Deltex2 represses MyoD expression and inhibits myogenic differentiation by acting as a negative regulator of Jmjd1c

Dan Luo[a,b,1], Antoine de Morree[a,b,1], Stephane Boutet[a,b], Navaline Quach[a,b], Vanita Natu[a], Arjun Rustagi[a], and Thomas A. Rando[a,b,c,d,2]

[a]The Glenn Center for the Biology of Aging, Stanford University School of Medicine, Stanford, CA 94305; [b]Department of Neurology and Neurological Sciences, Stanford University School of Medicine, Stanford, CA 94305; [c]Neurology Service, VA Palo Alto Health Care System, Palo Alto, CA 94304; and [d]Research Enhancement Award Program (REAP), Rehabilitation Research & Development Service, VA Palo Alto Health Care System, Palo Alto, CA 94304

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The myogenic regulatory factor MyoD has been implicated as a key regulator of myogenesis, and yet there is little information regarding its upstream regulators. We found that Deltex2 inhibits myogenic differentiation in vitro, and that skeletal muscle stem cells from Deltex2 knockout mice exhibit precocious myogenic differentiation and accelerated regeneration in response to injury. Intriguingly, Deltex2 inhibits myogenesis by suppressing *MyoD* transcription, and the Deltex2 knockout phenotype can be rescued by a loss-of-function allele for *MyoD*. In addition, we obtained evidence that Deltex2 regulates MyoD expression by promoting the enrichment of histone 3 modified by dimethylation at lysine 9 at a key regulatory region of the *MyoD* locus. The enrichment is attributed to a Deltex2 interacting protein, Jmjd1c, whose activity is directly inhibited by Deltex2 and whose expression is required for MyoD expression in vivo and in vitro. Finally, we find that Deltex2 causes Jmjd1c monoubiquitination and inhibits its demethylase activity. Mutation of the monoubiquitination site in Jmjd1c abolishes the inhibitory effect of Deltex2 on Jmjd1c demethylase activity. These results reveal a mechanism by which a member of the Deltex family of proteins can inhibit cellular differentiation, and demonstrate a role of Deltex in the epigenetic regulation of myogenesis.

Deltex2 | Jmjd1c | MyoD | myoblast | differentiation

During development, myogenic progenitors arise from a population of Pax3/7-expressing cells in the dermomyotome (1, 2) and begin to express the myogenic regulatory factor myogenic differentiation 1 (MyoD) in the somites and myotomes at embryonic day (E) 10.5 and in the limbs at E11.5 (3). During postnatal myogenesis, muscle stem cells (MuSCs, or "satellite cells") give rise to MyoD-expressing cells on activation in response to stimuli such as injury or degenerative diseases (4–6). MyoD-expressing myoblasts ultimately withdraw from the cell cycle and fuse to form multinucleated myotubes, which then develop into myofibers, the mature cells of skeletal muscle. During the process of myoblast differentiation, MyoD expression first increases and then decreases (7, 8). Although MyoD knockout mice have only a modest phenotype (9), likely because Myf5 can compensate, subsequent studies have revealed a delayed differentiation during development (10) and impaired differentiation of MyoD$^{-/-}$ myoblasts despite the expression of Myf5 (11-13). Because of the critical role of MyoD in developmental and regenerative myogenesis, the regulation of its expression has been studied in detail.

Three regulatory elements have been identified in the *MyoD* promoter: a core enhancer region (CER) located ∼20 kb upstream of the transcriptional start site that is active in early embryonic myoblast development, a distal regulatory region (DRR) in the 5′ proximal 6 kb, and a proximal regulatory region (PRR). These three elements function together to drive *MyoD* transcription in adult muscle fibers and cultured muscle cells (14–18). Both serum response factor and MEF2 bind to the DRR to regulate *MyoD* transcription (19, 20). In terms of the complexity of the *MyoD* promoter and the expression profiles of MyoD during development and postnatal myogenesis, additional regulatory factors clearly play roles in the regulation of *MyoD* transcription.

Our previous studies revealed that the Notch signaling pathway plays a critical role in postnatal myogenesis (21, 22), consistent with previous in vitro observations of the inhibition of myogenic differentiation by activation of the Notch pathway (23). This may be attributed to its effects on down-regulation of MyoD. Indeed, ectopic expression of the intracellular domain of Notch (NICD) represses myogenesis by targeting the MyoD basic helix-loop-helix domain (24). In addition, canonical Notch signaling suppresses MyoD expression (25), and forced expression of the active form of the Notch coactivator, RBP-J, inhibits muscle differentiation by blocking the expression of MyoD (25, 26). Given the complexity of the regulation of myogenic differentiation by Notch signaling, it is obvious that Notch signaling needs to be tightly regulated during myogenesis. Therefore, regulators of the Notch pathway may be critical for regulating steps in the myogenic process by their effects on MyoD.

Deltex is a Notch-binding protein that acts as a positive regulator of Notch signaling in *Drosophila* (27–29). Although only one *Deltex* gene has been found in *Drosophila* (27), a Deltex gene family, including *Deltex1*, *Deltex2*, *Deltex3*, and *Deltex4*, has been found in both human and mouse (28). Deltex2 is further divided into a long form and a short form (Deltex2L and Deltex2S, respectively) owing to alternative splicing (30). The mammalian Deltex1 is the homolog most closely related to *Drosophila* Deltex (31). The N-terminal portion of the Deltex protein is necessary and sufficient to bind the ankyrin repeats of Notch (28). Deltex3, lacking key domains in the N-terminal region of Deltex1 and 2,

Significance

The data presented address a fundamental mechanism controlling the expression of a master regulator of cellular differentiation, MyoD, by a member of the Deltex family of proteins. We show that MyoD expression is regulated by modulation of histone methylation in its promoter region by the histone demethylase, Jmd1c. These data provide insight into the epigenetic control of gene expression in the regulation of myogenic differentiation.

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[1]D.L. and A.d.M. contributed equally to this work.

[2]To whom correspondence should be addressed. Email: rando@stanford.edu.

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does not bind to Notch (30), suggesting a Notch-independent function at least for this isoform. The potential role of Deltex in regulation of myogenic differentiation in mammals has not yet been investigated in any detail (30). Other than a decrease in myogenin mRNA levels by the overexpression of Deltex2 in C2C12 cells (30), the regulation of myogenic differentiation by Deltex family members has not been studied either in relationship to Notch signaling or via any Notch-independent mechanisms in mammalian cells.

In studies of the regulation of myogenesis by Notch signaling, we examined the effects of different regulators of Notch signaling, including the Deltex family members, on myogenic differentiation. We found that Deltex2, but not Deltex1, is expressed in murine myogenic progenitors, and that Deltex2 inhibits myogenic differentiation independent of the canonical Notch signaling pathway. Rather, the mechanism of this inhibition is under the control of MyoD expression involving the regulation of H3K9 methylation by the histone demethylase, Jmjd1c. Deltex2 binds to Jmjd1c and inhibits its demethylase activity by monoubiquitination to regulate the pattern of methylation of histones associated with key regulatory regions of the MyoD gene, and thus is a critical determinant of the inducibility of this essential early myogenic regulator. These results reveal a mechanism by which a member of the Deltex family of proteins inhibits myogenic differentiation by regulating MyoD expression, and demonstrate a role of Deltex in epigenetic regulation to control those processes.

Results

Enhanced Muscle Regeneration in Deltex2 KO Mice. Notch signaling plays an important regulatory role in tissue morphogenesis both during development and during postnatal regeneration of skeletal muscle (32). That control is mediated by a series of regulatory proteins that enhance or inhibit Notch signaling by regulating protein processing, localization, activity, and stability (33). We have previously demonstrated a critical role of Notch signaling in postnatal regenerative myogenesis in mice (21). Given that *Drosophila* Deltex positively regulates Notch signaling (28), we were interested in exploring the potential regulation of myogenesis by members of the Deltex family of proteins in a mammalian system. We first examined which of the Notch-interacting Deltex family members (Deltex1, 2, and 4; Deltex3 does not have a Notch-interacting domain) are expressed in mouse myogenic progenitors. Published microarray data revealed the highest expression of Deltex2 in C2C12 myoblasts, which was decreased on differentiation, whereas Deltex1 was barely detectable and Deltex4 was expressed at low levels and increased with differentiation (Fig. S1*A*) (34).

