The transcription factor Prox1 is essential for satellite cell differentiation and muscle fibre-type regulation

Riikka Kivelä¹², Ida Salmela¹², Yen Hoang Nguyen³⁴, Tatiana V. Petrova⁵, Heikki A. Koistinen³⁴, Zoltan Wiener²,† & Kari Alitalo¹²

The remarkable adaptive and regenerative capacity of skeletal muscle is regulated by several transcription factors and pathways. Here we show that the transcription factor Prox1 is an important regulator of myoblast differentiation and of slow muscle fibre type. In both rodent and human skeletal muscles Prox1 is specifically expressed in slow muscle fibres and in muscle stem cells called satellite cells. Prox1 activates the NFAT signalling pathway and is necessary and sufficient for the maintenance of the gene program of slow muscle fibre type. Using lineage-tracing we show that Prox1-positive satellite cells differentiate into muscle fibres. Furthermore, we provide evidence that Prox1 is a critical transcription factor for the differentiation of myoblasts via bi-directional crosstalk with Notch1. These results identify Prox1 as an essential transcription factor that regulates skeletal muscle phenotype and myoblast differentiation by interacting with the NFAT and Notch pathways.
adult skeletal muscles regenerate after damage due to the proliferation and differentiation of muscle resident stem cells called satellite cells (SC). In healthy resting muscle these cells are in a quiescent state, which is characterized by a reversible mitotic arrest and slow metabolic activity indicative of many stem cell populations. In response to muscle injury, SCs can be activated, resulting in proliferation, differentiation and self-renewal. Members of the MyoD family of myogenic regulatory factors (MyoD, Myogenic factor 5 (Myf5), Myogenic factor 6/Mef4 and myogenin) control the formation of skeletal muscle and muscle regeneration in the adult. The paired box factors Pax3 and Pax7 are important upstream regulators of the myogenic regulatory factors in SCs and the differentiation of committed myoblasts require the downregulation of Pax7 (refs 3,4). However, the molecular mechanisms underlying SC activation and differentiation are not completely understood.

Skeletal muscle fibres can be classified by their contraction speed and their aerobic (oxidative) versus anaerobic (glycolytic) production of ATP. Slow type I fibres express myosin heavy chain I (Myh7, MyHC I), use aerobic metabolism, are rich in mitochondria and myoglobin, and have good endurance. In contrast, type IIB fast twitch fibres, which express Myh4/MyHC IIB, are primarily anaerobic and glycolytic, contain many glycogen granules, and contract quickly for short-term activity. In addition, the oxidative type IIA (Myh2/MyHC IIA) and intermediate type IIX (Myh11/MyHC IIX) fast twitch fibres have intermediate contraction properties compared with type I and IIB fibres.

The prospero-related homebox 1 (Prox1) transcription factor, especially its homeo-prospero domain, is highly conserved among vertebrates. Prox1 is essential for the development of several organs; Prox1-deficient mice die before birth due to multiple developmental defects. A role for Prox1 in the development of lymphatic vasculature, liver, pancreas, heart and lens has been shown in previous studies. Some studies have suggested a role for the Prox1 in skeletal muscle development and fibre type specification. In zebrafish, the U-boot (Ubo) gene, which regulates slow versus fast fibre differentiation pathways, activates Prox1 in slow myoblasts. This is crucial for the terminal stages of fibre development and for their proper organization in slow fibres. Recently, Petchey et al. demonstrated in mice that Prox1 is required for the repression of fast troponin T3, troponin I2, and myosin light chain 1 in both cardiac and skeletal muscle, indicating conservation of Prox1 function in vertebrates.

Here we assess the role of Prox1 in the regulation of skeletal muscle phenotype and function. We show that Prox1 is expressed in rodent and human slow muscle fibres and in SC. In SC, Prox1 expression is induced on differentiation, and Prox1 silencing results in Notch1 activation and inhibition of myoblast differentiation. Interestingly, Prox1 is also necessary and sufficient for the slow fibre type gene program via regulation of NFAT signalling.

