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STEM CELLS AND REGENERATION

RESEARCH ARTICLE

SMAD2 promotes myogenin expression and terminal myogenic differentiation

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ABSTRACT

SMAD2 is a transcription factor, the activity of which is regulated by members of the transforming growth factor β (TGFβ) superfamily. Although activation of SMAD2 and SMAD3 downstream of TGFβ or myostatin signaling is known to inhibit myogenesis, we found that SMAD2 in the absence of TGFβ signaling promotes terminal myogenic differentiation. We found that, during myogenic differentiation, SMAD2 expression is induced. Knockout of SMAD2 expression in primary myoblasts did not affect the efficiency of myogenic differentiation but produced smaller myotubes with reduced expression of the terminal differentiation marker myogenin. Conversely, overexpression of SMAD2 stimulated myogenin expression, and enhanced both differentiation and fusion, and these effects were independent of classical activation by the TGFβ receptor complex. Loss of *Smad2* in muscle satellite cells *in vivo* resulted in decreased muscle fiber caliber and impaired regeneration after acute injury. Taken together, we demonstrate that SMAD2 is an important positive regulator of myogenic differentiation, in part through the regulation of *Myog*.

KEY WORDS: SMAD2, TGFβ, Muscle development, Myogenin, Regeneration, Mouse

INTRODUCTION

After injury, skeletal muscle can regenerate owing to the presence of myogenic precursor cells, called satellite cells, found between the muscle fiber sarcolemma and the basal membrane (Mauro, 1961). Satellite cells are a heterogeneous population characterized by both their histological location and their expression of paired box protein 7 (PAX7). Normally quiescent, satellite cells are activated following muscle injury to proliferate and differentiate into myocytes that can fuse to one another to form myofibers, or to damaged myofibers to repair them (Charge and Rudnicki, 2004). Adult skeletal myogenesis is a well-organized process governed by the induction and expression of transcription factors known as the myogenic regulatory factors (MRFs). The MRFs, including MYF5, MYOD, myogenin (MYOG) and MRF4 (also known as Myf6), are part of the basic helix-loop-helix family of transcription factors, which bind E-boxes found in many myogenic promoters and are known for their ability to convert non-myogenic cells to the myogenic lineage by upregulating muscle specific genes (Wang and Rudnicki, 2012). MYF5 and MYOD are important myogenic commitment factors, while myogenin and MRF4 are induced later in differentiation and are necessary for the development of mature muscle. MYOD^{−/−} animals have normal skeletal muscle (Rudnicki et al., 1992), and loss of MYOD can be compensated for by prolonged MYF5 expression (Megeney et al., 1996). MYF5^{−/−} animals die shortly after birth of respiratory failure due to a malformed rib cage, but similar to MYOD^{−/−} animals, they have relatively normal skeletal muscle with unchanged expression of MYOD, myogenin and MRF4 (Braun et al., 1992). Myogenin^{−/−} mice die perinatally from respiratory failure due to a severe reduction in all skeletal muscle, characterized by an abundance of mononucleated cells and rare myofibers, with a failure to induce the contractile protein myosin heavy chain (MyHC), suggesting these myoblasts are committed to the myogenic lineage but fail to differentiate and form myofibers (Hasty et al., 1993; Nabeshima et al., 1993). MRF4^{−/−} mice have normal expression of muscle-specific genes, suggesting that myogenin has the crucial role in myogenic differentiation (Hasty et al., 1993).

Many signaling pathways regulate myogenic differentiation, including members of the transforming growth factor β (TGFβ) superfamily – TGFβ being a potent inhibitor (Liu et al., 2001, 2004). Both TGFβ and family member myostatin activate the transcription factors SMAD2 and SMAD3 through receptor-dependent phosphorylation of their C-termini, promoting their interaction with SMAD4, nuclear localization and interaction with response elements in the promoters and enhancers of target genes (Shi and Massague, 2003). As such, SMAD2 and SMAD3 are largely considered to mediate an anti-myogenic arm of TGFβ family signaling. However, despite the inhibitory role of myostatin and TGFβ signaling on myogenesis, loss of SMAD3 expression in myoblasts impairs myogenic differentiation (Ge et al., 2011, 2012), suggesting that SMAD3 has some pro-myogenic functions. In accordance with this, retinoic acid treatment, which promotes myogenic differentiation, can stimulate SMAD3 expression and rescue myogenesis in the presence of TGFβ, at least in part through a physical interaction between SMAD3 and C/EBPβ, a transcriptional factor that is present in undifferentiated cells and acts to inhibit myogenic differentiation (Lamarche et al., 2015).

SMAD2 and SMAD3 are highly conserved and are activated similarly; however, there is increasing evidence that these two transcription factors have divergent roles *in vivo* with functions beyond classical TGFβ signaling. Although SMAD3-null mice are viable and fertile (Yang et al., 1999; Zhu et al., 1998), deletion of SMAD2 is embryonic lethal as embryos fail to gastrulate and induce mesoderm (Nomura and Li, 1998; Waldrip et al., 1998; Weinstein et al., 1998), suggesting that, during development, SMAD2 is of greater importance. Although the role of SMAD3 in myogenesis has been investigated, little is known about the specific contribution of

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DEVELOPMENT

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SMAD2 to myogenic differentiation. Herein, we developed a conditional SMAD2 knockout mouse in which SMAD2 expression is abolished in PAX7^{+} muscle myogenic precursor (satellite) cells (Smad2[SC−/−]). We report that SMAD2 is required for efficient myogenic differentiation in a TGFβ-independent mechanism. In its absence, postnatal muscle growth and muscle regeneration after injury are impaired. We find that SMAD2 regulates the expression of key regulators of myogenesis to promote differentiation.

RESULTS

SMAD2 expression promotes myogenic differentiation

To characterize the role of SMAD2 during adult myogenesis, we first quantified Smad2 mRNA and protein expression in the myogenic cell line C2C12, cultured under growth conditions and after induction to differentiate. Smad2 mRNA expression was relatively stable during myoblast differentiation (Fig. 1A). Interestingly, SMAD2 protein expression was lower in proliferating myoblasts seeded at low density (low confluency, LC) but increased as culture density increased (high confluency, HC) and in early myogenic differentiation, coincident with the upregulation of myogenin expression (Fig. 1B,C). After 2 days of differentiation, SMAD2 protein levels returned to levels observed in sub-confluent cells (Fig. 1B,C).

To determine the role of SMAD2 during myogenic differentiation, C2C12 myoblasts were retrovirally transduced to express full-length SMAD2, or treated with empty virus (pLPCX), and overexpression of SMAD2 was confirmed by western blot and RT-qPCR in proliferating cells (Fig. 2A,B). After differentiation for up to 4 days under low serum conditions, overexpression of SMAD2 increased both the percentage of cells that differentiated as well as the average myotube size (fusion index), suggesting that SMAD2 positively regulates myogenic differentiation (Fig. 2C-E). The enhanced differentiation and fusion were not due to variations in cell numbers as this was unchanged by SMAD2 overexpression (Fig. 2F). Further, we did not observe any differences in the percentage of Ki67^{+} cells or BrdU uptake in SMAD2-overexpressing cells compared with controls (Fig. S1A,B).
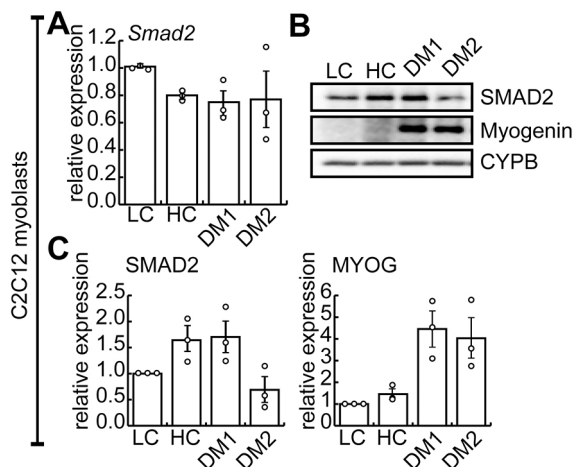

Fig. 1. SMAD2 expression is regulated during myogenic differentiation. (A) RT-qPCR analysis of Smad2 expression in C2C12 myoblasts that were cultured in growth medium at low confluency (LC), high confluency (HC) or following induction to differentiate in low serum conditions (differentiation medium, DM) for one day (DM1) or 2 days (DM2). n=3. (B) Representative western blot of SMAD2 and MYOG expression in C2C12 cells under growth conditions and after induction to differentiate for the indicated days. CYPB is a loading control. (C) Quantification of SMAD2 and MYOG expression from B compared with LC. n=3. Data are mean±s.e.m. (biological replicates).

Analysis of myogenic marker expression revealed that SMAD2 overexpression did not impact Smad3 expression but stimulated both Myog and Tmem8c (myomaker; Mymk) expression after 1 day of differentiation (DM 1) (Fig. 2G). Myogenin protein levels were also increased on DM 1 compared with control cells (Fig. 2H,I), consistent with enhanced myogenic differentiation and fusion.

Given the positive effect of SMAD2 overexpression on myogenic differentiation, we assessed the expression of markers associated with undifferentiated and differentiated cells under growth conditions (Fig. 2J). As in differentiating cells, Smad3 levels were not affected by Smad2 overexpression under growth conditions. However, the expression of Pax7 and Cebpb, two markers associated with the undifferentiated state, was downregulated in cells overexpressing Smad2, whereas Myod1 expression was unaffected (Fig. 2J). Further, Myog expression was greatly increased in cells overexpressing Smad2, suggesting that SMAD2 overexpression promotes precocious differentiation of myoblasts under growth conditions (Fig. 2J).

