in differentiated myotubes, whereas methylation of histone H3 at lysine 9, which is associated with transcriptional silencing, is reduced. These results support the notion that transcription of D2 in muscle cells is coupled to specific control of histone methylation.

LSD-1 controls D2 expression and local TH production

As D2 plays an obligate role in terminal muscle differentiation of muscle precursor cells, and the lysine-specific demethylase, LSD-1 or KDM 1 is induced during C2C12 differentiation (19), we measured the levels of LSD-1 in proliferating or differentiating primary myoblast progenitor cells (mpcs). As shown in Figure 3A and B, LSD-1 mRNA and protein levels are low in proliferating mpcs and significantly induced in differentiated cells, in agreement with the muscle differentiation markers, MyoD and myogenin, and concomitantly to the surge in D2 expression (Figure 3B and C). To find a mechanistic link between
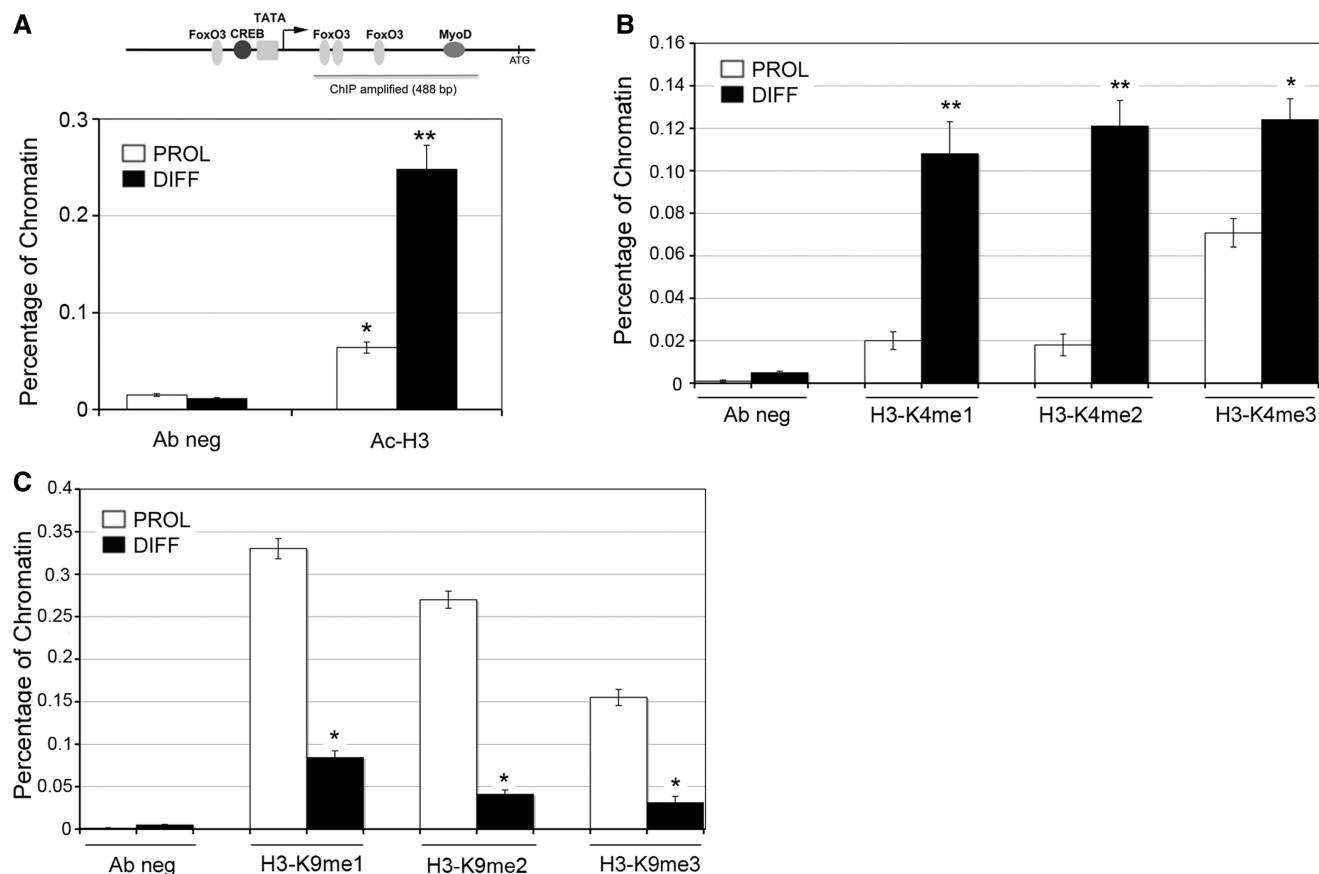
Figure 2. Dio2 promoter is regulated by histone acetylation and methylation at specific lysine residues. (**A**) Top: Schematic diagram depicts *Dio2* promoter with TATA box and binding sites for different transcription factors. Amplified region in the ChIP assayes is underlined. Bottom: The histone H3 acetylation profile of the *Dio2* promoter was evaluated by ChIP analysis in proliferating and differentiated muscle cells. mpcs were grown in proliferating or differentiating conditions (see 'Materials and Methods' section), and chromatin was immunoprecipitated using the H3-pan-acetylated antibody. The amount of precipitated chromatin was calculated relative to the total input chromatin and expressed as percentage of the total. (**B**) ChIP assays were carried out to evaluate the *in vivo* methylation status of histone H3 on the *Dio2* promoter. Chromatin extracted from proliferating and differentiated mpcs cells was immunoprecipitated using the indicated antibodies for detection of H3-K4 methylation. The amount of precipitated chromatin was calculated relative to the total input chromatin and expressed as percentage of the total. (**C**) ChIP analysis of H3-K9 mono-, di- and tri-methylated lysines on *Dio2* promoter were performed as indicated above. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