Using RT-PCR, Northern blot, Western blot, and immunofluorescence analyses, we confirmed robust expression of Deltex2 in proliferating C2C12 myoblasts and freshly isolated murine myogenic progenitors, which declined immediately on the onset of myogenic differentiation in C2C12 myoblasts (Fig. S1 *B* and *C*). In contrast, expression of Deltex1 was not detected, and Deltex4, although expressed, exhibited a distinct cytosolic localization when expressed as a GFP-fusion protein, indicating that it has a distinct function (Fig. S1*D*).

Based on these in vitro findings, we wanted to test for any functional role of Deltex2 in myogenesis by analyzing Deltex2 knockout (KO) mice (35). We observed no differences between KO and control mice in terms of muscle morphology, Pax7-positive cells, and transcript levels of the activation marker MyoD in MuSCs (Fig. S1 *E*–*G*), suggesting that developmental myogenesis does not depend on Deltex2 expression. As is the case with many important regulators of myogenesis, such as MyoD (9), the KO yields a negligible phenotype unless the tissue is stressed in some way (11); thus, we examined the regenerative response of the muscles of Deltex2 KO mice to injury. At 3 d postinjury, KO muscles exhibited on average significantly

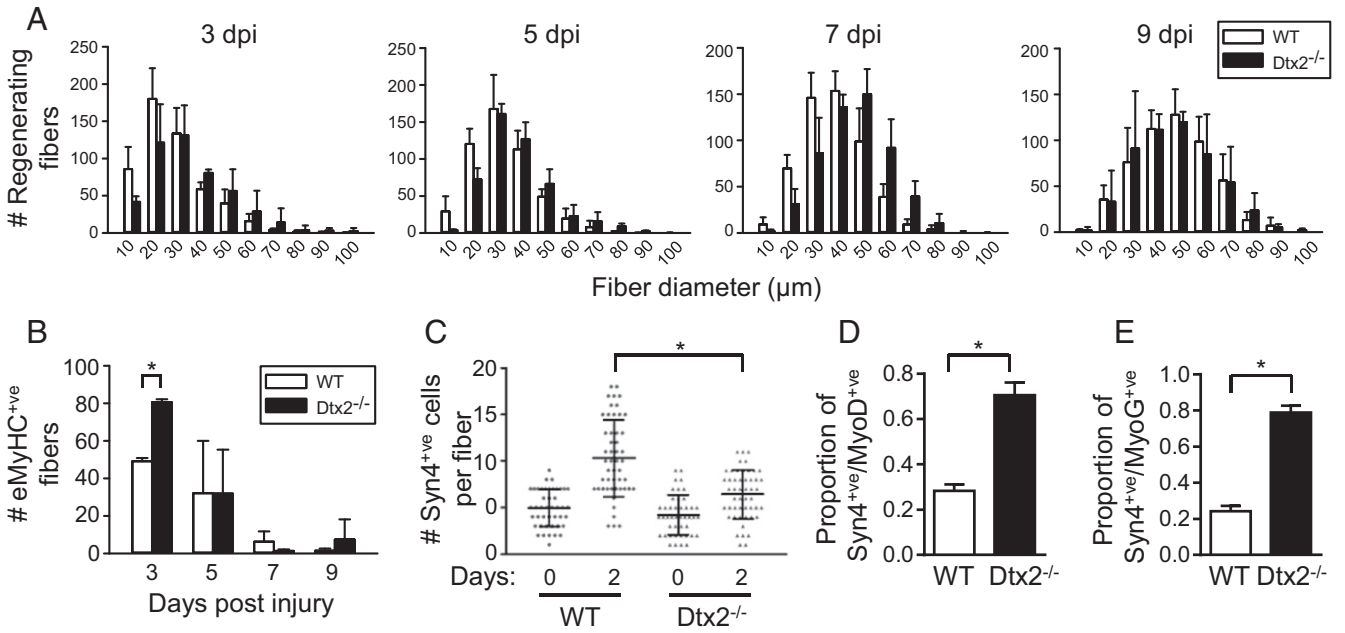

Fig. 1. Knockout of Deltex2 accelerates muscle regeneration and enhances satellite cell differentiation capacity. (*A*) The kinetics of muscle regeneration are enhanced in the absence of Deltex2. Tibialis anterior (TA) muscles of control and Deltex2 KO mice were injured by injection of $BaCl_2$. Cryosections of regenerating muscles were immunostained to detect early regenerating myofibers at 3, 5, 7, and 9 d postinjury, and the numbers of centrally nucleated regenerating fibers were quantified and graphed according to fiber diameter. $P < 0.0001$ for 3, 5, and 7 d postinjury, χ^2 test. (*B*) The numbers of regenerating myofibers expressing embryonic myosin heavy chain, assessed in the studies described in *A*. $^*P < 0.05$. (*C*) Number of $Syn4^{+ve}$ cells counted on single fiber explants from WT and KO ($Dtx2^{-/-}$) extensor digitorum longus (EDL) muscles immediately after dissociation (day 0) or cultured for 2 d (day 2). The gray line represents the mean. (*D* and *E*) Quantitative analysis of the proportion of $Syn4^{+ve}$ SCs expressing MyoD (*D*) or MyoG (*E*) per fiber in single fiber explants from WT or Deltex2 KO mice, cultured for 2 d. $n = 50$. $^*P < 0.0001$.

larger and more regenerating fibers compared with control muscles (Fig. 1 *A* and *B* and Fig. S1*H*). The differences in regenerating fiber sizes persisted through 7 d postinjury, but disappeared by 9 d postinjury (Fig. 1*A* and Fig. S1*H*). These data indicate that the processes of myogenic differentiation and muscle regeneration are accelerated in Deltex2 KO muscles, and suggest that Deltex2 may suppress the myogenic program in myogenic progenitors.

Enhanced Numbers of MuSC Progeny with High Levels of MyoD and MyoG in Deltex2 KO ex Vivo Cultures. To test whether Deltex2 also might regulate myogenesis by mediating MuSC proliferation, we evaluated EdU incorporation in vivo and in vitro in MuSCs from wild-type (WT) and Deltex2 KO mice. We observed no differences (Fig. S2 *A* and *B*), suggesting that proliferation is unperturbed in Deltex2 KO MuSCs (Fig. S2*B*). Likewise, we found comparable levels of quiescence and activation markers during the early stages of MuSC activation in vivo and in vitro (Fig. S2 *C* and *D*). We next isolated single fiber explants from WT and Deltex2 KO mice and analyzed MuSCs and their progeny over time to study myogenesis ex vivo. Immediately on isolation, there was no difference in the number of MuSCs associated with individual myofibers from WT mice compared with those from Deltex2 KO mice (Fig. 1*C*); however, over the next 2 d, the number of MuSC progeny showed a significantly greater increase in the WT cultures compared with the Deltex2 KO cultures (Fig. 1*C*), suggesting that MuSCs from Deltex2 KO muscles may enter the myogenic differentiation program earlier. Analysis of cultures for markers of myogenic differentiation revealed a dramatic increase in the number of cells expressing high levels of MyoD (Fig. 1*D*) and MyoG (Fig. 1*E*) in Deltex2 KO explants. These results suggest that Deltex2 allows for the proliferative expansion of MuSCs by suppressing myogenic differentiation, consistent with the precocious myofiber formation after injury in Deltex2 KO mice.

Knockdown of Deltex2 Enhances the Differentiation of Myogenic Progenitors. To further explore the role of Deltex2 in regulating myogenic differentiation, we isolated MuSCs by flow cytometry and differentiated them in culture. Differentiating Deltex2 KO cells expressed higher levels of MyoD and MyoG compared with control cells and gave rise to larger myotubes (Fig. 2 *A* and *B*). To confirm that this phenotype depends on Deltex2, we used siRNA duplex oligoribonucleotides to knock down endogenous Deltex2 in primary myoblasts. After 24 h of differentiation posttransfection, mRNA levels of Deltex2 (both Deltex2L and Deltex2S) were reduced by 90% (Fig. S3 *A* and *B*), and endogenous Deltex2 protein levels were markedly decreased (Fig. S3*C*). Cells with reduced levels of Deltex2 exhibited accelerated differentiation, as shown by the formation of larger myotubes compared with control cultures (Fig. 2*C*). In siRNA-treated cells, reduction of Deltex2 levels resulted in higher expression levels of MyoD and myogenin compared with control-transfected cultures (Fig. 2*D* and Fig. S3 *D* and *E*). These data clearly show that myoblasts with decreased Deltex2 expression levels have enhanced differentiation, indicating that Deltex2 plays an important inhibitory role in myogenic differentiation.