As myoblasts can produce TGFβ ligands and do express TGF receptors, we examined SMAD2 phosphorylation in proliferating and differentiating myoblasts. We found no detectable C-terminal phosphorylation in these cells in the absence of exogenous TGFβ (Fig. 2K) consistent with our previous observations (Lamarche et al., 2015), in which SMAD3 was also found to not be phosphorylated in untreated cells. We next generated pooled stable C2C12 cell lines expressing full length SMAD2 or a truncated SMAD2 in which the C-terminal SSMS motif, targeted by the activated TGFβ receptor complex, was deleted (SMAD2ΔSSMS) (Fig. 2L). The SMAD2ΔSSMS mutant is not responsive to TGFβ signaling (Choy et al., 2000). Upon differentiation, the SMAD2ΔSSMS was able to enhance myogenic differentiation and fusion similarly to full length SMAD2 (Fig. 2M-O) without impacting cell numbers (Fig. 2P), suggesting that the stimulation of myoblast fusion by SMAD2 does not depend on the presence of the C-terminal SSMS motif and therefore classical TGFβ signaling pathways.

We next characterized the myogenic potential of primary myoblasts deficient for Smad2. Primary myoblasts were isolated from the Smad2^{fl/fl} mouse (Ju et al., 2006) and retrovirally transduced to express a tamoxifen-regulated Cre recombinase (CreER) (Nishijo et al., 2009). Excision of Smad2 was achieved by tamoxifen (4OH-TAM) treatment of pooled stable cell lines to generate a Smad2-deficient cell line (+TAM), or with vehicle to generate controls (Veh). Tamoxifen treatment resulted in near complete loss of Smad2 mRNA expression (Fig. 3A). To determine the impact of Smad2 depletion on myogenic differentiation, TAM- and vehicle-treated cultures were induced to differentiate for 2 days under low serum conditions. Although the loss of Smad2 expression did not negatively impact myogenic differentiation as measured by the percentage of nuclei found within MyHC^{+} cells (differentiation index; Fig. 3B,C), myotube maturation was impaired, as evidenced by much smaller myotubes in cultures lacking Smad2 and a reduced fusion index (average number of nuclei/myotube; Fig. 3B,D). The culture density was unaffected by loss of Smad2 expression (Fig. 3E) and the percentage of Ki67^{+} cells was similarly unaffected (Fig. S1C). Although the expression of Tmem8c and Myod1 was highly variable and largely unchanged in tested cells, Myog and neonatal myosin heavy chain (Myh8), markers of later differentiation, were significantly reduced in Smad2-deficient cultures, consistent with impaired terminal differentiation (Fig. 3F). Expression of Smad3 was unchanged with knockdown of Smad2, suggesting that this factor does not increase its expression to compensate for the loss of Smad2 in myoblasts (Fig. 3F). Western blot analysis revealed that cells lacking SMAD2 also express less
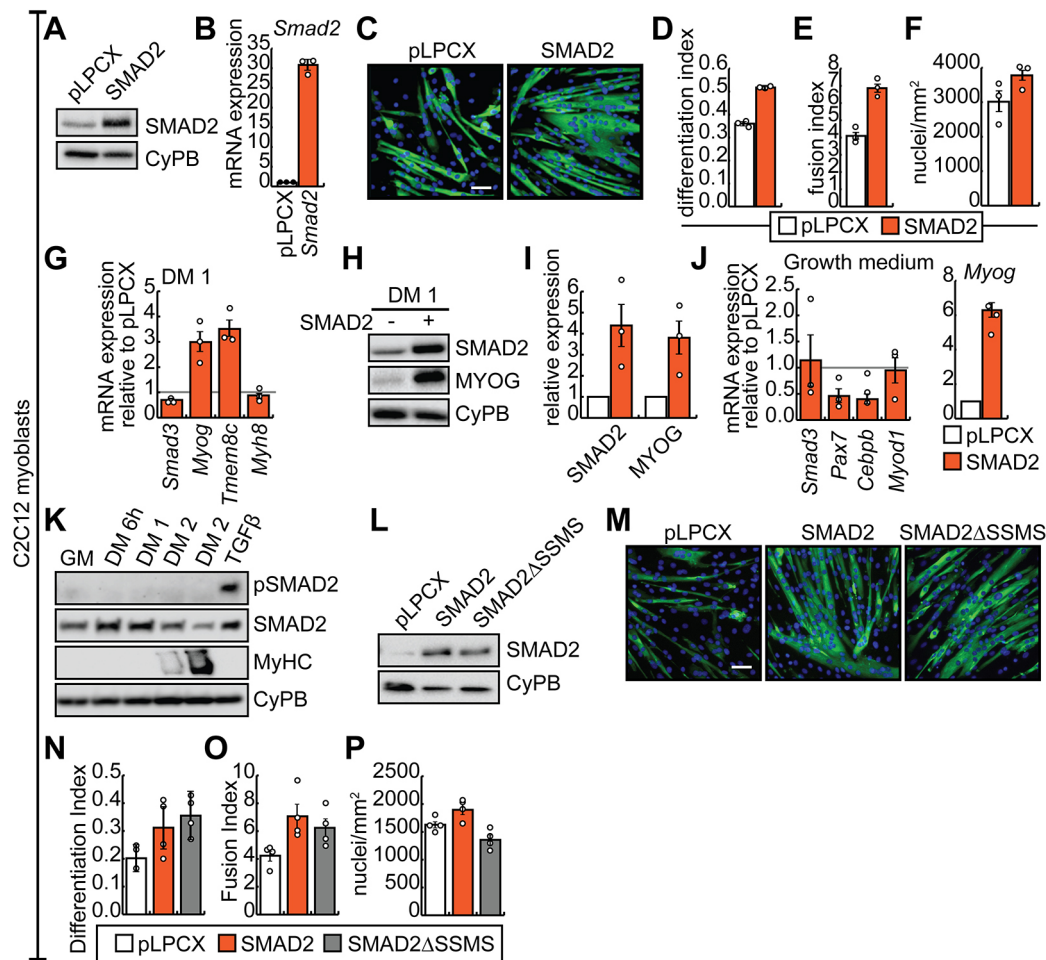
Fig. 2. Overexpression of SMAD2 enhances terminal differentiation and myoblast fusion. (A) Western blot of SMAD2 expression in C2C12 myoblasts retrovirally transduced to express SMAD2 or with empty virus (pLPCX) and differentiated for 24 h. CyPB is a loading control. (B) RT-qPCR analysis of *Smad2* expression in cells from A. $n=3$. (C) Immunostaining for MyHC (green) in cells transduced as in A and induced to differentiate for 4 days. DAPI (blue) counterstains the nuclei. (D) Differentiation index (number of nuclei in MyHC⁺ cells/total nuclei) from cells differentiated as in C. $n=3$. (E) Fusion index (number of nuclei found in MyHC⁺ cells with two or more nuclei/number of myotubes) from cells differentiated as in C. $n=3$. (F) Total nuclei per mm² for cultures differentiated as in C. $n=3$. (G) *Smad3* and myogenic marker mRNA expression in myoblasts transduced as in A after induction to differentiate for 1 day (DM 1). $n=3$. (H) Representative western blot of SMAD2 and MYOG expression in myoblasts transduced as in A after induction to differentiate for 1 day (DM 1). CyPB is a loading control. (I) Quantification of western blots represented in H. $n=3$. (J) RT-qPCR analysis of *Smad3*, *Pax7*, *Cebpb*, *Myod1* and *Myog* expression in myoblasts transduced as in A and cultured in growth medium. Data for *Smad2*-overexpressing cultures is shown as the mean relative to controls indicated by the gray line. $n=3$. (K) Representative western blot of C-terminally phosphorylated SMAD2 (pSMAD2), SMAD2 and MyHC expression in proliferating and differentiating C2C12 cells for the indicated time points. C2C12 cells treated for 7 h in the presence of 2 ng/ml TGFβ is included as a positive control. CyPB is a loading control. (L) Western blot of C2C12 cells transduced to express SMAD2 or a truncated SMAD2 lacking the C-terminal SSMS motif (SMAD2ΔSSMS). (M) Immunostaining for MyHC (green) cells from L differentiated for 4 days. (N) Differentiation index from cells differentiated as in M. $n=3$. (O) Fusion index from cells differentiated as in M. $n=3$. (P) Cell culture density expressed as nuclei/mm² in images used to calculate N and O. $n=3$. Data are mean±s.e.m. (biological replicates) for B,D-G,I,J,N-P. Scale bars: 50 μm.

MYOG protein compared with controls (Fig. 3G). Taken together, our data indicate that SMAD2 expression is required for efficient myogenic differentiation, at least in part, through the regulation of myogenin expression.