LSD-1 expression and D2 transcription, we over-expressed LSD-1 in proliferating myoblasts and measured D2 mRNA levels. Figure 3D shows that LSD-1 expression induced 8-fold increase in D2 mRNA and induced expression of differentiation markers (see later in the text). Conversely, inhibition of LSD-1 by specific siRNA or treatment with pargiline, a chemical inhibitor of mono-aminoxidases that inhibits LSD-1 *in vivo* (11), inhibited D2 expression resulting in a significant reduction of TH activity (Figure 3D–F). Furthermore, LSD-1 mutant proteins, which were either point mutated at the NH terminus (26) or deleted at the COOH terminus (LSD-1Δ), failed to induce D2 expression (Figure 3G). ChIP assay of muscle progenitor cells (mpcs) revealed that LSD-1 protein was recruited to the *Dio2* promoter in differentiated myotubes (Figure 3H).

Taken together, these data demonstrate that LSD-1 stimulates D2 expression and increases TH-dependent transcription. Conversely, inhibition of endogenous LSD-1 abrogates D2 surge and TH-induced transcriptional activation.

LSD-1 inversely regulates the expression of D2 and D3

D3, the TH inactivating enzyme, exerts an opposite effect on TH metabolism compared with D2, and the two enzymes are regulated in a reciprocal fashion in several systems (23,27–29). In keeping the ability of LSD-1 to control D2 expression, we sought to test whether LSD-1 might target and direct the expression of D3 in muscle cells. To this end, we measured D3 mRNA in myoblasts over-expressing LSD-1 or LSD-1-depleted (iLSD-1). As shown in Figure 4A, D3 was significantly inhibited by LSD-1 over-expression and stimulated by LSD-1 depletion, thus demonstrating that D3 is repressed by LSD-1. Moreover, LSD-1 was also recruited to the chromatin of the *Dio3* promoter (Figure 4B). To evaluate the impact of histone methylation on D3 expression in muscle cells, we measured the methylation marks on the *Dio3* promoter in proliferating and differentiated mpcs using ChIP assays with antibodies specific for H3K4me2 and H3K9me2. We found that the *Dio3* promoter chromatin was marked by high levels of H3K4me2 in proliferating cells
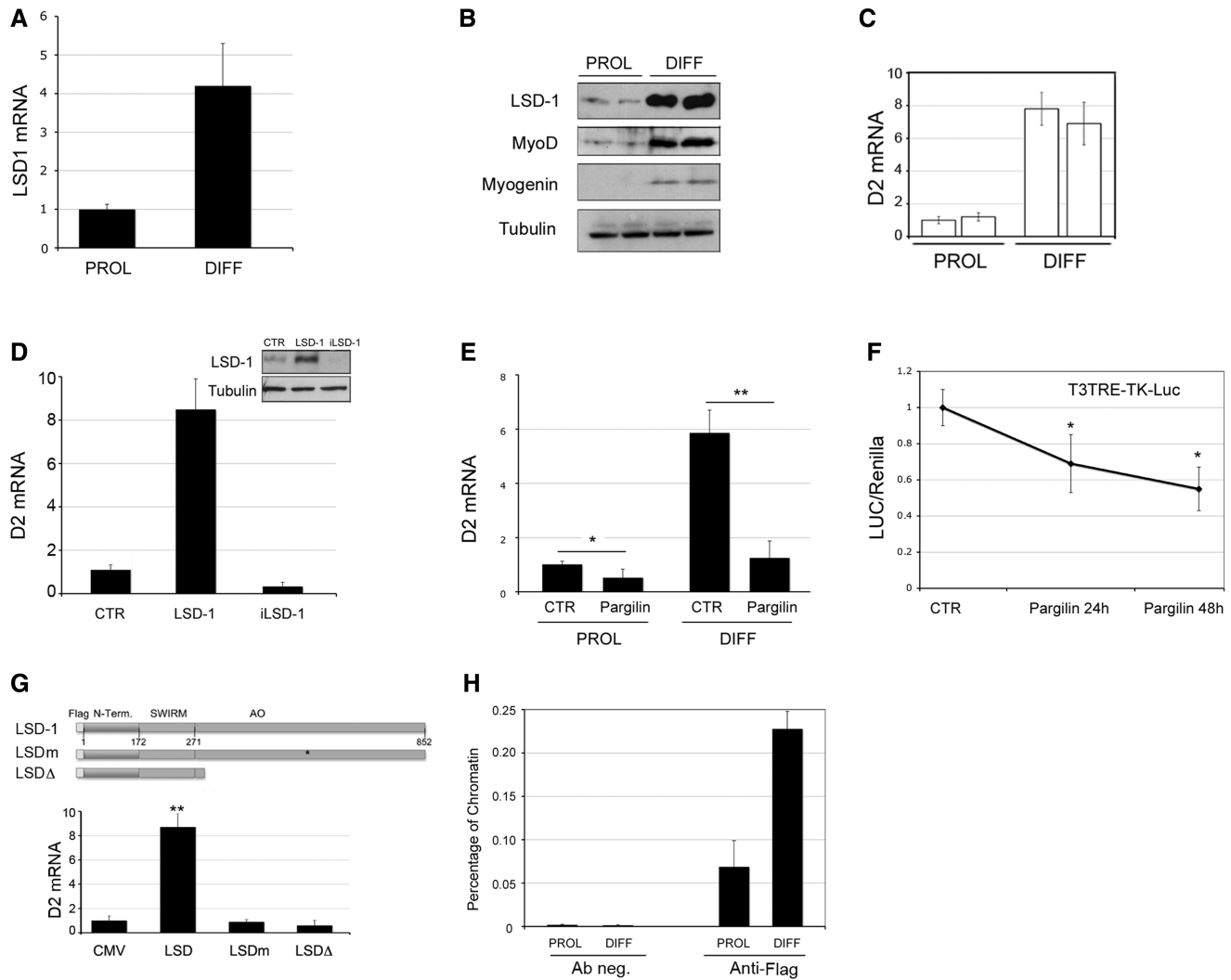
Figure 3. LSD-1 increases the expression of D2 in muscle cells. (**A**) LSD-1 mRNA levels were analysed by RT-PCR in proliferating and differentiated mpcs cells. (**B**) Western blot analysis of LSD-1, MyoD and myogenin levels in proliferating and differentiated mpcs. (**C**) D2 mRNA levels were measured by RT-PCR in the same cells as in (**B**). (**D**) C2C12 cells were transiently transfected with LSD-1-expressing plasmid or an siRNA for specific LSD-1 silencing, and D2 mRNA levels were measured by RT-PCR. Effective LSD-1 expression and silencing were evaluated by western blot (Top). (**E**) D2 mRNA was measured in proliferating and differentiated C2C12 cells treated with 3 mM pargilin for 24 h. (**F**) TH concentration was evaluated in proliferating C2C12 cells on pargilin treatment for 24 and 48 h by co-transfecting the T3TRETK-Luc promoter and CMV-Renilla as internal control. (**G**) Effect of LSD-1, LSD-1 m and LSD-1Δ on D2 mRNA levels in transiently transfected C2C12 cells. (**H**) mpcs were transfected with Flag-tagged LSD-1 plasmid, and chromatin extracted from proliferating and differentiated cells was immunoprecipitated using the anti-Ha antibody. RT-PCR was carried out with oligos amplifying *Dio2* promoter region on purified DNA to evaluate the amount of LSD-1 bound fragments. The amount of precipitated chromatin was calculated relative to the total input chromatin and expressed as percentage of the total. The results are shown as means ± SD of the LUC/Renilla ratios from at least three separate experiments, performed in duplicate. *$P < 0.05$, **$P < 0.01$.