Results
Prox1 is expressed in slow muscle fibres and SC. Prox1 expression in skeletal muscle has been reported in zebrafish and mice. Our results show that human skeletal muscle fibres express Prox1 and, interestingly, we observed nuclear Prox1 immunostaining only in the nuclei of muscle fibres that express the slow MyHC I in all mouse, rat and human skeletal muscles. The Prox1-positive nuclei were located under the basalm lamina, indicating that they were muscle fibre nuclei. Furthermore, Prox1 was not expressed in blood vascular endothelial cells. We compared Prox1 expression in different rat muscles, and observed the highest Prox1 mRNA levels in the soleus muscle (SOL), which predominantly consists of type I slow fibres, followed by the red part of the gastrocnemius muscle (RGA) enriched for type IIA fibres (Fig. 1c). In contrast, Prox1 expression was only marginal in the tibialis anterior (TA) and the white part of the gastrocnemius muscle (WGA), both containing mainly fast IIB fibres. Mouse and rat skeletal muscles contain fewer slow fibres than most human muscles and, in accordance with this, fewer Prox1-positive nuclei were observed in rodent muscles than in human muscles (Fig. 1).

Figure 1 | Prox1 expression in slow muscle fibres in mammalian skeletal muscle. (a) Prox1 expression in the nuclei (magenta) of slow muscle fibres stained for MyHC I (green) in human vastus lateralis muscle. Co-staining with laminin indicates that the Prox1 positive nuclei are under the basal lamina of the muscle fibres (arrows). Note that Prox1 staining does not co-localize with endothelial cells (CD31, arrows). (b) Prox1 positive nuclei (magenta) in MyHC I (green) positive slow muscle fibres in rat tibialis anterior muscle. Few Prox1 positive nuclei were found also in the proximity of fast fibres. (c) Prox1 mRNA expression in various rat skeletal muscles. Note the highest expression in the slow soleus (SOL) muscle. RGA, red gastrocnemius; TA, tibialis anterior; WGA, white gastrocnemius. Data is presented as mean ± s.e.m., n = 4, one-way analysis of variance with Tukey’s post hoc test, **P < 0.01, ***P < 0.001 compared with soleus. Scale bars, 50 μm.
In addition, we observed mononuclear MyHC I-negative cells expressing Prox1 in the skeletal muscle. Staining of rat TA muscle sections for the SC markers Pax3 and Pax7 with Prox1 indicated that these cells have SC identity (Fig. 2a), raising the possibility that Prox1 has a role in the regulation of muscle stem cells as well. We further confirmed our findings in human skeletal muscles, where Pax7 and NCAM (CD56) positive SCs expressed Prox1 (Fig. 2b), with 98% of Pax7+/ nuclei being Prox1 positive (81 out of 83 cells) (Fig. 2c).

**Prox1 expression is necessary for the slow muscle phenotype.** Since Prox1 was abundantly expressed in slow muscle fibres, we next assessed the function and downstream signalling of Prox1 in skeletal muscles. Adeno-associated viral vector (AAV8) encoding Prox1 was injected into mouse TA muscle, which is composed of fast muscle fibres with very low endogenous Prox1 expression. The contralateral muscle was injected with AAV encoding mCherry reporter protein equipped with a nuclear localization signal. Although the overall muscle structure was not affected by Prox1 overexpression, after four weeks we observed occasional cells expressing MyHC I (Fig. 3a), which were not found in the control TA muscles (MyHC I staining in 6.6 % of all fibres and 25.4 % of Prox1 overexpressing fibres).

Prox1 transduction to fast skeletal muscle resulted in the activation of the slow muscle fibre specific gene program involving contractile elements and calcium regulation (Myh7, TnnC1, TnnC11, Myoz2, Sl1), while downregulating fast MyHC genes (Myh2 and Myh4; Fig. 3b). Furthermore, Gene Set Enrichment Analysis (GSEA) from whole-genome microarray analysis revealed a strong induction of soleus/slow fibre-specific genes (Fig. 3c). However, Prox1 targets in skeletal muscle were tissue-specific without significant overlap with genes regulated by Prox1 in colorectal cancer.17,18 (Supplementary Fig. 1a).