Knockdown of SMAD2 inhibits muscle regeneration after acute injury

To determine the *in vivo* role for SMAD2 in the regulation of myogenesis, we generated a conditional knockout model in which *Smad2* is excised in muscle satellite cells by breeding the *Smad2*^fl/fl mouse (Ju et al., 2006) with a *Pax7*^CreER/+ driver line (Nishijo et al., 2009). To assess the myogenic potential of *Smad2*-deficient satellite cells *in vivo*, we treated *Smad2*^fl/fl*Pax7*^CreER/+ (*Smad*^SC−/−) and non-Cre expressing littermates (*Smad2*^fl/fl*Pax7*^+/+; WT) with tamoxifen at 6 weeks of age to induce excision of *Smad2*. One

week after tamoxifen treatment, we injured the tibialis anterior (TA) muscle in *Smad2*^SC−/− and controls with cardiotoxin and assessed the extent of repair 7 days after injury (Fig. 4). SMAD2 protein expression in freshly isolated myoblasts from *Smad2*^SC−/− and controls confirmed efficient knockdown in this model (Fig. 4A). One week after injury, WT muscle repaired efficiently, regaining a fiber cross-sectional area (XSA) of 50.5±0.07% (mean±s.e.m.) of uninjured controls (Fig. 4B,C, white bars). *Smad2*^SC−/− muscle, however, had impaired regeneration, with fiber XSA significantly smaller than those of injured WT mice and recovering only 26.0± 0.13% of the XSA of uninjured controls (Fig. 4B,C, blue bars). As regeneration is dependent on PAX7⁺ cells, immunostaining for PAX7 was performed in repairing muscle and uninjured control muscle to assess the population size. The number of PAX7⁺ cells per area was not different between genotypes in the uninjured TA
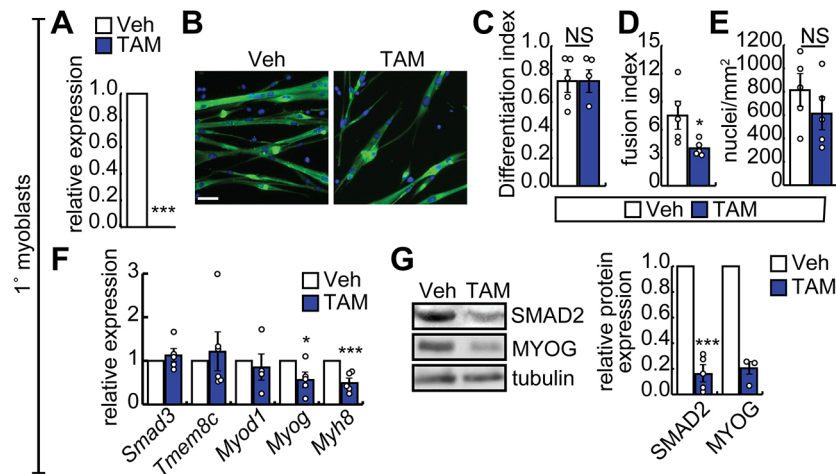
Fig. 3. SMAD2 regulates terminal myogenic differentiation. (A-G) Primary myoblasts isolated from *Smad2*^{fl/fl} mice were retrovirally transduced to express the tamoxifen-inducible Cre recombinase (CreER) and treated with 4-OH tamoxifen (TAM) for 3 days to induce excision or with vehicle (Veh) to generate controls. (A) *Smad2* mRNA expression following induction of *Smad2* excision with 4-OH tamoxifen after 48 h in differentiation medium. *n*=5. (B) Immunostaining for MyHC expression in TAM-treated and vehicle-treated myoblasts differentiated for 2 days and counterstained with DAPI (blue). (C) Differentiation index (number of nuclei in MyHC+ cells/total nuclei) for cells in B. *n*=5. (D) Fusion index (number of nuclei found in MyHC+ cells with two or more nuclei/number of myotubes) from cells differentiated as in B. *n*=5. (E) Number of nuclei per mm^2 counted in C. *n*=5. (F) RT-qPCR analysis of *Smad3*, *Pax7* and myogenic marker expression in cells transduced and differentiated as in B. *n*=4-6. (G) SMAD2 and MYOG expression in control and SMAD2-deficient primary myoblasts differentiated as in B (left) and quantification of protein expression (right). *n*=5 (SMAD2), *n*=3 (MYOG). Data are mean±s.e.m. (biological replicates). *$P<0.05$, ***$P<0.001$ (Student's *t*-test). NS, not significant. Scale bar: 50 µm.

muscles and both underwent expansion following injury (Fig. 4D). However, we did observe a small but significant decrease in the number of PAX7+ cells in *Smad2*[SC−/−] cardiotoxin-injured muscle.

To determine whether regeneration was impaired or simply delayed, we repeated the injury experiment and harvested TA muscle 14 days post-injury. The remaining hindlimb muscles were digested for
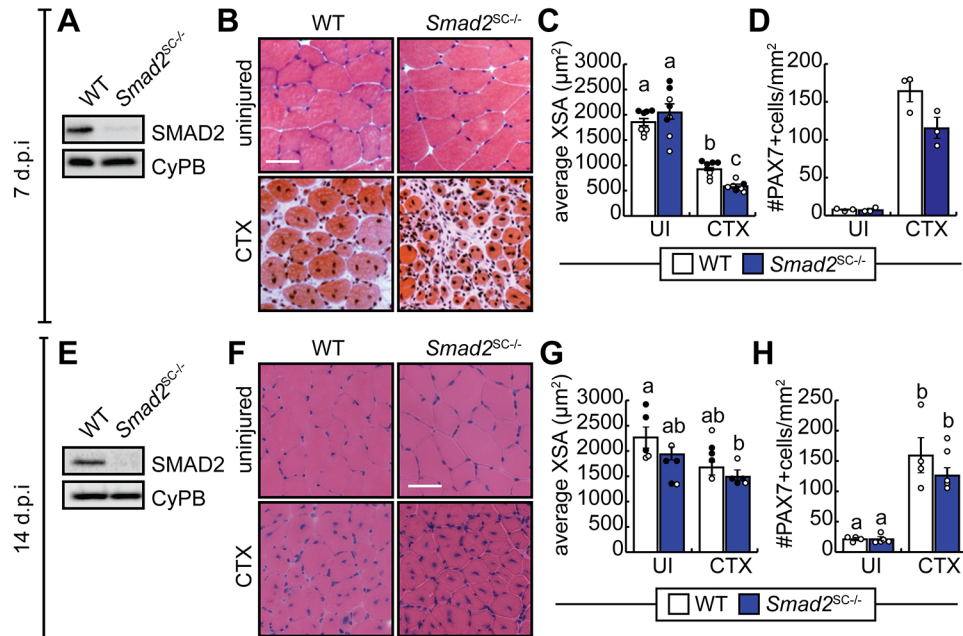

Fig. 4. SMAD2 is required for efficient muscle regeneration. (A) Representative western blot of SMAD2 protein expression in isolated satellite cells from WT and *Smad2*[SC−/−] hindlimb 7 days after injury with cardiotoxin to the left TA muscle (7 d.p.i.). CyPB is a loading control. (B) Representative images of cardiotoxin (CTX)-injured and uninjured TA muscle sections from WT and *Smad2*[SC−/−] mice after repair for 7 days. (C) Average XSA of muscle fibers in WT and *Smad2*[SC−/−] mice injured as in B. *n*=8 pairs for uninjured muscle (UI), and *n*=7 pairs for cardiotoxin-injured muscle. (D) Number of PAX7+ cells per area of uninjured and injured TA from B. *n*=3. (E) Representative western blot of SMAD2 protein expression in isolated satellite cells from WT and *Smad2*[SC−/−] hindlimb 14 days post-injury with cardiotoxin to the left TA muscle (14 d.p.i.). CyPB is a loading control. (F) Representative images of cardiotoxin (CTX)-injured and uninjured TA muscle sections from WT and *Smad2*[SC−/−] mice after repair for 14 days. (G) Average XSA of muscle fibers in WT and *Smad2*[SC−/−] mice injured as in F. *n*=5 pairs. (H) Number of PAX7+ cells per area of uninjured and injured TA from F. *n*=4 for WT and *n*=5 for *Smad2*[SC−/−]. Data are mean±s.e.m. (biological replicates). Means indicated with different letters are significantly different from one another at a minimum cut-off of $P<0.05$ (two-way ANOVA). Black dot data points are male mice and white dots represent female mice. Scale bars: 50 µm.

satellite cell isolation to confirm inactivation of SMAD2 (Fig. 4E). At this time point, the injured WT myofibers regained 76.9±0.24% of WT uninjured TA muscle XSA, whereas $Smad2^{SC-/-}$ injured muscle regained 78.1±0.10% of uninjured control XSA to levels comparable with WT muscle, suggesting that loss of SMAD2 causes a delay in muscle regeneration (Fig. 4F,G). The number of PAX7$^+$ cells was unchanged from control in both the injured and uninjured muscle, suggesting the mild reduction in satellite cell numbers is unlikely to underlie impaired regeneration (Fig. 4H).

Loss of SMAD2 *in utero* perturbs post-natal fiber growth

Knockout of *Smad2* is embryonic lethal, with perturbed formation of mesoderm, and thereby skeletal muscle (Nomura and Li, 1998; Waldrip et al., 1998; Weinstein et al., 1998). To assess the contribution of SMAD2 to the extensive satellite cell differentiation occurring during the postnatal period, $Smad2^{fl/fl}Pax7^{wt/wt}$ females were bred to $Smad2^{fl/fl}Pax7^{CreER/wt}$ males to generate $Smad2^{SC-/-}$ and WT progeny mice, and *Smad2* was excised *in utero* by gavage of the pregnant dams at embryonic day (E) 15.5 with tamoxifen. Pups were subsequently sacrificed at postnatal day (P) 21 and TA muscles were dissected and flash frozen for histological analysis and PAX7 immunostaining. The remaining hindlimb muscles were digested for satellite cell isolation to confirm loss of SMAD2 expression (Fig. 5A). Hematoxylin and Eosin (H&E) staining and analysis of the XSA revealed significantly smaller myofibers at P21 in $Smad2^{SC-/-}$ muscle compared with WT (Fig. 5B,C). Further, the TA muscle of $Smad2^{SC-/-}$ had an average of ~3000 fibers, whereas WT TA had ~2500 (Fig. 5D). Immunostaining revealed no difference in the number of PAX7$^+$ cells per mm^2 of tissue in the

TA muscle at P21 of $Smad2^{SC-/-}$ mice compared with WT muscle sections (Fig. 5E), suggesting that the smaller fiber caliber was not due to reduced satellite cell numbers.