that drastically decreased on differentiation, whereas H3K9me2 levels remained unchanged throughout differentiation (Figure 4C). Notably, the methylation profile of the permissive histone mark H3-K4 was inversely regulated on the *Dio2* and *Dio3* promoters, in accordance with the expression levels of D2 and D3 in mpcs [(24) and Dentice *et al.*, manuscript in preparation]. Thus, reciprocal regulation of D2 and D3 may be part of a novel, convergent mechanism operated by LSD-1 to regulate TH signalling during myogenesis.

To verify that LSD-1 directs activation of D2 and represses D3 during muscle differentiation via a direct effect on their local histone methylation marks, we

silenced LSD-1 in mpcs and evaluated the methylation profiles of the *Dio2* and *Dio3* promoters. Depletion of LSD-1 expression in mpcs cells by siRNA prevented the loss of H3-K9me2 on the *Dio2* promoter in differentiated cells, indicating that LSD-1 demethylated H3-K9m2 and, in doing so, it stimulated D2 expression in differentiated myotubes (Figure 4D). At the same time, in proliferating cells, LSD-1 stimulates D2 expression, as indicated by the significant increase in the H3-K9 methylation on LSD-1 depletion (Figure 4D). These results support the overall effect of LSD-1, which over-expression increases D2 mRNA levels in both proliferating and differentiated mpcs (Figure 3D). The H3-K4 methylation profiles of
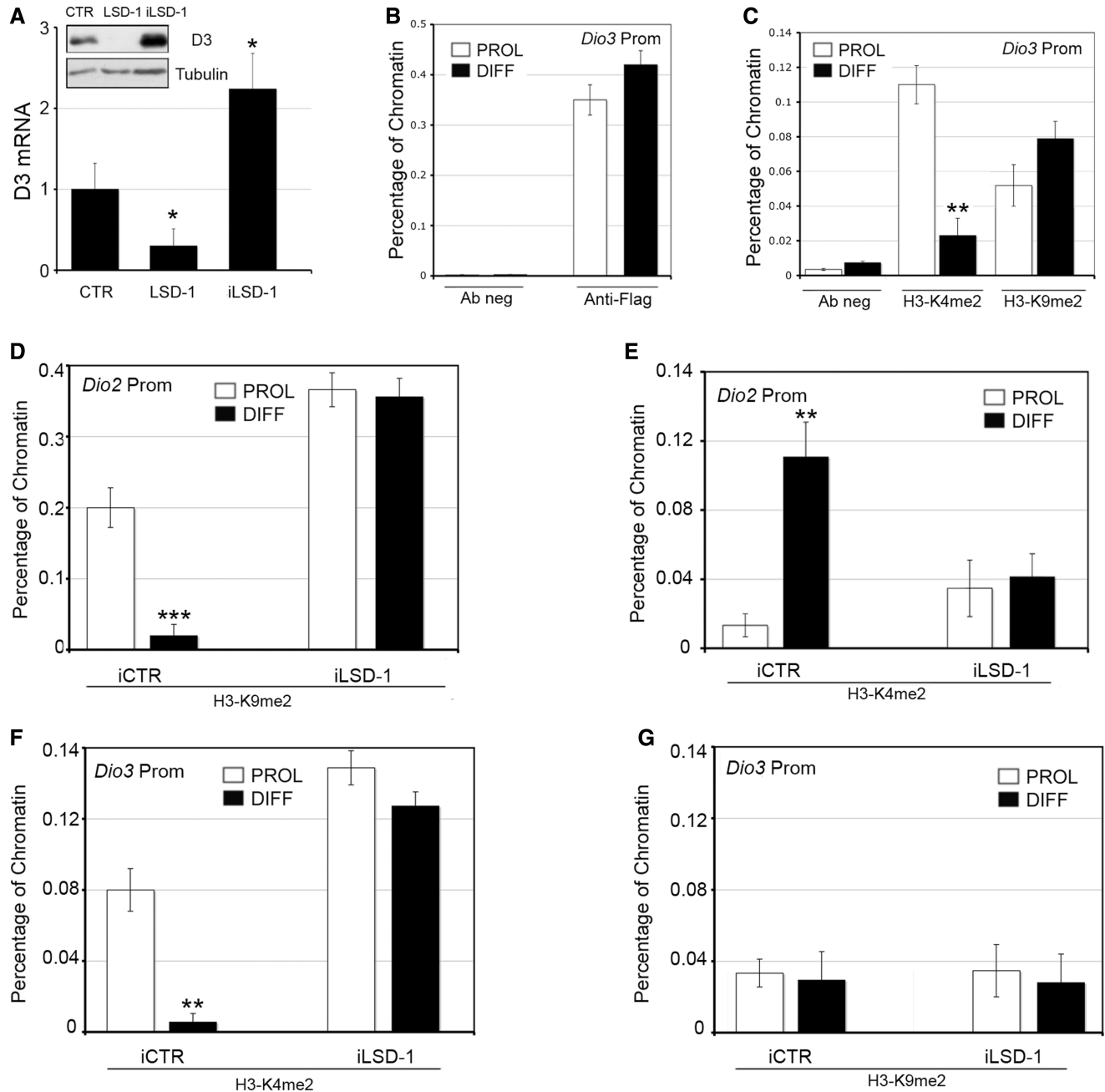
Figure 4. LSD-1 inversely regulates D3 expression. (**A**) D3 mRNA and protein levels in C2C12 cells transiently transfected with LSD-1-expressing plasmid or an siRNA for specific LSD-1 silencing (iLSD-1). (**B**) mpcs were transfected with Flag-tagged LSD-1 plasmid, and chromatin extracted from proliferating and differentiated cells was immunoprecipitated using the anti-Ha antibody. RT-PCR was carried out with oligos amplifying *Dio3* promoter region on purified DNA to evaluate the amount of LSD-1 bound fragments. The amount of