Deltex2 Inhibits Myogenic Differentiation. To directly test the sufficiency of Deltex2 for suppressing myogenic differentiation, we generated stable transfectants of Deltex2 fused with EGFP at its COOH terminus in C2C12 myoblasts. The Deltex2-expressing cells were induced to differentiate and analyzed for myogenin expression. At times when myogenin was clearly expressed in control cells, it was undetectable in Deltex2-expressing cells (Fig. 2*E*), suggesting an inhibition of myogenic differentiation by Deltex2. This is consistent with findings from previous studies of the role of Deltex2 in neurogenesis (30). Morphological analysis revealed that Deltex2-expressing cells did not form myotubes under differentiation-inducing conditions (Fig. 2*F*), even after maintenance of cultures in differentiation medium (DM) for 8 d. Thus, Deltex2 is a potent inhibitor of myogenic differentiation.

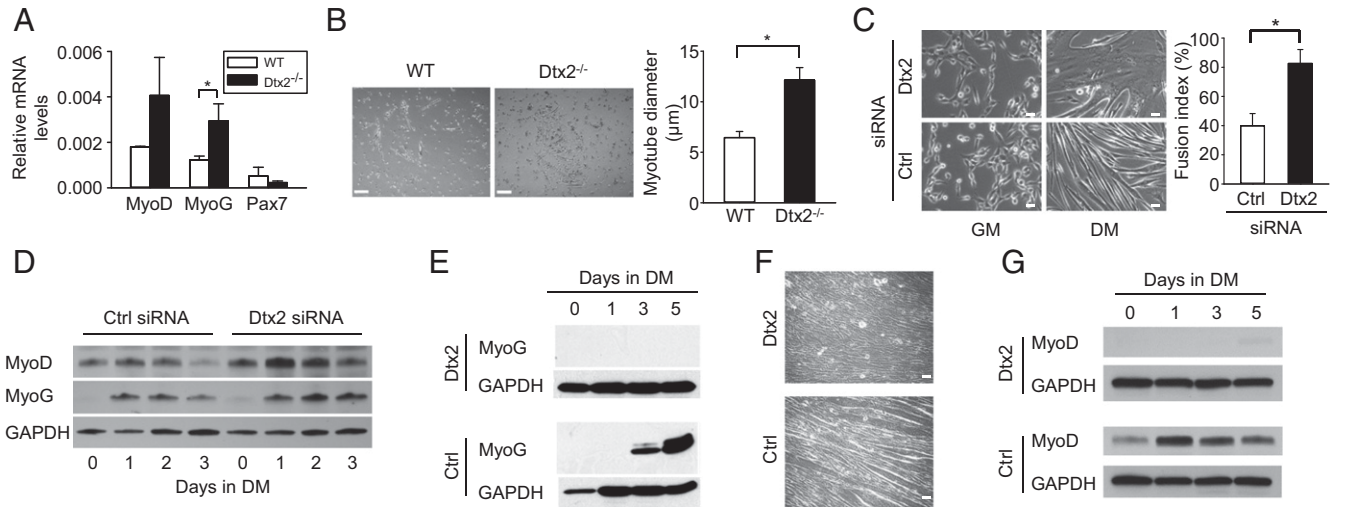

Fig. 2. The effects of Deltex2 on differentiation of primary myoblasts. (*A*) Primary myoblasts were differentiated in vitro and analyzed for differentiation markers by qRT-PCR. (*B*, *Left*) Representative images of primary myoblasts from WT and Deltex2 KO mice differentiated for 6 d in vitro. (Scale bar: 100 μ.) (*B*, *Right*) Graph depicting quantitation of myotube diameters from studies illustrated in the representative images. $n = 3$. $^*P < 0.05$. (*C*) Primary myoblasts were treated with Deltex2 (Dtx2) or control siRNA (Ctrl) and analyzed either in growth medium (GM) or after 3 d in differentiation medium (DM). (*Left*) Representative images of myoblasts and differentiated myotubes in response to siRNA treatment. (Scale bar: 40 μ.) (*Right*) Graph depicting fusion index of cells illustrated in representative images. $n = 3$. $^*P < 0.05$. (*D*) Endogenous MyoD and Myogenin protein levels analyzed by Western blot analysis of primary myoblasts treated with Deltex2 or control siRNA. (*E*) Myogenin levels detected by Western blot analysis of C2C12 cells stably transfected with EGFP-Deltex2 (Dtx2; *Upper*) or a control EGFP vector (Ctrl; *Lower*). Myogenin levels were analyzed as a function of time in DM, as indicated. (*F*) C2C12 cells stably transfected with Deltex2 or control vectors were cultured in DM for up to 8 d and analyzed microscopically for the formation of multinucleated myotubes. (Scale bar: 100 μ.) (*G*) MyoD levels detected by Western blot analysis of differentiating C2C12 cells stably transfected with Deltex2 or control vectors. MyoD levels were analyzed as a function of time in DM, as indicated.

To study the mechanism(s) by which Deltex2 inhibits myoblast differentiation, we analyzed the effect of Deltex2 on the expression of the early myogenic regulatory factors (MRFs), Myf5 and MyoD. Myf5 transcript levels were negligibly affected (Fig. S3*G*), but both MyoD mRNA and protein levels were dramatically decreased in Deltex2-expressing cells compared with control cell populations (Figs. S3 *G–I*). We further analyzed MyoD protein levels in Deltex2-expressing cells under differentiation culture conditions, and found that MyoD protein was not detectable in cultures maintained for up to 5 d in DM, whereas the control cells showed a normal MyoD expression pattern (Fig. 2*G* and Fig. S3*I*). Therefore, from both loss-of-function and gain-of-function studies, our results suggest that Deltex2 might inhibit myogenic differentiation by suppressing MyoD expression.

Mechanism of Deltex2 Inhibition of Myogenesis: Inhibition of MyoD Expression. The regulation of myogenesis is clearly controlled by MRFs with overlapping activities, and MyoD is a critical factor in regulating myogenic differentiation (8). As such, knockdown of MyoD by siRNA (Fig. 3 *A* and *B*) led to a decrease in the formation of multinucleated myotubes and reduced myogenin expression in primary myoblasts induced to differentiate (Fig. 3 *B–D*), phenocopying the effects of Deltex2 expression. Indeed, the expression pattern of endogenous MyoD protein was inversely correlated with that of endogenous Deltex2 protein during normal myogenic differentiation (Fig. 2*G* and Figs. S1*C* and S3*I*). MyoD levels were maximal at the onset of differentiation, whereas Deltex2 levels were minimal at that same time.

To test the hypothesis that the inhibition of differentiation by Deltex2 occurs through the down-regulation of MyoD, we performed rescue experiments by ectopic expression of MyoD in Deltex2-expressing myoblasts. Proliferating cells were transiently transfected with a MyoD expression vector and then induced to differentiate at 1 d after transfection. After incubation in DM for 5 d, Deltex2-expressing myoblasts with constitutive MyoD expression formed myotubes, whereas the cells transfected with a control vector did not (Fig. 3*E*). Myogenin expression could be detected in the MyoD-expressing cells 1 d after the cells were cultured in DM, whereas the control cells expressed myogenin only at day 5 after being cultured in DM (Fig. 3*F*), even though myotubes were still not detected at this time point (Fig. 3*E*). These data indicate that forced expression of MyoD can rescue the inhibition of myogenic differentiation by Deltex2.

To test whether Deltx2-dependent regulation of MyoD underlies the regeneration phenotype observed in vivo in Deltex2 KO muscles, we bred Deltex2 KO mice with a MyoD KO model to obtain Deltex2$^{-/-}$MyoD$^{+/-}$ mice. On muscle injury to these mice, we observed comparable proliferative activity but reduced levels of MyoD expression in activated MuSCs of Deltex2$^{-/-}$MyoD$^{+/-}$ mice compared with Deltex2$^{-/-}$ mice (Fig. S4 *A* and *B*); however, there was a significant decrease in the size of regenerating fibers in the Deltex2$^{-/-}$MyoD$^{+/-}$ mice, comparable to that in controls and significantly smaller than that in Deltex2 KO mice (Fig. 3*G*). Taken together, these data demonstrate that the reduction in MyoD expression can rescue the Deltex2 KO phenotype in vivo and suggest that Deltex2 inhibits myogenic differentiation in vivo by limiting MyoD expression.