SMAD2 promotes myoblast fusion

C2C12 myoblasts overexpressing SMAD2 have enhanced fusion and fewer unfused MyHC$^+$ myocytes in culture after differentiation (Figs 2F and 6A). As fusion is regulated by numerous factors, and myomaker expression was increased by SMAD2 overexpression (Fig. 2G), we examined the expression of the known pro-fusogenic gene *Klf4*, which promotes fusion through upregulation of *Npnt* (Sunadome et al., 2011). After 24 h of differentiation (DM 1), KLF4 protein expression was increased in cells overexpressing SMAD2 (Fig. 6B). To confirm that SMAD2 could directly regulate transcription from the *Klf4* promoter, we performed a reporter assay using a (−1481/+45) *Klf4*-luciferase construct in C2C12 myoblasts (Karpurapu et al., 2014). Expression of SMAD2 in C2C12 cells increased *Klf4* promoter activity by ~5-fold, suggesting that SMAD2 can directly regulate transcription of *Klf4* in myoblasts (Fig. 6C). We next explored the *Klf4* regulatory region, and identified putative SMAD binding elements in the *Klf4* promoter (pro) and enhancer regions (−10 kb) using published SMAD3 chromatin immunoprecipitation (ChIP)-seq data (Mullen et al., 2011) coupled to motif analysis. ChIP revealed that SMAD2 occupies the *Klf4* regulatory region under differentiation conditions, and thus likely directly regulates *Klf4* expression (Fig. 6D). Consistent with this, expression of the pro-fusogenic gene *Npnt*, a known KLF4 target gene, was enhanced by SMAD2 overexpression (Fig. 6E).

In *Smad2*-deficient primary myoblasts, fusion was reduced, and we observed an increase in the percentage of unfused MyHC$^+$ cells (Fig. 6F). Further, we found that the number of nascent myotubes, with a total of two nuclei, was also increased compared with controls, with a concomitant reduction in the percentage of larger myotubes (Fig. 6G). After 12 h of differentiation, *Smad2*-deficient cells had significantly reduced KLF4 protein expression and *Klf4* mRNA expression (Fig. 6H,I) consistent with the observed phenotype. Further, we observed reduced expression of the KLF4 target gene *Npnt* (Fig. 6I).

Given that *Klf4* is a transcriptional target of SMAD2 in myoblasts and that fusion is enhanced in cells overexpressing SMAD2 and perturbed in cells lacking SMAD2, we hypothesized that SMAD2 acts through KLF4 to enhance fusion. To test this, we retrovirally transduced WT or *Smad2*-deficient primary myoblasts to express KLF4 or with empty virus and induced their differentiation (Fig. 7). Introduction of KLF4 did not affect the differentiation of myoblasts of either genotype but enhanced the fusion of myoblasts isolated from WT cells (Fig. 7A-C). However, KLF4 overexpression in *Smad2*-deficient cells failed to rescue fusion (Fig. 7A,C). Consistent with these findings, although *Klf4* mRNA expression was increased in *Smad2*-deficient cells overexpressing *Klf4*, the expression of the downstream target *Npnt* was not rescued in the absence of SMAD2 6 h after induction to differentiate in low-serum conditions (Fig. 7D). Similarly, *Myog* expression, which was reduced at this time point in *Smad2*-deficient cells, was not rescued by addition of KLF4 (Fig. 7D). As myogenin expression was influenced by SMAD2 in our experiments, we verified whether SMAD2 could regulate *Myog* expression directly using a *Myog*-luc construct in a reporter assay in the presence of ectopic SMAD2 (Fig. 7E). Although the addition of SMAD2 did not increase reporter activity, KLF4 was found to be a potentiator of *Myog* promoter activity (Fig. 7E). Next, KLF4 and SMAD2 recruitment to the *Myog* promoter was assessed by ChIP, and both were found to be enriched
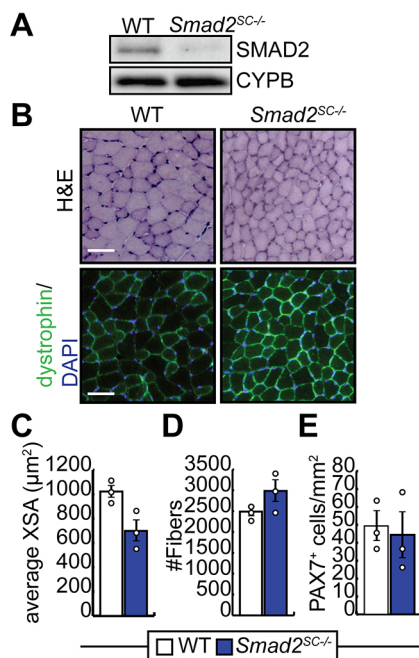

Fig. 5. SMAD2 regulates fiber size after birth. (A) Western blot analysis of SMAD2 and CYPB (loading control) from satellite cells isolated from WT and $Smad2^{SC-/-}$ pups on P21 produced from $Smad2^{fl/fl}Pax7^{wt/wt}$ pregnant dams bred with $Smad2^{fl/fl}Pax7^{CreER/wt}$ males and gavaged with tamoxifen at E15.5. (B) H&E staining (top) and anti-dystrophin immunostaining (bottom) of muscle sections from WT and $Smad2^{SC-/-}$ mice at P21. (C) Average XSA calculated from muscle sections as in A. *n*=3 pairs. (D) Average number of fibers in the TA muscle of WT and $Smad2^{cKO}$ pups from muscle sections as in A. (E) Number of PAX7$^+$ cells per area in muscle sections from B. Data are mean±s.e.m. (biological replicates). Scale bars: 50 µm.
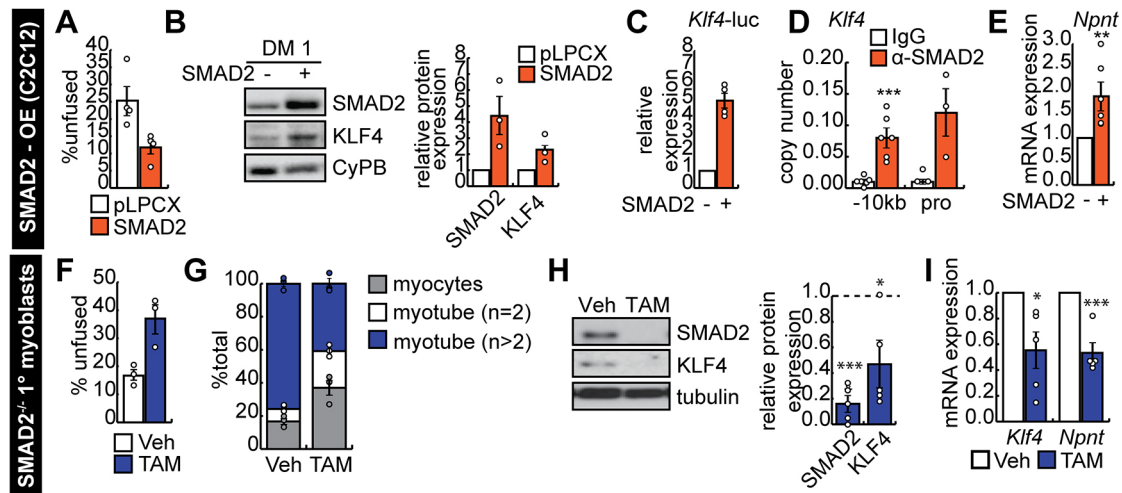
Fig. 6. SMAD2 regulates KLF4 expression to promote myoblast fusion. (A) Percentage of mononucleated MyHC⁺ cells in cultures of C2C12 cells transduced to express SMAD2 or with empty virus (pLPCX) and induced to differentiate for 4 days. *n*=4. (B) Representative western blot (left) and protein expression quantification (right) of SMAD2 and KLF4 expression in differentiating SMAD2-overexpressing primary myoblasts differentiated for one day (DM 1). *n*=3. (C) Luciferase reporter assay to measure *Klf4* promoter activity in the presence or absence of ectopic SMAD2, shown relative to reporter alone and corrected for transfection efficiency. *n*=4. (D) ChIP of SMAD2 recruitment to the *Klf4* promoter and −10 kb upstream region containing putative SMAD2 motifs in primary myoblasts isolated from C57BL/6 mice and differentiated for 1 day. Data are copy numbers as compared to pulldown with type-matched IgG as a control. *n*=6 (−10 kb) and *n*=3 (pro). (E) *Npnt* expression in cells cultured as in B. *n*=5. (F) Percentage of mononucleated MyHC⁺ in control (Veh) and *Smad2⁻/⁻* primary myoblasts (TAM) after differentiation for 48 h. *n*=3. (G) Percentage of myocytes, nascent myotubes (myotubes with two nuclei) and myotubes (>2 nuclei) in cultures from F. *n*=3. (H) Representative western blot (left) and quantification (right) relative to controls (dotted line) of KLF4 expression from cultures in F. *n*=5. (I) *Klf4* and *Npnt* mRNA expression in primary myoblasts differentiated as in F. *n*=5. Data are mean±s.e.m. (biological replicates). *P<0.05, **P<0.01, ***P<0.001 (Student's *t*-test).

(Fig. 7F). As an interaction between KLF4 and SMAD2 was reported in vascular smooth muscle cells (Li et al., 2010), we immunoprecipitated SMAD2 from C2C12 cells, which efficiently coprecipitated KLF4, suggesting that these proteins interact in myoblasts (Fig. 7G).