precipitated chromatin was calculated relative to the total input chromatin and expressed as percentage of the total. (**C**) ChIP assays were carried out to evaluate the *in vivo* methylation status of histone H3 on the *Dio3* promoter. Chromatin extracted from proliferating and differentiated mpcs was immunoprecipitated using the indicated antibodies. The amount of precipitated chromatin was calculated relative to the total input chromatin and expressed as percentage of the total. *$P < 0.05$, **$P < 0.01$. (**D–G**) ChIP assays were carried out to evaluate the K4 and K9 methylation status of histone H3 on the *Dio2* and *Dio3* promoter. Chromatin extracted from proliferating and differentiated mpcs, transiently transfected with control siRNA (iCTR) or specific LSD-1-targeting siRNA (iLSD-1), was immunoprecipitated using the indicated antibodies for detection of H3-K4 or H3-K9 methylation. The amount of precipitated chromatin was calculated relative to the total input chromatin and expressed as percentage of the total. **$P < 0.01$, ***$P < 0.001$.

Dio2 promoter revealed that the increase in this activation mark is also abolished in the absence of LSD-1, thus contributing to the impaired surge in D2 expression in LSD-1 depleted myotubes (Figure 4E). Conversely, depletion of LSD-1 inhibited demethylation of H3-K4me2 residues on the *Dio3* promoter, whereas H3-K9me2 marks remained unchanged, demonstrating that LSD-1 was required for efficient down-modulation

of D3 expression in differentiated myotubes (Figure 4F and G). Taken together, these data indicate that LSD-1 plays a dual role in the coordinated regulation of D3 and D2 expression during the differentiation of muscle cells: H3K4 m2, when present, is the preferred LSD-1 substrate (30) and as a direct consequence of its loss, LSD-1 inhibits transcription (D3); conversely, if only H3K9 m2 is present (H3K4m2 is low or absent), LSD-1 removes the repressive mark H3K9m2 and stimulates transcription.