Inhibition of Myogenic Differentiation by Deltex2 Is Independent of the Canonical Notch Signaling Pathway. To test whether the inhibition of MyoD expression and myogenic differentiation by Deltex2 is Notch-dependent (28), we analyzed expression levels of MyoD in Deltex2-expressing cells with or without concurrent inhibition of Notch signaling by L-685,458 (36). Like untreated

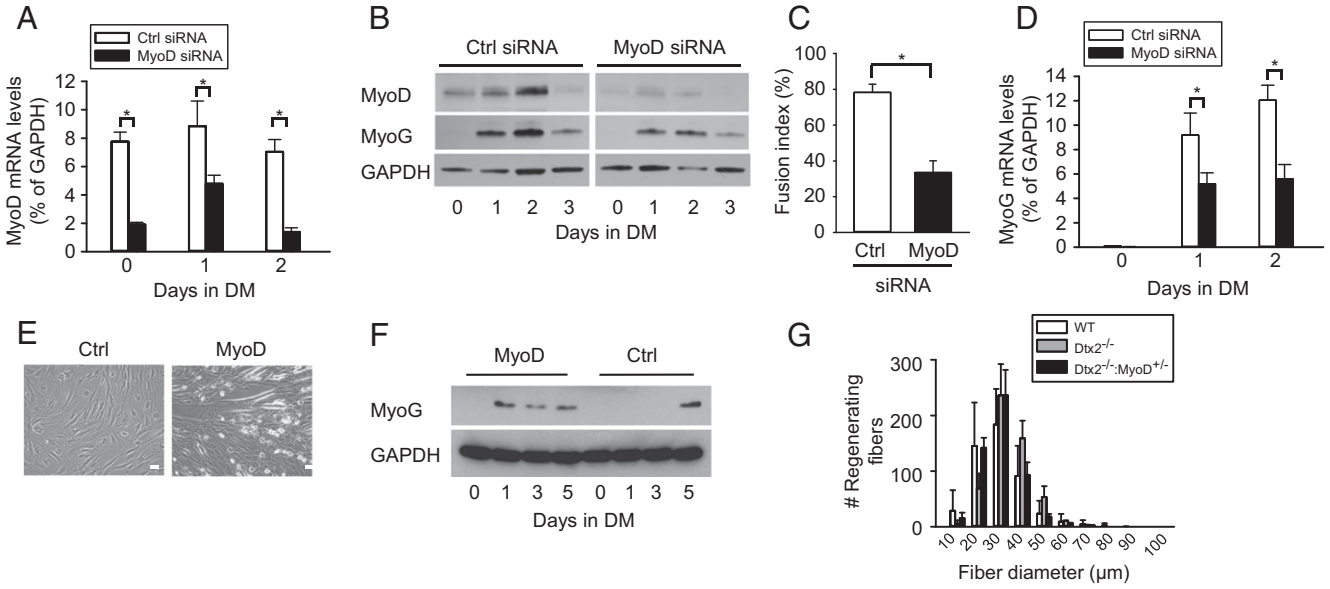

Fig. 3. The inhibition of differentiation by Deltex2 occurs through the down-regulation of MyoD. (*A*) MyoD or control siRNA were transiently transfected into primary muscle cell cultures. GM was replaced with DM at 24 h after transfection. Real-time PCR was used to measure endogenous MyoD mRNA levels. $n = 3$. $^*P < 0.05$. (*B*) MyoD and Myogenin protein levels were analyzed by Western blot analysis of primary myoblasts treated with MyoD or control siRNA. (*C*) Primary myoblast cultures were treated with siRNA as in *A*. Morphological changes were analyzed by quantification of fusion indices after 3 d of differentiation. $n = 3$. $^*P < 0.05$. (*D*) Endogenous myogenin mRNA levels were detected by real-time RT-PCR during differentiation of primary myoblasts treated with MyoD or control siRNA. $n = 4$, $^*P < 0.05$. (*E*) C2C12 cells stably transfected with Deltex2 were cultured in GM and transiently transfected with either MyoD or control vector (Ctrl). The medium was changed to DM at 1 d after transfection. The cells were cultured in DM for 5 d and then analyzed microscopically for the formation of multinucleated myotubes. (Scale bar: 50 μ.) (*F*) Experiments as in *E* were analyzed for Myogenin levels by Western blot analysis as a function of time in DM. (*G*) TA muscles of control, Deltex2$^{-/-}$, and Deltex2$^{-/-}$MyoD$^{+/-}$ mice were injured by injection of $BaCl_2$. Cryosections of regenerating muscles were immunostained to detect early-regenerating myofibers at 5 d postinjury, and the numbers of regenerating fibers was were quantified and graphed according to fiber diameter. (Scale bar: 20 μ.) $P < 0.0001$ comparing Deltext2$^{-/-}$ and Deltex2$^{-/-}$MyoD$^{+/-}$.

cells, the Deltex2-expressing cells treated with the Notch inhibitor for up to 3 d did not express MyoD protein (Fig. S5*A*), suggesting that Notch signaling is not required for the inhibition of MyoD expression by Deltex2. In contrast, after introducing Deltex1 into C2C12 cells, we observed similar inhibition of myogenic differentiation and MyoD expression (Fig. S5*B*), but this could be restored by treatment with L-685,458 (Fig. S5*B*). These results indicate that the inhibition of MyoD expression by Deltex2 is independent of canonical Notch signaling.

In a final test for an effect of Deltex2 on Notch signaling activity, we used a reporter construct (Hes1P wt) in which luciferase is driven by the mouse Hes1 promoter containing a CSL [named for CBF1 (also known as RBP-J), suppressor of hairless (SuH), and Lag-1] binding site. The luciferase activity of this reporter was enhanced by cotransfection with a Notch expression construct that expresses NICD and the associated transmembrane domain, thus requiring cleavage by γ-secretase to liberate the active form of the protein, NICD. This enhancement could be reduced by treating the cells with L-686,458 (Fig. S5*C*). A mutant reporter construct (Hes1P mut) in which the CSL binding site GTGGGAA was mutated to GTGAAAA did not show any activity, even in the presence of activated Notch (Fig. S5*C*). Therefore, luciferase activity from the WT construct is a reliable read-out of Notch signaling. Transfection of Deltex2 did not enhance the luciferase activity significantly (Fig. S5*D*). These data further suggest that Deltex2 acts in a Notch-independent fashion.

Deltex2 Binds to the MyoD Promoter and Enhances H3K9 Dimethylation. We sought to explore the mechanism by which Deltex2 may regulate MyoD expression. Because protein structure analysis did not reveal any potential DNA-binding domain within the Deltex2 protein (30, 37, 38), we tested whether Deltex2 binds to chromatin in myoblasts using a chromatin association assay (39). Both endogenous Deltex2 and exogenous Deltex2 transfected into 293T cells could be detected in the chromatin fraction together with histone H2B, as well as in the soluble nuclear wash (Fig. 4*A*). In contrast, a nuclear protein, DDX6, known to not be associated with chromatin (40), was detected in the soluble nuclear wash fraction but not in the chromatin fraction (Fig. 4*A*). These data support the idea that Deltex2 is a chromatin-associated protein.

To test whether Deltex2 is a component of protein complexes binding to any one of the three known regulatory regions—the CER, DRR, and PRR (16, 17)—on the MyoD promoter, we performed a chromatin immunoprecipitation (ChIP) analysis to screen the MyoD promoter sequence. An antibody against GFP was used to pull down Deltex2-DNA complexes from cells expressing Deltex2-EGFP, with cells expressing EGFP alone serving as controls. Within the 24-kb MyoD promoter sequence, Deltex2-EGFP was enriched in the DRR and the PRR, but not in the CER (Fig. 4*B*).

Because Deltex2 protein binds to both the DRR and PRR regions, we further explored whether this binding is correlated with modification of either Histone H3 or H4 associated with the MyoD promoter. The acetylation and methylation of Histones H3 and H4 play critical roles in regulating gene transcription (41–43). Although there are subtle changes in H3K27Ac and H4K16Ac, our ChIP assays on Deltex2-expressing and control cells did not reveal any significant changes in the acetylation status in histone H3 or H4 at the CER, DRR, and PRR (Fig. S6). Intriguingly, however, in Deltex2-expressing cells, histone H3 was dimethylated at lysine 9 (H3K9me2) and was highly enriched in the DRR region; all other modifications tested (histone H3 dimethylated at lysines 4 or 27 or trimethylated at lysines 4, 9, 27, or 36) remained unchanged in all three regions (Fig. 4*C*). Furthermore, the H3K9me2 enrichment in the DRR region was reduced in primary myoblasts in which the Deltex2 levels were knocked down compared with the control cells (Fig. 4*D*). Given that methylation of histone H3K9 is generally associated with gene repression (44), these data suggest that Deltex2 may inhibit myogenic differentiation by repressing MyoD expression by promoting a specific pattern of histone methylation.