To test if Prox1 is necessary for the slow fibre program, we deleted Prox1 in skeletal muscle fibres by administering tamoxifen to HSA-CreERT2,Prox1fl/fl adult mice (indicated as Prox1Δ in figures). We used the human α-skeletal actin (HSA) promoter-driven Cre-recombinase, which is expressed specifically in skeletal muscle fibres to delete Prox1 in skeletal muscles (Supplementary Fig. 2a). Prox1 deletion resulted in increased expression of fast MyHC genes and downregulation of typical slow fibre genes in soleus muscle (Fig. 3d). As additional evidence, we deleted Prox1 via injection of AAV8-Cre vector into...
Figure 3 | Prox1 regulates the slow muscle fibre gene program in skeletal muscle. (a) MyHC I (Myh7) expression in muscle fibres (arrows) in AAV8-Prox1 and AAV8-Ctrl transduced TA muscle. (b) Expression of myosin heavy chain and calcium-signalling genes in AAV8-Prox1 and AAV8-Ctrl transduced TA muscle. Note that the expression of slow fibre-specific genes are upregulated and fast MyHC genes downregulated in Prox1 overexpressing muscles. (c) Heat map from GSEA analysis showing that Prox1 overexpression in TA muscle leads to expression of many soleus specific myofibrillar genes (red colour indicates positive and blue negative enrichment). (d) Effect of Prox1 deletion on soleus muscle gene expression (HSA-CreERT2; Prox1fl/fl mice). Note the increased expression of fast MyHCs Myh2 and Myh4 RNAs, whereas slow troponin isoform RNAs and Myh7 RNA are decreased. (e) Deletion of Prox1 in slow soleus muscle with AAV-Cre leads to marked downregulation of MyHC I protein. Data is presented as mean ± s.e.m., n = 5 + 5 in a,b, n = 3 + 3 in c, n = 4 + 4 in d,e. Student’s two-tailed unpaired t-test, *P < 0.05, **P < 0.01, ***P < 0.001. Both overexpression and deletion experiments were conducted two times on independent mouse cohorts. WT, wild-type. Scale bars, 50 μm.
the soleus muscles of Prox1fl/fl mice. This led to a robust decrease of MyHC I protein level in the soleus muscle after three weeks (Fig. 3e, Supplementary Fig. 6a). Collectively, these results showed that Prox1 expression is necessary and sufficient for the slow muscle fibre gene expression program. Furthermore, Prox1-deficient mice had a significantly impaired exercise capacity, as they ran only half of the distance compared with wild-type littermates in a maximal graded exercise test (Supplementary Fig. 2b). In the gastrocnemius muscles of wild-type mice, the expression of Prox1 was decreased after an acute maximal exercise (Supplementary Fig. 2c).

There is a dramatic change in the expression levels of different MyHCs during the first weeks after birth, which was also detected in our experiments (Supplementary Fig. 2d). Surprisingly, however, Prox1 expression in mouse skeletal muscle remained constant during the first three weeks of postnatal life (Supplementary Fig. 2d), suggesting that Prox1 expression is relatively stable during postnatal muscle growth and development.

**Prox1 activates NFAT signalling in vivo and ex vivo.** Calcineurin/ Ca²⁺-dependent nuclear factor of activated T cells (NFAT) pathway is a key regulator of slow muscle fibre phenotype. GSEA analysis of the whole-genome transcriptome from Prox1 overexpressing muscles showed a highly significant induction of the genes that are upregulated by NFAT activity, suggesting that Prox1 exerts its effects via this pathway. Consistently, we found that genes repressed by NFAT signalling were downregulated in muscles with Prox1 overexpression (Fig. 4a). In particular, a significant upregulation of Rcan1, which is specifically increased on activation of calcineurin, was
of the Prox1-positive mononuclear cells in skeletal muscle, proliferation and differentiation. First, to test the stem cell properties of Prox1-positive SCs, we set out to assess the importance of Prox1 in SC proliferation and differentiation. Since Prox1 was detected in SCs in addition to mature slow muscle fibres, the Prox1-positive SCs act as stem cells after muscle injury. The Prox1-positive muscle fibres originating from Prox1-positive SCs were MyHC I negative in fast tibialis anterior muscle. The Prox1-positive SCs display stem cell behaviour under regenerative conditions.