FoxO3a and LSD-1 cooperatively induce D2 in muscle cells

A critical issue in studies of epigenetic control of gene expression is to identify specific transcription factor(s) able to direct or attract epigenetic factors to specific DNA sites. LSD-1 interacts with chromatin remodelling factors and transcription factors controlling gene expression and muscle cell differentiation (19). As we previously identified D2 as a downstream target of FoxO3a in myoblasts (24), we tested whether LSD-1 physically interacts with FoxO3a. To this aim, we performed Co-IP experiments with ectopically expressed Flag-LSD-1 and Ha-FoxO3a proteins (Figure 5A). Co-IP experiments showed that LSD-1 specifically interacts with FoxO3a in HEK293 cells. This interaction was confirmed by Co-IP experiments conducted with endogenous FoxO3 and LSD-1 proteins isolated from proliferating and differentiated myoblasts (Figure 5B). Finally, a Re-ChIP assay demonstrated that endogenous FoxO3 and LSD-1 proteins interact and are recruited together to the *Dio2* promoter (Figure 5C, upper). On the other hand, on the *Dio3* promoter chromatin, LSD-1 alone is recruited to the *Dio3* promoter, as we did not find FoxO3 associated with LSD-1 on *Dio3* proximal region (Figure 5C, bottom). Therefore, FoxO3 binding is neither directly involved in the LSD-1/*Dio3* interaction nor is it essential for the transcriptional regulation of the *Dio3* gene expression.

We previously demonstrated that both FoxO3a and LSD-1 physically associate with the *Dio2* promoter [(24) and Figure 3H]. To test the role of FoxO3a in the regulation of D2 by LSD-1, we performed ChIP assay in myoblasts expressing wild-type LSD-1 or FoxO dn. The latter is unable to bind the DNA and acts as dominant negative protein on wild -type FoxO3 by preventing its association with DNA. ChIP assay demonstrates that in the absence of efficient FoxO3 binding to DNA, LSD-1 is not recruited to the *Dio2* promoter (Figure 6A). This effect was confirmed by the analysis of D2 mRNA in myoblasts co-transfected with LSD-1, FoxO3a and FoxO dn or combination of these as shown in Figure 6B. Taken together, these data demonstrate a functional link between LSD-1 and FoxO3a: FoxO3a recruits LSD-1 to the *Dio2* promoter, and this interaction is essential for the biological activity of LSD-1 on the *Dio2* promoter-driven transcription. To address the functional relevance of this cooperation for myogenesis, we transiently transfected myoblasts with siRNA for LSD-1 (iLSD-1), with FoxO3 dn or a combination of both. As shown in Figure 6C, myoblast differentiation was negatively affected in the absence of either FoxO3 or LDS-1 or of both.

Conversely, over-expression of D2 or the treatment with TH efficiently rescued inhibition of differentiation caused by the absence of FoxO3 and LSD-1.

These data confirm the existence of a direct functional link *in vivo* between FoxO3 and LSD-1 and the myogenic differentiation programme and support the critical role of D2-induced T3 for the proper differentiation of muscle cells.

Based on the aforementioned results, we propose the following working model: (i) In proliferating myoblasts, low levels of LSD-1 and FoxO3 maintain high the ratio between methylated meH3K9 and meH3K4 and repress D2 transcription, thereby attenuating TH molecular availability; (ii) conversely, higher levels of LSD-1 and FoxO3 during differentiation attack H3K9me2 leading to reduced ratio meH3K9/me H3K4 and transition to a more permissive meH3K4 and AcH3 signal, which stimulates transcription of D2 promoter resulting in increased production of TH (Figure 6D). We suggest that LSD-1 demethylation of H3K9me2 or H3K4me2 is influenced by the relative concentration of the enzyme.

DISCUSSION

In this study, we have dissected the molecular determinants regulating the epigenetic control of D2 and D3 expression during myogenesis. Our data highlight LSD-1 as a central player in such a regulation and provide insights into the molecular mechanism of how chromatin remodelling enzymes work in concert with specific transcription factors to control *Dio2* and *Dio3* gene expression, thus allowing muscle gene expression.

D2 is part of the differentiation programme induced by de-acetylating agents

Type 2 deiodinase is expressed in myoblast precursor cells and is sharply induced during differentiation. We previously showed that D2, by increasing intracellular T3, promotes MyoD gene expression and allows terminal muscle differentiation. Lack or inhibition of D2 impairs the correct differentiation of primary muscle precursor cells *in vitro* and causes a delay in the regeneration process *in vivo*, which suggests that a D2-dependent, cell-autonomous amplification of TH signalling in myoblasts is critical for the myogenic programme and for muscle repair (24). Conversely, D3 is highly expressed in proliferating myoblasts (Dentice *et al.*, manuscript in preparation).