To determine whether SMAD2 and KLF4 cooperate to regulate *Npnt* expression, the *Npnt* gene regulatory region was analyzed for putative KLF4 and SMAD binding sites. Primers were designed to amplify three specific regulatory regions of the *Npnt* gene: 'pro1', which contains one KLF4 motif; 'pro2', which has two KLF4 motifs; and the −12 kb region, which contains three KLF4 motifs and one SMAD motif (Fig. 7H). Using publicly available ChIP-seq data, the −12 kb region was found to have H3K27Ac histone marks in myoblasts (GSE37525), corresponding with an active enhancer (Blum et al., 2012). ChIP revealed that SMAD2 and KLF4 occupy all three *Npnt* regulatory regions examined under differentiation conditions in C2C12 myoblasts (Fig. 7I). Taken together, these results suggest that SMAD2 is required for the regulation of *Npnt* by KLF4 during myogenic fusion.

SMAD2 negatively regulates the expression of inhibitors of myogenic differentiation

To explore the molecular mechanism by which SMAD2 promotes myogenic differentiation, we performed an RT-qPCR array comparing the expression of 84 genes involved in myogenesis and myopathy in differentiating C2C12 myoblasts overexpressing SMAD2 and in primary myoblasts lacking SMAD2 along with their respective controls. We found 23 upregulated genes (≥1.5-fold change) in C2C12 cells overexpressing SMAD2 after 2 days of differentiation compared with empty virus controls (Fig. 8A, cluster 1). As expected, the majority of upregulated genes were positive regulators of myogenic differentiation and maturation, such as the structural genes *Acta1*, *Neb* (nebulin) and the troponin genes (*Tnni2*,

Tnnc1, *Tnnt1* and *Tnnt3*). In addition to these regulators, 18 genes were downregulated by ≥1.5 fold in cells overexpressing SMAD2 compared with controls (Fig. 8A, cluster 2). Among these downregulated genes, we found known inhibitors of myogenic differentiation such as myostatin (*Mstn*), basic fibroblast growth factor (*Fgf2*), bone morphogenetic protein 4 (*Bmp4*), interleukin 6 (*Il6*) and mitogen-activated protein kinase 3 (*Mapk3*) (Fig. 8A, cluster 2). By comparison, knockout of SMAD2 in primary myoblasts resulted in the downregulation of four genes (*Myh2*, *Hk2*, *Myf6* and *Myf5*) at a threshold of ≥1.5-fold reduction compared with control cells (Fig. 8A, cluster 3). *Myh2* is a marker of terminal differentiation, which is in line with the requirement of SMAD2 for efficient myogenic differentiation. Interestingly, 18 mRNAs were upregulated in *Smad2*^SC−/−-derived primary myoblasts by at least 1.5-fold (Fig. 8A, cluster 4). Genes that failed to meet the fold change cutoff are indicated in Fig. 8B. Interestingly, only five genes were found among the genes downregulated in SMAD2-overexpressing cells and upregulated with loss of SMAD2 (Fig. 8A, genes in red). These genes, *Bmp4*, *Fgf2*, *Mstn*, *Igf1* and insulin growth factor binding protein 3 (*Igfbp3*) are, with the exception of *Igf1*, known potent inhibitors of myogenic differentiation. To validate our findings, we performed RT-qPCR analysis in SMAD2-overexpressing C2C12 myoblasts that had been differentiated for 1 day (Fig. 8C). Although *Mstn* expression was not significantly downregulated (highly variable) with SMAD2-overexpression (Fig. S2), all of the other candidate genes were consistently downregulated (Fig. 8C). In differentiating *Smad2*^SC−/−-derived primary myoblasts, although variability was increased, we observed upregulation of all factors, with *Bmp4* showing the most robust result (upregulated in all trials) (Fig. 8D). Taken together, these data suggest that SMAD2 regulates myogenic differentiation by inhibiting the expression of anti-myogenic factors that modulate the expression of myogenin and thus promotes myogenic differentiation.
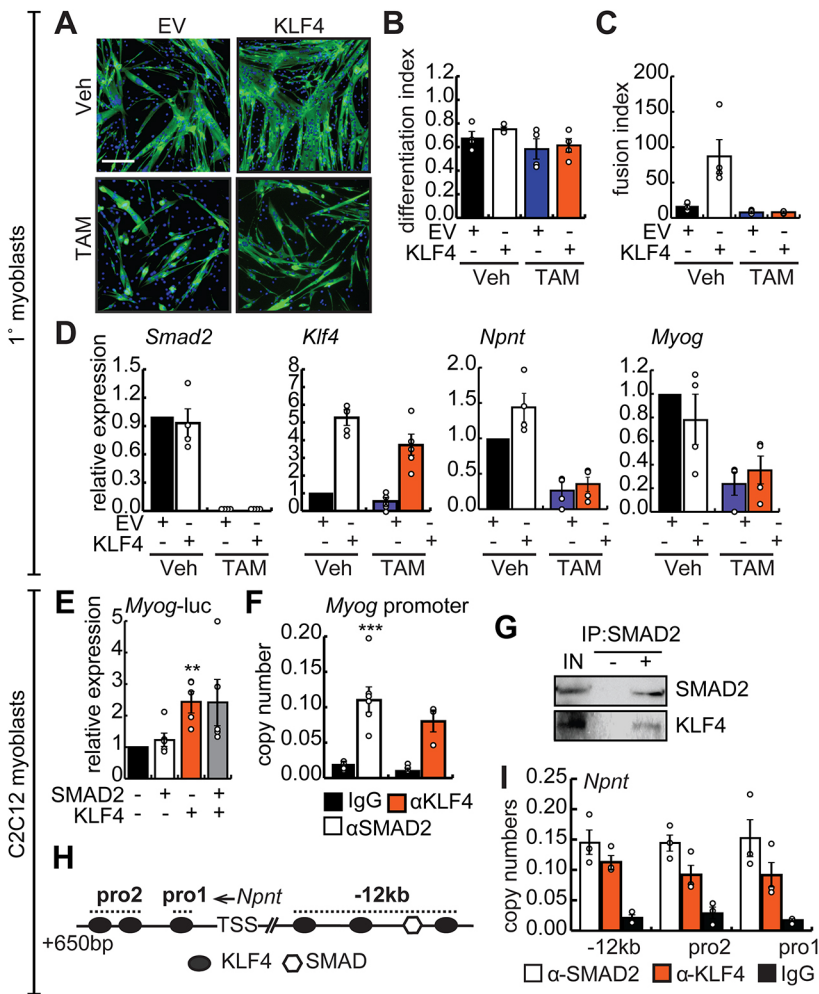
Fig. 7. Forced expression of KLF4 cannot rescue fusion, *Myog* or *Npnt* expression in *Smad2*-deficient myoblasts. (A) Immunostaining for MyHC showing myotubes generated from primary myoblasts isolated from *Smad2*^{fl/fl} mice retrovirally transduced to express CreER that were treated with 4-OH tamoxifen to excise *Smad2* (TAM) or vehicle-treated (Veh), transduced to express KLF4 or with empty virus (EV) and differentiated for 2 days. (B) Differentiation index (number of nuclei in MyHC^{+} cells/total nuclei) from cells differentiated as in A. *n*=4. (C) Fusion index (number of nuclei/myotube) of cells cultured, transduced and differentiated as in A. *n*=4. (D) RT-qPCR analysis of *Smad2*, *Klf4*, *Myog* and *Npnt* in cells transduced as in A and induced to differentiate for 6 h in low-serum conditions. *n*=4. (E) Reporter assay measuring activity of the *Myog* promoter in C2C12 cells in the presence of SMAD2 and KLF4 relative to controls. *n*=5. (F) Occupancy of the *Myog* promoter by SMAD2 and KLF4 by ChIP compared with non-specific antibody (IgG). *n*=6 for SMAD2, *n*=3 for KLF4. (G) Coimmunoprecipitation of KLF4 with SMAD2 from whole-cell extracts from differentiating C2C12 myoblasts 3 days after induction. IN is 10% of input used for IP. (H) Schematic of the regulatory region of the *Npnt* gene including putative SMAD2 and KLF4 motifs. (I) ChIP of SMAD2 and KLF4 occupancy of two promoter regions in the *Npnt* promoter and −12 kb upstream region performed in C2C12 cells differentiated for 1 day in low serum conditions. Data are copy numbers in comparison to pulldown with type-matched IgG as a control. *n*=3. Data are mean±s.e.m. (biological replicates). **P<0.01, ***P<0.001 (Student's *t*-test). Scale bar: 50 μm.

DISCUSSION

Herein, we identify SMAD2 as a powerful regulator of terminal myogenic differentiation and fusion using overexpression in C2C12 myoblasts, primary myoblasts isolated from floxed mice and *in vivo* using a conditional null model. Overexpression of SMAD2 in C2C12 cells enhanced myogenic differentiation, increased myotube size and promoted myogenin and myomaker expression, whereas knockout of SMAD2 decreased myotube and myofiber size and reduced myogenin expression without changes in myomaker expression. There was strong concordance between in culture and *in vivo* models: we noted that overexpression of SMAD2 enhanced the differentiation index in C2C12 myoblasts, whereas loss of SMAD2 in primary myoblasts did not reduce it. This discrepancy, and the results of the *in vivo* regeneration experiments, led us to conclude that SMAD2 is involved in late myogenic differentiation and fusion and that, although high levels of SMAD2 can enhance differentiation, its loss does not prevent differentiation from occurring. Indeed, SMAD2 gain- and loss-of-function experiments revealed that *Klf4*, *Myog* and *Npnt* are SMAD2 target genes. Our mRNA expression screen revealed that SMAD2 negatively regulates the expression of four genes: *Igf1*, *Igfbp3*, *Fgf2* and *Bmp4*. Although the mechanism by which SMAD2 inhibits the expression of these genes in skeletal muscle remains unknown, *Fgf2* was identified as a TGFβ target gene in stromal cells (Strand et al., 2014) and *Igfbp3* is a known TGFβ target during epithelial-to-mesenchymal transition (Natsuizaka et al., 2010). Interestingly, both IGF1 and IGFBP3 can enhance activation of TGFβ receptors and can stimulate TGFβ activity (Fanayan et al.,

2002; Kuemmerle et al., 2004; Natsuizaka et al., 2010; Peters et al., 2006; Rosendahl and Forsberg, 2006), suggesting that SMAD2 may act to inhibit classical TGFβ-mediated responses. Indeed, coupled with the inhibition of both myostatin and BMP4, the actions of SMAD2 during myogenic differentiation effectively reduce both receptor activation as well as autocrine production of TGFβ family ligands. These findings raise the intriguing possibility that SMAD2 may function in two distinct pathways. In the absence of TGFβ or myostatin, SMAD2 acts in a pro-myogenic pathway, promoting regulators of late myogenesis while inhibiting the expression of a potent inhibitor of myogenic differentiation (BMP4) and stimulators of TGFβ action (IGF1, IGFBP3). However, in the presence of TGFβ or myostatin, SMAD2 acts to inhibit myogenic differentiation. It remains unclear whether SMAD2 is acting on different gene targets in these two proposed mechanisms, or rather its activity is altered by phosphorylation and complexing with SMAD4, resulting in unique gene expression patterns regulated by SMAD2 in the presence and absence of TGFβ.