To test whether Prox1 regulates NFAT activity, we transduced C2C12 myoblasts with a retroviral Prox1 expression vector and subsequently the cells were transplanted into mouse muscles. Prox1-induced luciferase activity in C2C12 cells, indicating that it activates NFAT signalling in myoblasts (Fig. 4c). Immunostaining showed that Prox1 induces nuclear translocation of Nfatc1, reflecting its activation (Fig. 4d). Consistent with this finding, western blotting demonstrated increased cytoplasmic Nfatc1 in Prox1 silenced cells when compared with controls, indicating reduced nuclear translocation of Nfatc1 (Supplementary Figs 3 and 6b). Furthermore, the expression levels of Nfatc1, Nfat2 and Nfatc3 were down-regulated in Prox1 deleted soleus muscles (Fig. 4e). We conclude that Prox1 is involved in the activation of the NFAT pathway and several of its downstream genes in skeletal muscle.

The Prox1-positive SCs act as stem cells after muscle injury. Since Prox1 was detected in SCs in addition to mature slow muscle fibres, we set out to assess the importance of Prox1 in SC proliferation and differentiation. First, to test the stem cell properties of the Prox1-positive mononuclear cells in skeletal muscle, we used a lineage tracing approach in Prox1-CreERT2; Rosa26tdTomatoCreERT2/STOP/fl mice. In this model, a single tamoxifen injection induces Cre activity and results in the removal of a transcriptional stop cassette in front of the tdTomato reporter coding sequence, thus leading to the expression of the red fluorescent protein exclusively in the Prox1-positive cells and their progenies. Cardiotoxin was injected into the TA muscles to induce muscle injury. As the mouse TA does not contain slow fibres, the Prox1-positive cells in this muscle are SC or lymphatic endothelial cells. After 1 and 4 days, we detected a marked inflammation and degenerating muscle fibres (Fig. 5a). At day 4, the number of TdTomato-positive mononuclear cells was increased in the damaged area, and some of these cells were positive for the proliferation marker Ki67 (Supplementary Fig. 4). At day 22, the cardiotoxin-treated muscles had regenerated and several fibres expressed red fluorescence, demonstrating that they originated from Prox1-positive SCs (Fig. 5b). However, the nuclei of these fibres became Prox1 negative and they did not stain for MyHC I, indicating that they had acquired the fast fibre phenotype typical for TA muscle (Fig. 5c). Muscle fibres with red fluorescence were not found in any of the PBS injected muscles. To increase the labelling efficiency, we injected tamoxifen twice before CTX, which resulted in tdTomato labelling of most of the regenerated fibres three weeks after injury (Fig. 5d). Altogether, these data show that Prox1-positive SCs display stem cell behaviour under regenerative conditions.

Prox1 is critical for myoblast differentiation. To further test the role of Prox1 in SCs, we studied the mouse C2C12 myoblast cell line and primary human myoblasts. These cells can differentiate into myotubes with multiple nuclei, thus displaying stem cell features. Prox1 expression was strongly increased during mouse and human myoblast differentiation both at the mRNA and protein level (Fig. 6a,b). Silencing Prox1 by lentiviral shRNA...
Figure 6 | Prox1 regulates myoblast differentiation. (a) Analysis of Prox1 mRNA and protein during C2C12 myoblast differentiation (indicated by the increase in MyoD1 mRNA). (b) Prox1 protein and mRNA in human myoblasts before and after differentiation. Primary myoblast lines from two different individuals were analysed. (c-f) Analysis of myoblast proliferation and differentiation-related CyclinD1, Myf5 and MyoD mRNAs in shProx1 and shScr transduced primary human myoblasts before (c) and after differentiation for 7d (d), as well as in mouse C2C12 myoblasts before (e) and after (f) differentiation. (g) Myosin staining of C2C12 cells after 7d of differentiation. Note that Prox1 silencing completely blocked myotube development, as only occasional cells expressed myosin and these cells were negative for shProx1-GFP. Data is presented as mean ± s.e.m., n = 3 ± 3 biological replicates in a–g (all experiments were repeated three times). One-way analysis of variance with repeated measures followed by Tukey’s posthoc test and Student’s two-tailed unpaired t-test, *P < 0.05, **P < 0.01, ***P < 0.001. Scale bars, 50 μm.