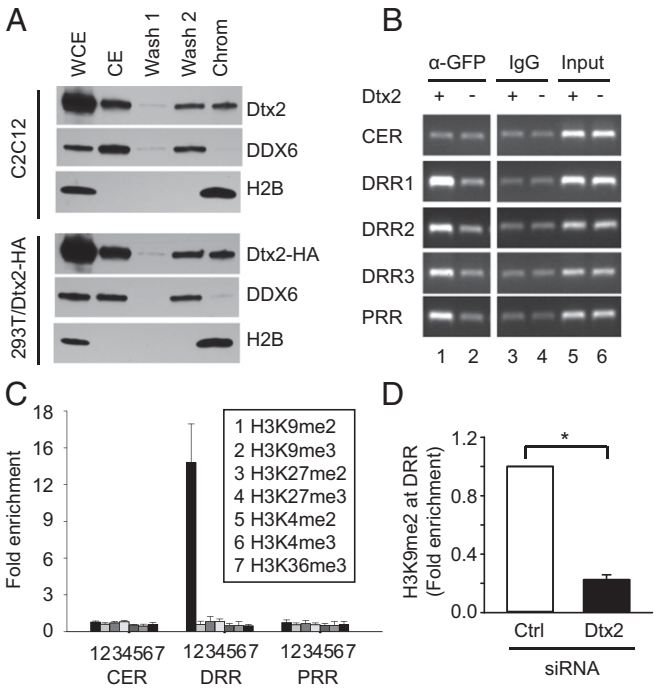

Fig. 4. Deltex2 is a MyoD promoter-binding protein that causes enrichment of dimethylated Histone H3K9 in the DRR of the MyoD promoter. (*A*) C2C12 and 293T cells transfected with HA-Deltex2 were fractionated. The indicated fractions were probed for endogenous Deltex2 or exogenous HA-Deltex2 by Western blot analysis. The non–chromatin-associated protein DDX6 was detected with an anti-DDX6 antibody as a control. An anti-H2B antibody was used to detect chromatin protein. (*B*) C2C12 cells were stably transfected with Deltex2-EGFP (lanes 1, 3, and 5) or EGFP (lanes 2, 4, and 6). Anti-GFP (lanes 1 and 2) or IgG control (lanes 3 and 4) antibodies were used to precipitate protein/DNA complexes. PCR products amplified from the CER, DRR, and PRR regions were separated in 1.5% agarose gels. Three consecutive primers—DRR1, DRR2, and DRR3 (primer sequences listed in Table S1)—were used to analyze the DRR region. (*C*) ChIP assays were performed with indicated antibodies on cell lysates from C2C12 cells stably transfected with Deltex2-EGFP or EGFP alone. ChIP-enriched DNA was quantified by real-time PCR (primer sequences listed in Table S1). H3 antibody served as a control. Values are expressed as fold-change of DNA in Deltex2-EGFP–transfected cells compared with EGFP-transfected control cells (set to 1). (*D*) Primary muscle cell cultures were treated with Deltex2 or control siRNA for 2 d cultured in GM. ChIP assays were performed to detect H3K9me2 enrichment at the DRR region of MyoD promoter in primary myoblasts. The primers and data analysis are the same as in *C*. $n = 3$. $^*P < 0.05$.

Deltex2 Binds to Jmjd1c, a Histone H3K9 Demethylase, and Inhibits Its Activity. Because Deltex2 has no known intrinsic histone-modifying activity, we performed a yeast two-hybrid assay to find proteins that bind to Deltex2, which could explain the enrichment of H3K9me2 at the DRR of the MyoD promoter by Deltex2. We used the second domain of Deltex2 protein as bait (amino acids 192–405; NP_001243025) (30). Among the first clones identified in the screen, a putative histone demethylase, Jmjd1c (also known as Jhdm2c), was identified. Jmjd1c belongs to a subfamily of Jumonji domain-containing protein members that includes Jmjd1a–c and Hairless (45), and both Jmjd1a and Jmjd1c have been shown to have demethylase activity specific for H3K9me2 (46, 47).

To confirm the interaction between Deltex2 and Jmjd1c, we performed coimmunoprecipitation (co-IP) and GST pull-down studies. Because all available antibodies are unsuitable for co-IP

of endogenous Deltex2 and Jmjd1c, we used several independent approaches to test for direct interactions between Deltex2 and Jmjd1c. First, EGFP-fused Jmjd1c and HA-tagged Deltex2 or EGFP-fused Deltex2 and HA-tagged Jmjd1c were cotransfected in HEK293T cells and then subjected to co-IP using an anti-GFP antibody. HA-tagged Deltex2 and HA-tagged Jmjd1c were detected by an anti-HA antibody. Deltex2 or Jmjd1c protein was detected in the immunoprecipitated complexes from Jmjd1c and Deltex2 cotransfected cells, but not in control cells (Fig. 5*A*). Second, lysates of HEK293 cells expressing EGFP-Jmjd1c were run over agarose resins to which bacterially produced GST-Deltex2 was bound. Full-length Jmjd1c was pulled down by GST-Deltex2 agarose but not by control glutathione agarose alone (Fig. 5*B*). These results strongly suggest that Deltex2 binds to Jmjd1c, consistent with the results of the yeast two-hybrid assay.

Given that Deltex2 interacts with Jmjd1c and is enriched at the MyoD promoter regulatory regions, we performed ChIP assays to test whether Jmjd1c protein is similarly enriched at MyoD regulatory regions. Among the three regulatory regions, Jmjd1c protein was most highly enriched at the DRR and even more enriched during differentiation (Fig. 5*C*), suggesting that Deltex2 and Jmjd1c act together at this MyoD promoter region to regulate MyoD transcription by modifying the methylation status of H3K9 there.

Knockdown of Jmjd1c Leads to Enrichment of H3K9me2 at the MyoD Promoter and Decreases MyoD Levels in Myogenic Progenitors. To test whether this Deltex2/Jmjd1c/H3K9me2 axis is a key regulator of MyoD expression and myogenic differentiation, we sought to directly test whether manipulating endogenous Jmjd1c levels would affect the enrichment of H3K9me2 at the MyoD promoter and the expression of MyoD. When Jmjd1c siRNAs were transfected into primary myoblasts, Jmjd1c mRNA and protein levels were reduced to 20% of control levels 1 d later (Fig. 5*D* and Fig. S7*A*). Jmjd1c knockdown led to enhanced H3K9me2 levels in the DRR region (Fig. 5*E*), whereas H3K9ac and H3K9me3 levels were unchanged. In these cells, the expression of MyoD was clearly repressed compared with control cells (Fig. 5*D* and Fig. S7*B*). In contrast, when a Jmjd1c cDNA expression vector was transfected into Deltex2-expressing myoblasts, MyoD mRNA levels and protein levels were significantly increased (Fig. 5*F* and Fig. S7*C*), suggesting that ectopic expression of Jmjd1c can rescue MyoD expression that was inhibited by the expression of Deltex2.