The interaction with, and apparent cooperation with, KLF4 may represent a mechanism by which SMAD2 activity can be modulated. Both SMAD2 and SMAD3 are weak DNA binders on their own, and given that SMAD binding motifs are very frequent, specificity and transcriptional activity appear to be mediated by interactions with other transcription factors such as SMAD4 and, in skeletal muscle, MYOD (Mullen et al., 2011). As such, phosphorylation by the TGFβ receptor complex and interaction with SMAD4 is considered essential for occupancy of regulatory
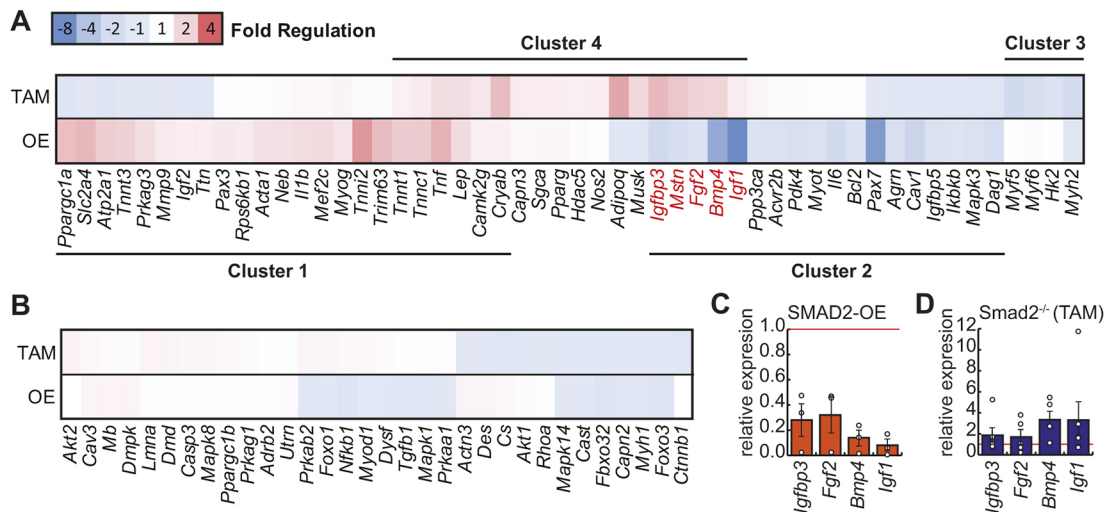
Fig. 8. SMAD2 regulates inhibitors of myogenesis. (A-C) C2C12 myoblasts retrovirally transduced to express SMAD2 and empty vector controls as well as primary myoblasts from *Smad2*^{fl/fl} mice retrovirally transduced to express CreER and treated with TAM or vehicle were induced to differentiate, and isolated mRNA (pooled from three trials) was analyzed by RT2 Profiler Array (Qiagen) for 84 myogenesis- and myopathy-related genes. Heatmaps are shown as relative to controls for both OE and TAM, displayed as fold-change. (A) Heatmap of differentially regulated genes by SMAD2 overexpression (OE) and SMAD2 knockout (TAM). Cluster 1 contains genes that are upregulated by ≥1.5-fold in C2C12 cells overexpressing SMAD2. Cluster 2 regroups genes that are downregulated by ≥1.5-fold with overexpression of SMAD2. Cluster 4 contains genes that are upregulated by ≥1.5-fold in *Smad2*[SC−/−] (TAM). Cluster 3 contains genes downregulated by ≥1.5-fold in Smad2[SC−/−]. Genes that are in red are upregulated with OE and downregulated in Smad2[SC−/−] with a threshold of ≥1.5-fold. (B) Genes not significantly regulated in either condition. (C,D) RT-qPCR analysis of *Igbp3*, *Fgf2*, *Bmp4* and *Igf1* expression relative to controls (red horizontal line) in cells transduced as in A and differentiated for 24 h. *n*=3 for SMAD2 OE and *n*=4 for *Smad2*[SC−/−]. Data are mean±s.e.m. (biological replicates).

elements. Interestingly, mutations in or deletion of *Smad4* are found in many cancers, and in the absence of SMAD4 protein, a subset of TGFβ target genes are unaffected, suggesting that SMAD4-independent gene transcription downstream of TGFβ occurs (Levy and Hill, 2005). In our system, effects of SMAD2 on myogenic differentiation are independent of the C-terminal serine residues targeted by the TGFβ type I receptor. Thus, during normal myogenic differentiation, SMAD2 recruitment to target genes involved in terminal differentiation and fusion may occur through interaction with lineage-specific factors such as MYOD and/or KLF4 much in the way that SMAD4 brings SMAD2 to gene targets. As such, activation of TGFβ receptors and SMAD2 C-terminal phosphorylation may divert SMAD2 activity towards anti-myogenic targets, and act as a switch between an anti-myogenic program and a pro-differentiation program. Consistent with this notion, TGFβ1 and TGFβ2 expression is highest in proliferating myoblasts and decreases with differentiation, precisely when the pro-myogenic role described herein is active. This raises the possibility that SMAD2 phosphorylation marks proliferative cells that cannot differentiate, and that suppression of this phosphorylation allows SMAD2 to assume a pro-myogenic role, driving the expression of KLF4, *Myog* and *Npnt*.

A pro-myogenic role has also been identified for SMAD3 (Ge et al., 2011, 2012; Lamarche et al., 2015). As our work focuses on the function of SMAD2 during myogenic differentiation, we cannot exclude a role for SMAD3 in our studies. Indeed, SMAD3 has some pro-myogenic functions (Ge et al., 2011, 2012), and thus a knockdown of both SMAD2 and SMAD3 could potentially impair myogenic differentiation to a greater extent than SMAD2 alone. Indeed, given the known collaboration of SMAD3 with master transcription factors such as MYOD and OCT4, it remains possible that transcription factors such as MYOD could also recruit both SMAD2 and SMAD3 to target genes (Mullen et al., 2011).

To influence gene expression, SMAD2 must gain entry into the nucleus, a process that, in the context of TGFβ signaling, requires

both phosphorylation of SMAD2 and its interaction with the co-SMAD SMAD4. Although TGFβ has been shown to regulate the interaction of SMAD2 with SMAD4 in a phosphorylation-dependent mechanism, the transcriptional output from SMAD2-dependent genes appears to be mediated more by the retention of phosphorylated SMAD2 in the nucleus, rather than its import (Schmierer and Hill, 2005). Indeed, TGFβ signaling does not appear to regulate the nuclear import rate for SMAD2, but rather decreases its export from the nucleus (Xu et al., 2002). However, phosphorylation of C-terminal serine residues by the ligand-bound TGFβ receptor is believed to induce a conformational change that allows both interaction with SMAD4 and more efficient interaction with DNA response elements in target promoters, a situation that is unlikely to happen in our current model. As such, in the absence of C-terminal phosphorylation, interaction with transcription factors such as MYOD may direct SMAD2 to gene targets promoting efficient myogenic differentiation, whereas TGFβ signaling, and downstream interaction with SMAD4 would be predicted to drive a different, anti-myogenic, gene expression program.

MATERIALS AND METHODS
Mice and animal care
All animal work was performed in accordance with the guidelines set out by the Canadian Council on Animal Care and was approved by the University of Ottawa Animal Care Committee. Smad2[tm1.1Epb] (Smad2^{fl/fl} mice) (Ju et al., 2006) were obtained from the Jackson Laboratory and crossed with mice bearing the Pax7-CreER[tm] (Pax7[CreER]) (Nishijo et al., 2009). Smad2^{fl/fl}*Pax7*^{+/+} (control) and conditional null Smad2^{−/−}Pax7[CreER−/+] (Smad2[SC−/−]) mice were generated and activation of CreER[tm] *in utero* was achieved by a single gavage of 2.5 mg tamoxifen (dissolved in corn oil) of pregnant dams at E15.5 or by five daily intraperitoneal injections of 1.5 mg tamoxifen dissolved in corn oil. For injury experiments, 1 week after the last dose of tamoxifen, mice were injured by injecting 30 μl of 10 μM cardiotoxin (Latoxan) to the left TA muscle, and TA muscles were harvested and sectioned 7- or 14-days post-injury. All animals were housed in a controlled facility (22°C with 30% relative humidity on 12 h light/dark cycle) and provided with food and water

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ad libitum. Sample size (animal numbers) and statistical power were estimated using the mean percent XSA recovery of injured fibers and its standard deviation in C57BL/6 male and female mice aged 4-6 weeks following cardiotoxin injury. Based on previously calculated means and error for controls, a sample size of less than six pairs was required.