Bidirectional regulation between Prox1 and Notch1. Notch signalling activity is crucial in maintaining SC quiescence and preventing premature muscle differentiation. Since our results showed that Prox1 is needed for the differentiation of myoblasts that have stem cell features, we studied if Prox1 modulates Notch signalling. We first tested if Prox1 regulates Notch signalling activity in C2C12 cells transfected with a CSL-luciferase reporter (for monitoring general Notch activity) or Notch1-specific luciferase reporter. Prox1 silencing significantly increased Notch activity in both assays, indicating that Prox1 represses Notch1 signalling (Fig. 7a). To test the functional domains of Prox1 that are needed for repression of Notch signalling, we transduced myoblasts with lentiviral constructs for wild-type Prox1 or the N-terminal domain of Prox1 lacking the DNA binding domain, and tested their effects on CSL- and Notch-luciferase activities. The results indicated that the N-terminal part of Prox1 was sufficient to suppress Notch1-activity, thus direct interaction with DNA was not required (Fig. 7b). Similar results were also obtained with transient transfection of these constructs (Supplementary Fig. 5a). We then investigated if this regulation occurs in vivo in skeletal muscle. Prox1 overexpression in mouse skeletal muscle resulted in significant downregulation of several Notch signalling-related genes (Fig. 7c).

Notch and its downstream effectors Hey1 and Hey2 were shown to regulate Prox1 in lymphatic endothelial cells. Thus, we next tested if Notch activity regulates Prox1 expression in myoblasts. Human primary myoblasts transduced with Ad-hNotch1 exhibited decreased Prox1 expression, whereas inhibition of Notch signalling by administration of the gamma-secretase/Notch cleavage inhibitor DAPT tended to increase Prox1 expression (Fig. 7d and Supplementary Fig. 5b). Notch1 also increased CyclinD1 and decreased MyoD1 expression, which
are needed to sustain cells in proliferating and undifferentiated state, respectively. To test if Prox1 expression is regulated by canonical Notch signalling, we silenced both Hey1 and Hey2 in human myoblasts, and found that this abolished the Notch1-induced decrease of Prox1 expression (Fig. 7e). These data suggest that high Notch activity in quiescent and proliferating SCs inhibits Prox1 expression, and that during differentiation, when Notch activity decreases, Prox1 expression is induced. Collectively, these results identify Prox1 as an essential transcription factor required for myoblast differentiation via bidirectional interaction with Notch1.

**Discussion**

In this study we identified the transcription factor Prox1 as a critical regulator of SC differentiation and muscle fibre phenotype in rodents and humans. Prox1 was necessary and sufficient for the slow fibre type gene program by regulating NFAT signalling and essential for myoblast differentiation by reciprocal regulation, with Notch, (see the schematic in Fig. 8).