As a further test of the Deltex2/Jmjd1c/H3K9me2 axis, and to test whether Deltex2 protein functionally inhibits the activity of Jmjd1c, we performed histone demethylase activity assays (48) using HA-tagged Jmjd1a and Jmjd1c proteins expressed in HEK293T cells, with or without cotransfection of Myc-tagged Deltex2 and incubated with a bulk preparation of histones from calf thymus as a substrate. Because the size of mouse Jmjd1c (285 kDa) precludes protein purification, we tested a partial Jmjd1c (amino acids 1560–2530) containing the Zn finger and Jumonji C domains, as described previously (47). When this partial Jmjd1c was added into the reaction, the H3K9me2 levels were decreased, albeit to a lesser extent than with the positive control Jmjd1a (Fig. 5*D* and Fig. S7*D*); however, when Deltex2 was present, the ability of Jmjd1c to demethylate H3K9me2 was inhibited (Fig. 5*G*). In contrast, Deltex2 had no effect on the demethylase activity of Jmjd1a (Fig. 5*G*). These results clearly demonstrate that Jmjd1c has H3K9me2 demethylase activity, and that Deltex2 can

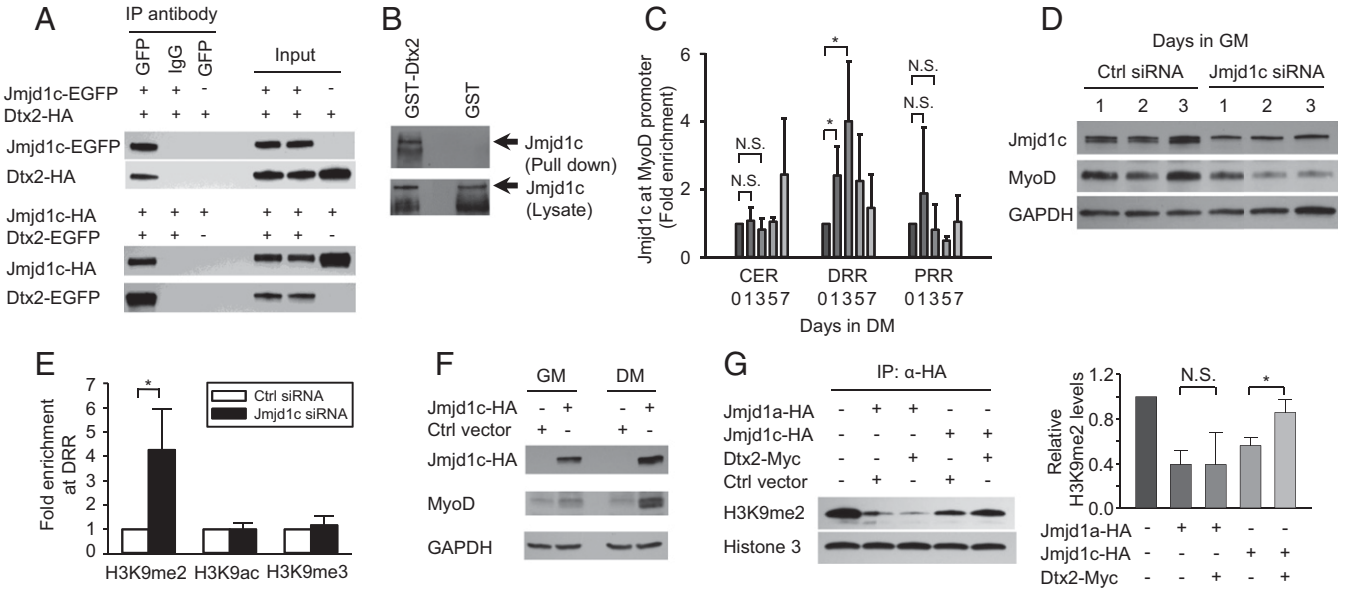

Fig. 5. The inhibition of myogenic differentiation by Deltex2 is mediated through Jmjd1c. (*A*) Jmjd1c-EGFP or EGFP vectors were cotransfected with HA-Deltex2 into HEK293T cells. After 24 h, proteins were immunoprecipitated with an anti-GFP or control antibody. Deltex2 and Jmjd1c were detected by Western blot analysis using antibodies against HA and GFP, respectively. The reciprocal IP is shown as well. (*B*) Lysates of HEK293T cells transfected with EGFP-Jmjd1c were incubated with glutathione-Sepharose 4B bound with GST-Deltex2 or GST only. Jmjd1c was detected by an anti-GFP antibody. Arrows indicate the position of Jmjd1c-EGFP. The Jmjd1c bands from the lysate blot indicate equal loading. (*C*) The enrichment of Jmjd1c at MyoD promoter regions was analyzed with ChIP assays in differentiating human primary myoblasts. During differentiation, the enrichment of Jmjd1c at the DRR was significantly greater than at the CER and PRR. $n = 4$. $^*P < 0.05$. (*D*) Endogenous Jmjd1c and MyoD proteins were analyzed by Western blot analysis in primary myoblasts treated with control or Jmjd1c siRNA. (*E*) Primary myoblasts were treated with Jmjd1c or control siRNA for 2 d. Antibodies against H3K9me2, H3K9me3, and H3K9Ac were used for ChIP analysis. The DRR region of the MyoD promoter was analyzed by real-time PCR. The primers and data analysis are the same as in *C*. $n = 3$. $^*P < 0.05$). (*F*) MyoD protein levels in Deltex2-expressing C2C12 cells transfected with either a Jmjd1c or control vector were analyzed by Western blot analysis. (*G*) HA-Jmjd1a, partial HA-Jmjd1c, Myc-Deltex2, and control vectors were transiently transfected into HEK293T cells. Jmjd1a and Jmjd1c proteins were immunoprecipitated with an anti-HA agarose and eluted with an HA peptide for in vitro histone demethylase activity assays. (*Right*) H3K9me2 and H3 were detected by Western blot analysis and quantified as shown. $n = 3$. $^*P < 0.05$; N.S., not significant.

inhibit that activity specifically. In addition, because H3K9me2 is highly enriched only in the DRR region in Deltex2-expressing myoblasts (Fig. 4*C*), and because both Deltex2 and Jmjd1c proteins are enriched in the DRR region, these observations support the idea that Deltex2 inhibits the demethylase activity of Jmjd1c at the DRR region, leading to high H3K9me2 levels locally, repression of MyoD transcription, and inhibition of myogenic differentiation.

To test whether Jmjd1c is essential for the regenerative phenotype observed in Deltex2 KO muscles, we injured Deltex2 KO muscles and injected siRNA against Jmjd1c into the muscles after 30 and 54 h. We killed the animals at 5 d postinjury and measured the diameters of regenerating fibers. Injured Deltex2 KO muscles treated with siRNA against Jmjd1c exhibited a modest but significant reduction in large regenerating fibers compared with muscles treated with control siRNA, demonstrating that transient knockdown of Jmjd1c can at least partially rescue the regenerative phenotype in Deltex2 KO animals (Fig. S7*E*). These data are consistent with the model in which Deltex2 inhibits myogenic differentiation by inhibiting Jmjd1c and thereby suppressing MyoD expression.

Deltex2 Inhibits Jmjd1c Activity by Mediating Its Monoubiquitination. To examine the mechanism by which Deltex2 negatively regulates Jmjd1c, we focused on the reported enzymatic activity of Deltex2 as an E3 ubiquitin ligase (37), postulating that perhaps Deltex2 inhibits Jmjd1c by causing its ubiquitination and degradation. After ectopic expression of Deltex2 in 293T cells, we found that ubiquitinated Jmjd1c was undetectable in control cells but steadily increased as the amount of Deltex2 was increased (Fig. 6*A*). Interestingly, only monoubiquitination of Jmjd1c was observed. In myoblasts, as in 293T cells, we observed monoubiquitinated, but not polyubiquitinated, Jmjd1c (Fig. 6*B*), and the level of monoubiquitinated protein was greater in cells expressing Deltex2 (Fig. 6*C*). This enhancement was not observed when the cells were transfected with Deltex2 expression constructs mutated in either the WWE domain (Mut1, Dtx2$^{R93A\text{-}R170A}$) or the RING domain (Mut2, Dtx2$^{I411R\text{-}M454R}$) (Fig. S8 *A*–*C*), suggesting that both domains are important in Deltex2-mediated monoubiquitination of Jmjd1c. Of note, the catalytic Deltex2 mutants were unable to reduce MyoD and MyoG expression, demonstrating that Deltex2 inhibits differentiation through its enzymatic activity (Fig. S8*D*).