Constructs and reagents

MSCV CreERT2 puro was a gift from Tyler Jacks (Addgene plasmid 22776) (Kumar et al., 2009). The LPCX-SMAD2 (Addgene plasmid 12636) and LPCX Smad2 deltaSSMS (Addgene plasmid 12637) were gifts from Rik Derynck (Choy et al., 2000). pMXs-KLF4 was a gift from Dr Toshio Kitamura (Addgene plasmid 13370) (Takahashi and Yamanaka, 2006) and the control vector pMXs-RFP was a gift from Dr William Stanford (Ottawa Hospital Research Institute, Canada). The pGL3-Klf4-Luc promoter was a kind gift from Dr Christman at the Ohio State University (Karpurapu et al., 2014). The −2 kb myogenin-luc reporter construct was a gift from Dr Alexandre Blais (Liu et al., 2010).

Isolation of primary myoblasts and cell culture

Primary myoblasts from C57BL/6 and *Smad2* conditional knockout mice were obtained as previously described (Marchildon et al., 2012). Briefly, lower hindlimb muscles from C57BL/6 mice (both sexes) aged 6-8 weeks were dissected and digested with collagenase (Roche). Isolated cells were plated on Matrigel-coated dishes and allowed to grow in DMEM containing 20% fetal bovine serum (FBS) and 10% horse serum (HS) in the presence of 10 ng/ml basic FGF and 2 ng/ml HGF (Peprotech). Differentiation was induced when cells reached 70-80% confluence by culturing in DMEM containing 2% FBS and 2% HS (differentiation medium, DM).

C2C12 cells (ATCC) were cultured in DMEM containing 10% FBS. To induce differentiation, growth medium was replaced with DMEM containing 2% HS when cells were 80-90% confluent. C2C12 are tested for contamination on a quarterly basis.

Retroviral infection

Retrovirus for expression of SMAD2 or KLF4 was generated by retroviral expression plasmids or empty vector controls into Phoenix cells, and virus was captured from supernatants after 2 days. For viral infection, growth medium was replaced with a medium containing virus when C2C12 cells or primary myoblasts reached 30%-40% confluency. Then, 48 h after infection, 2 μM puromycin was added to culture medium to select positive cells. Phoenix cells are tested for contamination on a quarterly basis.

Western blot analysis

Western blotting was performed as previously described (Fu et al., 2015). Briefly, whole-cell lysate of primary myoblasts was prepared by lysing buffer containing protease inhibitor cocktail (Roche). Protein concentration was determined using the BCA Protein Assay Reagent (Pierce Biotechnology) using bovine serum albumin as standard. Samples were subjected to SDS-PAGE gels and transferred to PVDF membranes (Millipore). Proteins were detected using the following antibodies: anti-Smad2 antibodies (Cell Signaling Technology, 5339S), anti-GKLF (KLF4) antibodies (Santa Cruz Biotechnology, sc-20691), anti-myogenin antibodies [Developmental Studies Hybridoma Bank (DSHB), F5D], anti-MF20 (DSHB), anti-phospho-SMAD2 antibodies (Cell Signaling Technology, 138D4), anti-cyclophilin B (Abcam, ab16045) and anti-αTubulin antibodies (Santa Cruz Biotechnology, sc-5286). Details on antibody validation and dilutions can be found in Table S3.

Immunofluorescence

Immunofluorescence staining was performed as previously described (Marchildon et al., 2012). Cultured cells were fixed by ice-cold methanol and permeabilization with PBS containing 0.5% Triton X-100. Detection was performed following standard procedures (www.abcam.com/protocols/immunocytochemistry-immunofluorescence-protocol) using anti-MF20 (DSHB) and anti-mouse-Cy3 (Jackson ImmunoResearch, 715-166-150, 1:500) antibodies. The differentiation index (DI) of myoblasts and fusion index (FI) of myotubes were calculated as previously described (Lamarche et al., 2015). Cryosections were fixed with 4% paraformaldehyde and

processed as previously described (Lala-Tabbert et al., 2016). Primary antibodies used were PAX7 (DSHB, Pax7-c, 1:100), MYOG (DSHB, F5D) and dystrophin (Abcam, ab15277, 1:100) followed by the secondary anti-mouse-Cy3 (Jackson ImmunoResearch, 715-166-150, 1:500) and Alexa-488 (Jackson ImmunoResearch, 711-546-152, 1:500).

Reporter assay

C2C12 cells were transiently transfected with reporter construct, mammalian expression constructs and control *Renilla* plasmid using FuGENE HD transfection reagent according to the manufacturer's instructions (Promega). Cells were supplemented with growth medium for 6 h and collected 48 h post-transfection. Extracts were analyzed using the Dual-Luciferase Reporter Assay Kit (Promega). The ratio of Luciferase/*Renilla* was calculated and normalized to experimental control (promoter in absence of experimental plasmid).

qPCR analysis

Cells were harvested and RNA was extracted using the RNA Easy Mini Kit (Qiagen) according to the manufacturer's instructions. Quantification was performed using a Nanodrop (Thermo Fisher Scientific) and 1 μg RNA was treated with RNase-Free DNase (Qiagen). First strand cDNA was made using the iScript cDNA Synthesis Kit (Bio-Rad). CT values from quantitative PCR were analyzed using the delta delta CT method, using 18S as an internal control (Livak and Schmittgen, 2001). The primer sequences used can be found in Table S1.

Assessment of cell cycle

Myoblasts cultured in growth medium were fixed in 2% paraformaldehyde (PFA) in PBS and blocked with 0.3% Triton X-100 and 10% goat serum. For BrdU incorporation, 10uM BrdU (Sigma-Aldrich) was added 6 h before cell fixation. Cells were incubated with anti-BrdU (Biotin; Abcam, ab2284, 1:500) followed by incubation with Cy3-conjugated streptavidin (Jackson ImmunoResearch, 016-160-084, 1:500). For Ki67 staining, anti-Ki67 (Abcam, ab15580, 1:100) and Alexa 488-conjugated donkey anti-rabbit (Jackson ImmunoResearch, 711-546-152, 1:500) were used for detection. Nuclei were counterstained with DAPI (0.5ug/ml) and BrdU+ or Ki67+ cells were scored.

ChIP

ChIP analysis was performed as previously described (Lala-Tabbert et al., 2016). Briefly, cells were cross-linked with 1% formaldehyde for 30 min at room temperature then sonicated for 30 cycles (30 s on/30 s off) with a Diagenode Bioruptor. Equal amounts of chromatin were incubated with antibodies against KLF4 (Abcam, 6 μg per reaction), SMAD2 (Abcam, 1:100) or rabbit IgG (Invitrogen, 10500C, 6 μg per reaction) as a negative control. Immunoconjugates were captured using protein G magnetic Dynabeads (Invitrogen), and DNA fragments were then purified with QIAquick PCR kit (Qiagen). A 10% input sample of each condition was used to generate a standard curve and the copy numbers of each immunoprecipitate is presented relative to the standard curve. Primer sequences and genome coordinates for qPCR-ChIP are listed in Table S2.

RT^2 profiler PCR array

cDNA from Smad2-null and -overexpressing cells and their relative controls was tested using the real-time RT^2 Profiler PCR Array (QIAGEN, PAMM-099Z, Skeletal Muscle: Myogenesis and Myopathy RT^2 Profiler PCR Array) in combination with SsoAdvanced Universal SYBR Green Supermix (Bio-Rad). Ct values were exported to a Microsoft Excel file to generate a Ct value table. The table was then uploaded on to the data analysis web portal at http://www.qiagen.com/geneglobe and fold change/regulation was calculated using the delta delta Ct method. The average of reference genes was used as internal control. The heatmap of fold regulation was prepared using Excel.

Statistical analysis

Statistical analysis was conducted using GraphPad Prism 6. A two-tailed unpaired Student's *t*-test was used when comparing two conditions. One-way

ANOVA was performed when comparing three or more treatments in one cell type. Two-way ANOVA was used when comparing two conditions in an experimental and control cell line. *Post-hoc* tests followed only statistically significant ANOVA results ($P<0.001$). Asterisks are used to indicate statistically significant changes from a control group as follows: *$P<0.05$, **$P<0.01$, ***$P<0.001$ (Student's *t*-test). For multiple comparisons (ANOVA), lower case letters are used to label means, such that bars bearing different letters are statistically different from one another with a minimum *P*-value of <0.05.