Our results show that Prox1 is expressed only in the nuclei of MyHC I positive slow muscle fibres, but not in the fast muscle fibres. In a recent study, Prox1 was shown to suppress fast fibre genes in the heart and skeletal muscle, but the authors suggested that this occurs in all muscle fibre types. We demonstrate here that Prox1 is able to initiate the slow muscle fibre program when transduced into fast muscles and that Prox1 deletion suppresses slow and induces fast fibre genes. Plasticity for fibre type switch can be activated in adult muscles by the previously identified Mef2, NFAT and myogenin transcription factors, which play a role in the determination of fibre-type characteristics. The NFAT pathway, especially Nfatc1, has been demonstrated to be essential for slow fibre-type determination. Whole-genome RNA expression analysis of Prox1 overexpressing muscles indicated NFAT pathway activation, thus accounting for the ability of Prox1 to induce slow muscle fibre genes.
Furthermore, Prox1 deletion resulted in decreased expression of Nfatc1−3 in skeletal muscle. Prox1 acts mainly as a transcriptional repressor, which is in line with the observed downregulation of the main inhibitors of NFAT signalling in Prox1 overexpressing muscles. These findings were further substantiated by results from the cultured C2C12 myoblasts, where Prox1-induced Nfatc1 nuclear translocation and NFAT transcriptional activation. In line with our results, Prox1 has been shown to be essential for shear stress-induced NFAT activation in lymphatic endothelial cells. Thus, our results provide novel evidence that Prox1 regulates muscle fibre phenotype via NFAT signalling.

In addition to slow muscle fibres, Prox1 is expressed in most, if not all, SCs and its expression is induced during myoblast differentiation. With lineage tracing we obtained evidence that Prox1-positive cells regenerate into muscle fibres after cardioxinduced muscle injury. To reveal the significance of Prox1 in SCs, we silenced Prox1 in human primary myoblasts and in mouse C2C12 myoblasts. This resulted in dysregulated expression of myogenic factors and in complete inhibition of differentiation. Several factors, such as PAX3, PAX7 and MyoD family members, have been shown to be involved in the regulation of SC properties (reviewed in ref. 2). They act in concert at different stages of SC self-renewal and differentiation. Our findings identify Prox1 as a new important regulator of SC differentiation.

Notch signalling is critical for skeletal myogenesis and it has an important role in maintaining the muscle stem cell pool by blocking myogenic differentiation and preventing premature muscle differentiation. After muscle injury, SCs start to proliferate and some of them will differentiate into myofibres concomitantly with downregulation of Notch, while others resume a quiescent state. Our results indicate a bidirectional negative regulation between Prox1 and Notch1 in myoblasts and in skeletal muscle, which fits nicely with their sequential expression patterns during the transition from SC quiescence to differentiation. Deletion of the C-terminal DNA binding domain (homeobox and prospero domains) of Prox1 was not sufficient to relieve the Prox1-mediated suppression of Notch promoter activity, indicating that Prox1 influences Notch1 as transcriptional co-repressor via (an)other factor(s).

In neuronal progenitor cells, Prox1 has been shown to regulate the Notch1-mediated inhibition of neurogenesis and to directly interact with chromatin at the Notch1 promoter. Similarly to our findings, the DNA binding domain of Prox1 was not needed for the repression of Notch1 activity in neuronal progenitor cells, and the nuclear receptor LHR-1/NR5A2 was identified as a binding partner of Prox1 on the Notch1 promoter. LHR-1/NR5A2 is also expressed in C2C12 cells and skeletal muscles (unpublished findings of RK and KA), thus it may serve as a binding partner for Prox1 in SC as well. We have recently shown that Prox1 is essential for the expansion of the stem cell pool in intestinal tumours, where its deletion reduced the number of stem cells. Together, our results and previous findings suggest that Prox1 is an important regulator of stem cell behaviour in various tissues.

The present results identify Prox1 as an important factor in skeletal muscle SC differentiation and show that Prox1 is an integral part of the slow muscle fibre program. Our results open up new directions to study and to manipulate SC behaviour during regeneration, and to understand better the role of Prox1 in muscle diseases and aging.

Methods

Animal experiments. All mice used in the experiments were maintained in the C57Bl/6 background. Animal experiments were approved by the Regional State Administrative Agency for Southern Finland. Mice were housed in individually ventilated cages with enrichment materials in a facility monitored by the Federation of European Laboratory Animal Science Associations guidelines and recommendations.