We next combined purified HA-tagged partial Jmjd1c and GST-fused Deltex2 proteins with a commercial E1-E2 mixture in an in vitro ubiquitination assay, and again observed increasing levels of monoubiquitinated Jmjd1c as the amount of recombinant Deltex2 was increased (Fig. 6*D*), further confirming that

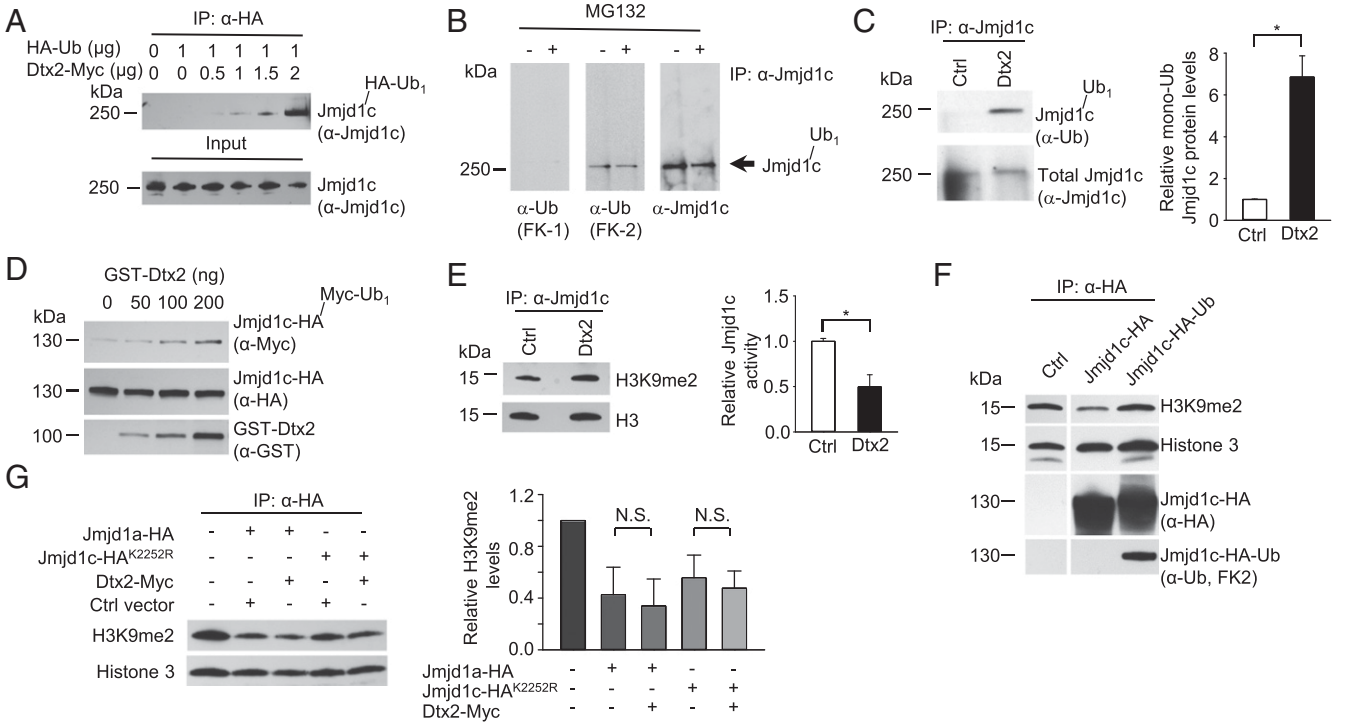

Fig. 6. Deltex2 inhibits Jmjd1c activity by mediating its monoubiquitination. (*A*) Analysis of Jmjd1c ubiquitination. Lysates of 293T cells expressing HA-ubiquitin and Myc-Deltex2 were immunoprecipitated with an anti-HA antibody and analyzed by immunoblotting using an anti-Jmjd1c antibody. (*B*) C2C12 cells were treated with MG132 or DMSO for 6 h and then lysed. Proteins were immunoprecipitated with an anti-Jmjd1c antibody and then analyzed by immunoblotting for Jmjd1c, polyubiquitinated proteins (FK-1), and monoubiquitinated or polyubiquitinated proteins (FK-2). The arrow indicates the positions of nonubiquitinated and monoubiquitinated Jmjd1c, which are not resolved because of the small difference in molecular weights. No polyubiquitinated Jmjd1c protein was detected. (*C*) Analysis of of Jmjd1c monoubiquitination levels. C2C12 cells were transfected with Deltex2 or control vectors for 24 h and then lysed. (*Left*) Proteins were immunoprecipitated with an anti-Jmjd1c antibody and then analyzed by immunoblotting for anti-ubiquitin (FK-2) and Jmjd1c. (*Right*) The levels of monoubiquitinated Jmjd1c (shown quantitatively) were determined as the ratio of monoubiquitinated to total Jmjd1c protein, normalized to control. $n = 3$. $^*P < 0.05$. (*D*) In vitro ubiquitination assay. Purified HA-Jmjd1c and GST-Deltex2 were incubated with Myc-ubiquitin and an E1 and E2 mixture and then analyzed by immunoblotting for Myc. The blot was then stripped and reprobed for HA and GST. (*E*) Nuclei of C2C12 cells transfected with Deltex2 (Dtx2) or empty (Ctrl) vectors were isolated and lysed under nondenaturing conditions. Total (native and monoubiquitinated) Jmjd1c was immunoprecipitated and used in demethylation activity assays. (*Left*) The assays were immunoblotted for H3K9me2. The blot was then stripped and reprobed for H3. (*Right*) The relative Jmjd1c activity was designated as the inverse value of H3K9me2 protein levels (shown quantitatively) standardized to total H3, and then normalized to control. $n = 3$. $^*P < 0.05$. (*F*) Jmjd1c-HA or Jmjd1c-HA-Ub proteins were immunoprecipitated from lysates of transfected 293T cells and used in Histone demethylation assays in vitro. H3K9me2, H3, and HA were detected by Western blot analysis. The blot was then stripped and reprobed for anti-ubiquitin (FK2). (*G*) Purified HA-Jmjd1a or HA-Jmjd1c^{K2252R} proteins from transfected 293T cells were tested for histone demethylase activity in vitro. H3K9me2 and H3 were detected by Western blot analysis and quantified as shown. $n = 3$. N.S., not significant.

Deltex2 acts as an E3 ligase leading to the monoubiquitination of Jmjd1c. Taken together, the data both in cells and with purified proteins demonstrate that Deltex2 can directly monoubiquitinate Jmjd1c.

Although monoubiquitination is typically associated with altered protein function (49, 50), it also has been reported to lead to proteasomal degradation, similar to the effects of polyubiquitination (51). Thus, we asked whether the E3 ligase activity of Deltex2 could lead to the ubiquitination and proteasomal degradation of Jmjd1c. Treating C2C12 cells with the proteasome inhibitor MG132 did not lead to an accumulation of Jmjd1c protein (Fig. S9*A*), suggesting that Jmjd1c protein levels are not regulated by proteasomal degradation. In addition, levels of endogenous Jmjd1c were not affected by either overexpression or knockdown of Deltex2 in myoblasts, suggesting that Deltex2 does not regulate steady-state Jmjd1c protein levels (Fig. S9 *B–D*).

Given that the ubiquitination of Jmjd1c by Deltex2 did not appear to inhibit demethylase activity due to Jmjd1c degradation, we asked whether the monoubiquitination of Jmjd1c might directly inhibit the enzymatic activity. We found that the histone demethylase activity of endogenous Jmjd1c present in C2C12 cells was reduced to <50% of control values in cells with elevated levels of monoubiquitinated Jmjd1c induced by the expression of Deltex2 (Fig. 6*E*). We tested for a causal relationship between Jmjd1c monoubiquitination and reduced demethylase activity through two independent approaches. The use of ubiquitin fusion proteins has proven to be a valuable approach to examining the effects of monoubiquitination on protein function (52). Along that line, we created a fusion protein to mimic the monoubiquitinated Jmjd1c protein and to test the effects on histone demethylase activity. Consistent with the finding that Jmjd1c histone demethylase activity is decreased when it is monoubiquitinated by Deltex2, enzyme activity was completely absent in the Jmjd1c-ubiquitin fusion protein (Fig. 6*F*).