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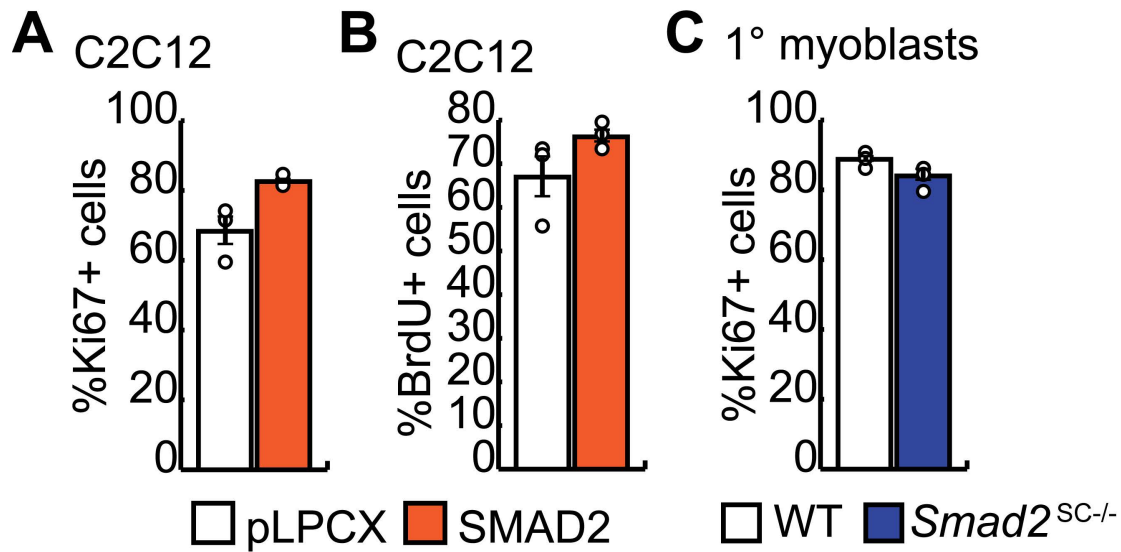
Supplemental Figure 1

Fig. S1. Changes in SMAD2 expression in myoblasts does not impact cell proliferation.
(A) C2C12 myoblasts retrovirally transduced to express SMAD2 or with empty virus (pLPCX)"
in growth medium were subjected to Ki67 immunostaining and Ki67+ cells were scored as a
percentage of total cells. (B) BrdU incorporation in cells as in (A) after a 6 h pulse with BrdU.
Data is shown as the percentage of BrdU+ cells relative to total cells. (C) Percent of Ki67+"
primary myoblasts isolated from *Smad2*^{fl/fl} mice and retrovirally transduced to express the"
tamoxifen-inducible Cre recombinase (CreER) and treated with 4-OH tamoxifen (TAM) for 3 days
to induce excision or with vehicle (Veh) to generate controls. Bars are the mean of three biological
repeats ± s.d.
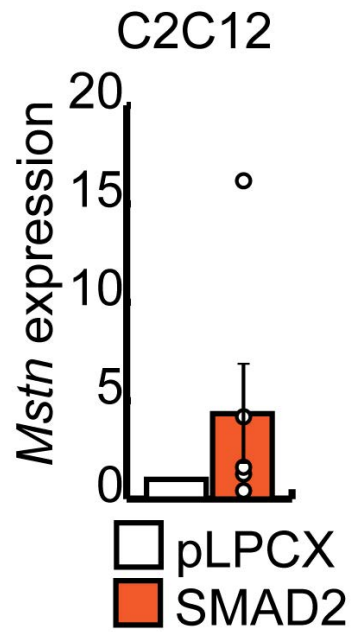
Supplemental Figure 2

Fig. S2. Changes in *Mstn* expression in C2C12 myoblasts overexpressing SMAD2 or treated with empty vector (pLX), n=6.

Table S1. Primers used for qPCR.

Gene	Forward primer	Reverse primer
Smad2	5'-ATGTCGTCCATCTTGCCA-3'	5'-AACCGTCCTGTTTTCTTTAG-3'
Smad3	5'-CGTAATTCATGGTGGCTGTG-3'	5'-ACCAAGTGCATTACCATCCC-3'
Cebpb	5'-TCGAACCCGCGGACTGCAAG-3'	5'-CGACGACGACGTGGACAGGC-3'
Pax7	5'-GACGACGAGGAAGGAGACAA-3'	5'-CGGGTTCTGATTCCACATCT-3'
18S	5'-CGCCGCTAGAGGTGAAATC-3'	5'-CCAGTCGGCATCGTTTATGG-3'
Myod1	5'-TGGCATGATGGATTACAGCG-3'	5'-CCACTATGCTGGACAGGCAGT-3'
Myog	5'-ATCGCGCTCCTCCTGGTTGA-3'	5'-CTGGGGACCCCTGAGCATTG-3'
Myh8	5'-TCGCTGGCTTTGAGATCTTT-3'	5'-ACGAACATGTGGTGGTTGAA-3'
Klf4	5'-GCAGTCACAAGTCCCCTCTC-3'	5'-TAGTCACAAGTGTGGGTGGC-3'
Npnt	5'-TGGAGGCAAACCCAGATCAC-3'	5'- GCAGCGACCTCTTTTCAAGC-3'
Tmem8c	5'-ATCGCTACCAAGAGGCGTT-3'	5'-CACAGCACAGACAAACCAGG-3' (Millay et al., 2013)
Bmp4	5'-TTCCTGGTAACCGAATGCTGA-3'	5' CCTGAATCTCGGCGACTTTTT -3' primerBank ID : 6680796a1
Igf1	5'-AGAGCCTGCGCAATGGAATA-3'	5'-TGCTGATTTTCCCCATCGCT-3'
Igfbp3	5'-CCAGGAAACATCAGTGAGTCC-3'	5'-GGATGGAACTTGGAATCGGTCA-3' primerBank ID : 6680385a1
Mstn	5'-AGTGGATCTAAATGAGGGCAGT-3'	5'-GTTTCCAGGCGCAGCTTAC-3' primerBank ID: 6754752a1

Table S2 . Primers used for chromatin immunoprecipitation.

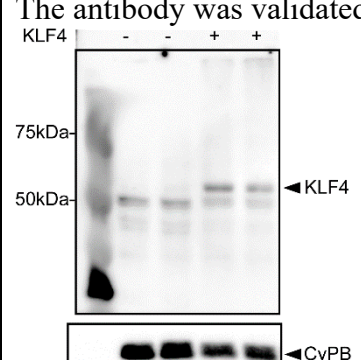| Region	Forward primer	Reverse primer
Klf4 **pro1**	(chr4:55532503-55532525, mm10) 5'-TATAACTTCTCGCTCGCTTGCTC-3'	(chr4:55532632-55532653, mm10) 5'-TGCGCGGAGTTTGTTTATTTAG-3'
Klf4 **-10kb**	(chr4:55542941-55542961, mm10) 5'- CAGGAATGCCTGTGGGGATAG-3'	(Chr4:55543026-55543045, mm10) 5'-TGACCGGCTGAAGCTTTGTC-3'
Myog **promoter**	(chr1:134289663-134289681, mm10) 5'-GAGGCCCGGGTAGGAGTAA-3'	(chr1:134289763-134289782, mm10) 5'-GCCGTCGGCTGTAATTTGAT-3'

Npnt **pro1**	(chr3:132949265-132949284, mm10) 5'-GCTTTTCCTCTGGTCCCCTC-3'	(chr3:132949336-132949355, mm10) 5'-GATGCCGCACCTGTTTTACC-3'
Npnt **pro2**	(chr3:132949284-132949303, mm10) 5'-ATTGGCTTTCCTGTCCCTGG-3'	(chr3:132949107-132949126, mm10) 5'-TTAGCCTCTGGCTGCTTTCC-3'
Npnt **-12kb**	(chr3:132962692-132962711, mm10) 5'- GGTTGGAACCGCAGTGAGTA-3'	(chr3:132962807-132962826, mm10) 5'- GCAGTGACAACAGGGAGACA-3'

Table S3. Antibody validation and dilutions used.

Antibody: Anti-SMAD2

Source and catalogue/clone number	Cell signaling, 5339S
Dilution used	1:1000 in 1% skim milk
Validation data	Antibody validation data is provided on the manufacturer's website using a SMAD2 knockout (https://www.cellsignal.com/products/primary-antibodies/smad2-d43b4-xp-rabbit-mab/5339?site-search-type=Products&N=4294956287&Ntt=5339s&fromPage=plp&_requestid=1257133) and in Figure 2A (overexpression), Figure 3G, 4A, 4E, 5A (knockdown).

Antibody: anti-GKLF (KLF4)

Source and catalogue/clone number	Santa Cruz BioTech, sc-20691
Dilution used	1:200 in 1% skim milk
Validation data	The antibody was validated using overexpression in primary myoblasts.

Antibody: anti-Myogenin

Source and catalogue/clone number	Developmental Studies Hybridoma Bank (DSHB), F5D. F5D was deposited to the DSHB by Wright, W.E. (DSHB Hybridoma Product F5D).
Dilution used	1:100 in 1% skim milk
Validation data	Information about this antibody can be found at: https://dshb.biology.uiowa.edu/F5D and https://antibodyregistry.org/search.php?q=AB_2146602.

Antibody: anti-Myosin Heavy Chain

Source and catalogue/clone number	DSHB, MF-20
Dilution used	used 1:100 in 1% skim milk
Validation data	Information about this antibody can be found at: https://dshb.biology.uiowa.edu/MF-20 and https://antibodyregistry.org/search.php?q=AB_2147781

Antibody: anti-phospho-SMAD2

Source and catalogue/clone number	Cell signaling, 138D4
Dilution used	1:1000 in 5% BSA
Validation data	Antibody validation is available on the manufacturer's website in the presence and absence of exogenous TGFβ (https://www.cellsignal.com/products/primary-antibodies/phospho-smad2-ser465-467-138d4-rabbit-mab/3108?site-search-type=Products&N=4294956287&Ntt=138d4&fromPage=plp&_requestid=1258035) and in Figure 2K

Antibody: anti-cyclophilin B

Source and catalogue/clone number	Abcam, ab16045
Dilution used	1:2000 in 1% skim milk
Validation data	Antibody validation using knockouts is available on the manufacturer's website (https://www.abcam.com/cyclophilin-b-antibody-ab16045.html)

Antibody: anti-αTubulin

Source and catalogue/clone number	Santa Cruz BioTech, sc-5286
Dilution used	1:500 in 1% skim milk
Validation data	Validation data in transfected 293T cells is provided on the manufacturer's website: https://www.scbt.com/p/alpha-tubulin-antibody-b-7?requestFrom=search . The antibody has been cited by 1,246 publications to date.