Several studies have shown that class I and class II deacetylase HDACs repress muscle differentiation by inhibiting the activity of the MRFs family in proliferating myoblasts, whereas their repression is relieved in differentiating myocytes (31–34). Accordingly, HDACIs have been demonstrated to promote the formation of myotubes—a process that results in an increase in both the number of nuclei and in cell size (35,36). Our results show that the *Dio2* promoter region and D2 mRNA expression are significantly induced by a wide spectrum of HDACIs in muscle cells, which suggests that the switch between slightly expressed and highly upregulated D2 in
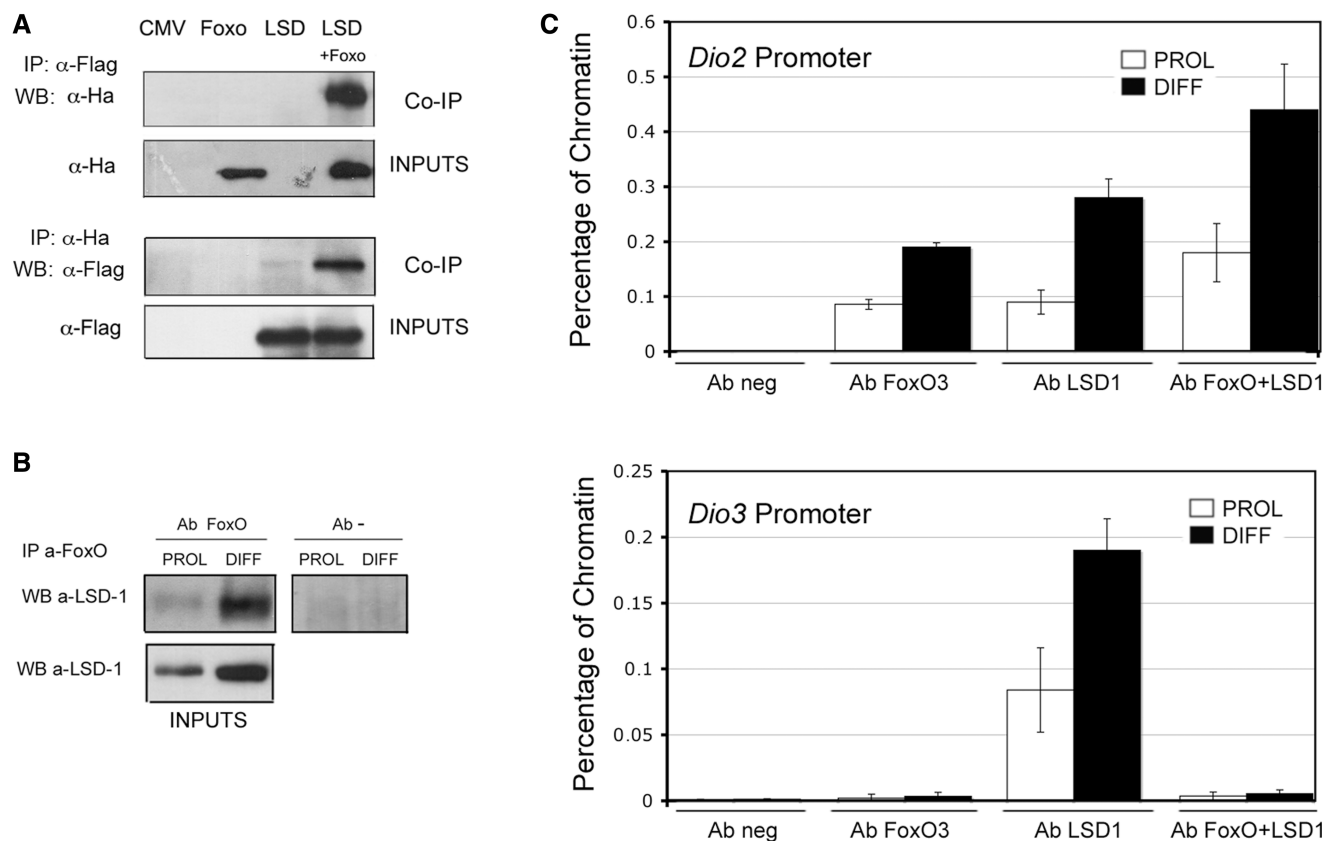
Figure 5. LSD-1 interacts with FoxO3 on Dio2 promoter region. (**A**) Co-IP assay of transfected Flag-LSD-1 and Ha-FoxO3 proteins. Extracts from transiently transfected HEK-293 cells were immunoprecipitated with anti-Flag antibody, and anti-Ha antibody for reverse Co-IP. The western blot was decoded using anti-Ha and anti-Flag antibodies, respectively. Inputs were controlled for FoxO3 and LSD-1 expression by performing anti-Ha and anti-Flag immunoblot. (**B**) Co-IP assay on endogenous FoxO3 and LSD-1 proteins. Two milligrams of total extracts from proliferating and differentiated mpcs were subjected to immunoprecipitation with FoxO3 antibody or a negative antibody as control, and the western blot was decoded using the anti-LSD-1 antibody. (**C**) Re-ChIP experiments were performed to verify the binding of FoxO3/LSD-1 complex to *Dio2* and *Dio3* promoters. Chromatin extracted from proliferating and differentiated mpcs was immunoprecipitated using the indicated antibodies. The amount of precipitated chromatin was calculated relative to the total input chromatin and expressed as percentage of the total.

proliferating and differentiated myoblasts is dynamically controlled by a balance between HATs and HDACs. Thus, the control of D2 expression is a novel aspect of the differentiation action of these two classes of enzymes. It remains to be established which HATs and HDACs are involved in the modulation of D2 expression. It is noteworthy that D2 mRNA is negatively regulated by Sir2 (37). Sir2, a class III deacetylase, represses muscle gene expression and differentiation by associating with K(lysine) acetyltransferase 2B (PCAF) and MyoD (37). Thus, the inhibition of D2 by Sir2 increases the possibility that D2 repression is part of a global programme aiming to block muscle differentiation.

In addition, da-Silva *et al*. (38) demonstrated that D2 expression is potently induced by the small polyphenolic molecule, kaempferol, which in turn enhances the D2-mediated T3 production, cellular oxygen consumption and increases a number of metabolically relevant genes. The authors tested several other related xenobiotic compounds, namely fisetin, piaceatannol and resveratrol. The latter is a constituent of red wine, a sirtuin activator, and a potent inhibitor of D2 levels. Overall, these data hint to acetylation and to multiple agents involved in the acetylation pathway as critical player in regulating D2 expression.