To further test whether Deltex2 negatively regulates Jmjd1c demethylase activity by monoubiquitination, we sought to identify and mutate the lysine residue targeted for ubiquitination by Deltex2 on Jmjd1c. The 285-kDa size of the Jmjd1c protein confounds purification for mass spectrometry analysis; thus, we turned to structural modeling to identify which of the 184 lysines in Jmjd1c might be targeted by Deltex2. Because the yeast two-hybrid experiments showed that Deltex2 binds to a part of the Jmjd1c protein that includes the demethylase domain, and because monoubiquitination affects demethylase activity, we generated and tested a structural model of the demethylase domain in Jmjd1c (Fig. S10*A*). In this structural model, a single lysine residue, lysine 2252 (NP_997104.2), protrudes from the rim of the enzymatic pocket with its side chain facing outwards. If ubiquitinated, it would sterically block substrates from entering the enzymatic pocket and thus inhibit demethylase activity. Moreover, multiple sequence alignments of vertebrate Jmjd1 demethylase domain sequences revealed that lysine 2252 is the only conserved lysine close to the enzymatic pocket (Fig. S10*B*). Thus, we mutated lysine 2252 to arginine to examine the potential role of lysine 2252 in the Deltex2-mediated inhibition of Jmjd1c function. The mutant protein is soluble (Fig. S7*D*) and retains its demethylase activity (Fig. 6*G*), indicating that it is properly folded. When transfected into 293T cells, the level of ubiquitinated mutant Jmjd1c was almost undetectable compared with that of WT Jmjd1c (Fig. S10*C*), indicating not only that lysine 2252 is a monoubiquitination target in Jmjd1c, but also that it appears to be the predominant site for ubiquitination. Furthermore, Deltex2 was not able to promote ubiquitination of the mutant Jmjd1c, confirming that lysine 2252 is the site monoubiquitinated by Deltex2 (Fig. S10*D*). Most importantly, the demethylase activity of the Jmjd1c mutant was not affected by Deltex2 (Fig. 6*G*), strongly suggesting that monoubiquitination at lysine 2252 is the mechanism by which Deltex2 negatively regulates the demethylase activity of Jmjd1c. Taken together, these date provide a mechanistic link between Deltex2 activity and enrichment of H3K9 dimethylation in the MyoD promoter by virtue of the ability of Deltex2 to inhibit the histone demethylase activity of Jmjd1c by monoubiquitination (Fig. S10*E*).

Discussion

In the present study, we have explored the role of the Deltex family of proteins in muscle cell differentiation. Surprisingly, we could detect strong expression of Deltex2, but not of Deltex1, in primary mouse muscle cells, C2C12 myoblasts, and skeletal muscle tissue. Interestingly, Deltex2 inhibited the differentiation of myogenic progenitors independent of Notch signaling; rather, it inhibited MyoD transcription. We found that Deltex2 is part of a chromatin complex that binds to the DRR and PRR regions of the MyoD promoter, leading to enrichment of H3K9me2 in the DRR region. Deltex2 binds to a member of the Jumonji family of histone demethylase proteins, Jmjd1c, and inhibits its demethylase activity. Manipulation of Jmjd1c levels leads to changes in MyoD expression levels associated with corresponding changes in the enrichment of H3K9me2 at the DDR region of the *MyoD* gene. Finally, we have demonstrated that monoubiquitinated Jmjd1c mediated by Deltex2 exhibits reduced its histone demethylase activity. Taken together, these results demonstrate a mechanism by which a member of the Deltex family of proteins regulates cellular differentiation epigenetically, a mechanism that is independent of the canonical Notch pathway and associated with H3K9me2 enrichment at the DRR of the *MyoD* promoter.

Although three key regulatory elements—the CER, DRR, and PRR—have been identified in the *MyoD* promoter, the mechanisms that control *MyoD* transcription are complex and remain incompletely defined (55). CpG demethylation in the CER might be required for enhancer activity in myogenic cells during development (56), but the CER appears not to be an essential regulatory region postnatally (18). Moreover, the CER of *MyoD* does not confer muscle specificity in cultured cells (17). Reporter assays using the DRR and PRR in C2C12 cells found that the DRR must be integrated to reflect endogenous gene activity (16). These observations suggest that MyoD may be regulated by epigenetic mechanisms, and that chromatin remodeling may be required. Because Deltex2 does not have any obvious DNA-binding domain, our finding that Deltex2 binds to histones associated with the *MyoD* promoter at the DRR and PRR suggests that Deltex2 associates with proteins that bind directly to DNA. Using ChIP, we found that in the presence of Deltex2, there was enrichment of H3K9me2 at the DRR, which demonstrates a direct link between histone methylation in the *MyoD* promotor and myogenic differentiation.

Histone methylation, a reversible process, is an important mechanism in the control of gene transcription (43, 44). In general, methylation of histone H3 at K9 and K27 and methylation of histone H4 at K20 are associated with repressed regions of chromatin, whereas methylation of histone H3 at K4, K36, and K79 is associated with active regions (44). Histone methylation is reportedly involved in the regulation of myogenic differentiation. The methylation of histone H3 lysine 9 by Suv39h occurs at several Rb/E2F target promoters and appears to be part of the control switch for C2C12 cells to exit the cell cycle and undergo differentiation (57). The removal of H3K27 methylation, regulated by dissociation of an active Ezh2-containing protein complex from the regulatory regions of myogenic genes, is required for muscle cell differentiation (58). The association of the histone methyltransferase Suv39h with MyoD, as well as methylation of H3K9 on the myogenin promoter by Suv39h, is essential for the inhibition of myogenic differentiation (59). Demethylation of H3K9me2 by the histone demethylase LSD1 at promoters of myogenic regulatory genes is associated with myogenic differentiation (60). Our results indicate that overexpression of Deltex2 changes the methylation status of H3K9 at the DRR of the *MyoD* promoter, a change that

results in suppression of *MyoD* transcription and a resulting block of differentiation.

Recent observations indicate that Deltex acts as an E3 ligase, leading to the ubiquitination of its targets. Indeed, Deltex1 degrades MEKK1 through ubiquitination (61), Deltex4 targets a kinase TBK1 for degradation (62), Deltex forms a trimeric protein complex with Notch and Kurtz that mediates the degradation of the Notch receptor through a ubiquitination-dependent pathway (63), Deltex monoubiquitinates Notch in $S2R^+$ cells, and Deltex is capable of self-ubiquitination (37, 64). However, no target of Deltex2 as an E3 ligase has been identified to date, nor has any study linked Deltex2 E3 ligase activity to myogenic differentiation.

Our data demonstrate that Deltex2 can inhibit Jmjd1c activity by monoubiquitination, suggesting that Deltex2 may function as an E3 ubiquitin ligase leading to either monoubiquitination or polyubiquitination. Similar functionalities have been found in other E3 ligases. Nedd4 regulates epidermal growth factor receptor pathway substrate clone 15 (Eps15) by monoubiquitination (65), but it also polyubiquitinates certain PPxY motif-containing proteins (66). Another well-studied example is Mdm2, which monoubiquitinates p53 but polyubiquitinates p53 when bound to MdmX (67). Recent studies suggest that specific E2 proteins are critical determinants that control protein monoubiquitination or polyubiquitination (68). Which E2 controls Deltex2 specificity of monoubiquitination remains to be determined. Polyubiquitination typically leads to proteasome degradation, whereas protein monoubiquitination plays important roles in regulating protein activity for such processes as protein trafficking, DNA repair, and gene transcription (69, 70). Our observation that monoubiquitination of Jmjd1c results in inhibition of enzymatic activity is consistent with this function.

Whereas Jmjd1a and Jmjd1b have been convincingly shown to have histone demethylation activity on H3K9me2 (46, 71), reports of the demethylase activity of Jmjd1c have been mixed. On the one hand, Jmjd1c overexpression was found to result in a global decrease in H3K9me2 (47), whereas knockdown of Jmjd1c led to an increase in H3K9me2 at specific genes (72), consistent with our findings at the *MyoD* promoter. On the other hand, recombinant Jmjd1c protein was shown to be ineffective at demethylating H3K9 in one study (71), but to be effective after supplementation with 293T cell nuclear extract in another study (72). This variable in vitro activity may reflect a dependency of the enzyme on specific cofactors, which is a topic for future studies.

In summary, we found that Deltex2 negatively regulates myogenic differentiation in a Notch-independent manner by inhibiting *MyoD* transcription. Deltex2 is a nuclear protein that causes enrichment of H3K9me2, associated with gene silencing, at a key regulatory region of the *MyoD* promoter. This effect is due to the binding of Deltex2 to Jmjd1c, a histone demethylase targeting H3K9me2, and to the negative regulation by Deltex2 of the demethylase activity of Jmjd1c via monoubiquitination. These data provide evidence for a mechanism of the regulation of myogenic differentiation by the epigenetic modulation of MyoD expression and present a paradigm for a novel role of the Deltex family of proteins in gene regulation.

Materials and Methods

All animal handling practices and procedures, including animal health monitoring, diet, primary enclosures, environmental control, and means of identification used in these studies, were in accordance with the current recommendations of the American Veterinary Medical Association. Animal protocols were approved by the Administrative Panel on Laboratory Animal Care of VA Palo Alto Health Care System. Detailed descriptions of the materials and methods used in this work are provided in *SI Materials and Methods*, including a descriptive list of chemicals and materials used, and details on the experimental protocols used to carry out in vitro and in vivo experiments.

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