Specific methylation of lysines in histone H3 dynamically modulates D2 expression

Herein, we demonstrate that the differential D2 expression in proliferating and differentiated muscle cells is coupled with a well-orchestrated switch of histone code modifications. ChIP assays demonstrated that the *Dio2* promoter is physically bound to H3 acetylated lysines, and that this binding, which generally corresponds to gene activation, is strongly induced in differentiated cells. Accordingly, methylated lysine 4 (H3-K4), a further mark of active transcription, is enriched in differentiated myotubes (Figure 2). Interestingly, the repressive histone mark H3-K9 goes in the opposite direction, being sharply downmodulated in differentiated muscle cells, whereas the methylation of histone H3-K27 that results in gene repression is absent on the *Dio2* promoter. Overall, the levels of mono-, di- and tri-methylated histone residues are consistent with earlier findings that D2 expression,
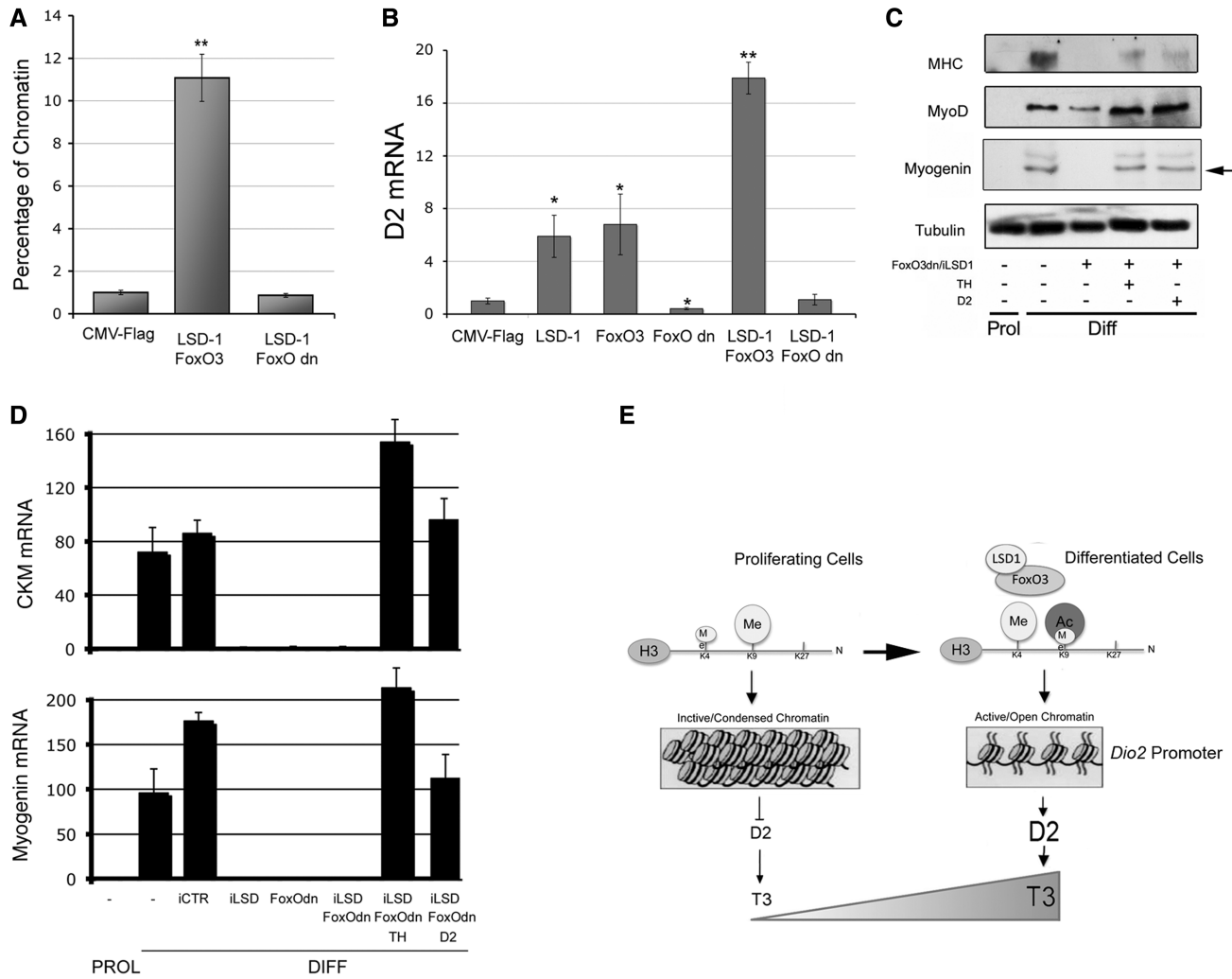
Figure 6. FoxO3/LSD-1 complex regulates D2 expression. (**A**) mpcs were transfected with CMV-Flag, Flag-LSD-1 and Ha-FoxO3 or Ha-FoxO3 dn plasmids, and ChIP assay was performed using the anti-Flag antibody to verify effective LSD-1 binding to the *Dio2* promoter. (**B**) D2 mRNA was measured by RT-PCR from transiently transfected C2C12 cells with the indicated expression vectors. (**C**) Differentiation markers Myogenin and MHC were measured by western blot analysis in mpcs transiently transfected with the indicated plasmids or siRNA constructs. (**D**) RT-PCR was carried out on the same cells to measure mRNA levels of Myogenin and muscle creatin kinase (CKM) levels. (**E**) Schematic diagram illustrating the LSD-1-FoxO3-regulated remodelling of chromatin in the *Dio2* promoter region in proliferating and differentiated muscle cells.

as well as TH signalling and TH target genes, such as MyoD and Myogenin, are highly upregulated during muscle cell differentiation. These data indicate that the state of histone modifications associated with the *Dio2* promoter in muscle cells can have a major impact on the activation kinetics of its transcription.

LSD-1 regulates the balance between D2 and D3 expression

What are the molecular determinants that govern the various *Dio2* promoter transcriptional states during muscle cell differentiation? In the attempt to discover the chromatin modifier enzymes endowed with the ability to control the complex execution of such a remodelling process on the *Dio2* promoter, we identified LSD-1 demethylase as a key component of the gene regulatory network that drives D2 expression and muscle-specific TH production. We demonstrate this in the myogenic cell line

C2C12, as well as in the more physiologically relevant primary culture of muscle stem cells (mpcs).

Notably, we also demonstrate that inhibition of LSD-1 activity by different means (siRNA, dominant negative over-expression or chemical block) potently prevents the upregulation of D2 in differentiating myoblasts. Accordingly, TH production, a crucial step for correct muscle cell differentiation, is abolished on LSD-1 inhibition, which suggests that LSD-1 plays a pivotal role in D2-mediated TH production and, most importantly, that specific de-methylase inhibition can modify TH signalling within a cell. Notably, it has been recently shown that LSD-1 participates in myoblast differentiation by removing the repressive histone codes during C2C12 mouse myoblast differentiation (19). The same study showed that, by associating with Mef2 and MyoD promoters, LSD-1 is required for correct regulation of histone marks during myogenesis. Our data demonstrate that,

besides its upregulation in C2C12 cells, LSD-1 expression increases during the differentiation of mpcs thereby resulting in LSD-1 accumulation 48 h after induction of differentiation. Our study provides further support to the concept that specific modulation of LSD-1 activity might be essential in skeletal muscle differentiation by modulating epigenetic marks.

It has been shown that D2 and D3, which play opposite roles in modulating TH availability at cellular level, often share regulating pathways that, by inversely balancing their expression, rapidly respond to different cellular TH requirements (23,27,28). Such dual control occurs in muscle cells, in which increasing levels of TH are necessary to fulfill muscle terminal differentiation. Our data demonstrate that, besides induction of D2- and D2-mediated TH production, LSD-1 binds to *Dio3* promoter and negatively regulates D3 expression, whereas LSD-1 silencing enhances D3 at mRNA level. Accordingly, the *Dio3* promoter region is bound to methylated H3-K4 lysine in proliferating myoblasts and H3-K9 in differentiated myotubes. This finding is in agreement with the observation that D3 is highly expressed in proliferating muscle cells and repressed during differentiation (Dentice *et al.*, manuscript in preparation).

Thus, by chromatin remodelling action, LSD-1 functions as a novel master regulator of D2–D3 balanced expression during muscle differentiation. In this scenario, it is tempting to speculate that the coupled D2–D3 modulation may be part of a differentiating programme triggered by LSD-1 action in muscle cells.

LSD-1 directly interacts with FoxO3

We previously identified D2 as a FoxO3 target gene in muscle cells (24). Here, we report the first evidence of an interaction between LSD-1 and FoxO3 at endogenous level in muscle cells (Figure 5A–C). Re-ChIP experiments demonstrated that FoxO3 and LSD-1 assemble together on the *Dio2* promoter, and that this interaction results in the concomitant synergistic induction of D2 mRNA (Figures 5C and 6B). Our results establish that the sequential interaction of FoxO3 and LSD-1 is a necessary step in the control of D2 expression. Indeed, blockage of FoxO3 DNA binding displaces LSD-1 from the *Dio2* promoter in muscle cells, thereby impairing D2-mediated TH production and the regulation of the downstream muscle-specific target of TH.

Given the novel role of the LSD-1–FoxO3 axis in *Dio2* gene expression and in muscle differentiation, it is noteworthy that both LSD-1 and FoxO3 have been implicated in muscle differentiation and are strongly upregulated in differentiated myotubes. In this scenario, it is relevant that D2 expression levels are significantly reduced in FoxO3-null muscle as well as in FoxO3-null mpcs (24). Both FoxO3 and LSD-1 have been shown in independent studies to bind to and induce the promoter of MyoD, which is the key transcription factor underlying muscle-specific gene expression and muscle differentiation. Therefore, it is feasible that FoxO3 and LSD-1 might associate on the MyoD promoter as well as on other promoters that are regulated by these two factors. We provide evidence that the interplay between FoxO3 and LSD-1 is an essential step in muscle differentiation, as the absence of FoxO3, LSD-1 or both impairs full differentiation of muscle cells (Figure 6C). This is in agreement with previous studies showing that FoxO3 as well as LSD-1 are required for myoblast differentiation (19,39). Forced expression of D2 or TH treatment rescue the differentiation programme, demonstrating that the FoxO3–LSD-1 and D2 axis is a requirement for proper myogenesis.

In conclusion, we have discovered a functional link between local modulation of TH action and epigenetic remodelling of the chromatin driven by LSD-1 demethylase. The FoxO3–LSD-1 axis, which controls TH signalling in muscle, could offer a specific set of action to affect TH signalling within muscle for therapeutic purposes.

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