The Prox1fl/fl (from Dr G. Oliver) and Human Skeletal Actin-MerCreMer (HSA-CreERT2, from Dr K. Eiser) mice were crossed to generate skeletal muscle specific Prox1-deficient mice (Prox1ΔA). These mouse lines have been described previously. To delete Prox1 from skeletal muscles, 8–10 weeks old male mice were given tamoxifen i.p. (80 mg/kg) for five consecutive days. The muscles were collected and analysed after two months of deletion. The experiment was performed twice with independent cohorts (n = 4–6 and n = 6–8). To delete Prox1 only in soleus muscle, AAV9-Cre or empty vector (30 µl per muscle, 6.96 × 10³ vp ml⁻¹) were injected into soleus muscles of Prox1ΔA mice (n = 3–4 in a second experiment n = 5–6). For overexpression of Prox1 in fast skeletal muscle, 8 weeks old C57Bl/6J male mice (C57Bl/6/OlaHsd from Harlan) were anaesthetized and recombinant adeno-associated viral vector (AAV8) encoding Prox1 was injected into mouse TA muscle (30 µl per muscle, 6.08 × 10⁶ vp ml⁻¹). The contralateral muscle was injected with AAV8 encoding mCherry reporter protein equipped with a nuclear localization signal. The experiment was performed twice with independent cohorts (n = 3–4 and n = 5–5).

Whole calf muscle complex was collected from wild-type mice at postnatal days 0, 7 and 21 to study the expression of Prox1 during postnatal development (n = 3 for each time point).

To investigate the role of Prox1 in SC, we performed lineage-tracing experiments. Prox1-CreERT2 mice (from Dr G. Oliver) were crossed with Rosa26-tdTomatoSTOPfl (Jackson Laboratories, Stock 007914) reporter mice. To label a subset of Prox1 positive cells, mice were given one tamoxifen dose (3 mg) at the same time when cardiotoxin (50 µl of 10 µM) was injected into TA muscle. PBS was injected into the contralateral muscle. Muscles were collected and analysed 1, 4 and 22 days after the injection (n = 4 muscles/time point/treatment).

In another experiment, we increased the tamoxifen dose to get more efficient labelling and gave two injections one and two days before cardiotoxin administration.

Maximal endurance running exercise test was performed on a rodent treadmill (Panlab) according to the following protocol: 9 min min⁻¹ for 5 min, 12 min min⁻¹ for 5 min, 15 min min⁻¹ for 5 min, 18 min min⁻¹ for 5 min and 20 min min⁻¹ until exhaustion (10° incline). Adaptation training session was performed two times before the experiment.

In all experiments using wild-type mice, mice were randomized into the experimental groups based on their body weight. The investigators were blinded to group allocation during experiments.

Soleus, gastrocnemius and tibialis anterior muscles were collected from three months old male wild-type rats for RNA and immunohistochemical analyses (n = 4).

Cell culture. The mouse myoblast cell line C2C12 (ATCC) was cultured in growth medium (DMEM high glucose) containing 10% fetal bovine serum (FBS), 2 mM L-glutamine, penicillin (100 U ml⁻¹) and streptomycin (100 µg ml⁻¹). Cells were differentiated for 4, 6, 7 and 8 days for further experiments. Differentiation medium contained (DMEM high glucose), 1–2% horse serum (HS), 2 mM L-glutamine, penicillin (100 U ml⁻¹) and streptomycin (100 µg ml⁻¹).

The isolation of SC and preparation of primary human myoblasts were conducted as described previously. In brief, a muscle biopsy of about 100 mg was taken under local anaesthesia with lidocaine (10 mg ml⁻¹, without ephinephrine) from the vastus lateralis muscle of healthy men. The tissue was dissected into small pieces followed by trypsinization in a shaking water bath at 37 °C. SC were then isolated and maintained in DMEM-F12 culture medium containing 20% FBS, 1% penicillin, 1% streptomycin, and 1% fungizone. Primary myoblasts were selected via magnetic cell separation using the CD56 antibody (Miltenyi Biotec). To obtain...
myotubes, myoblasts of 80% confluence were switched to differentiation medium containing 2% FBS for 7 days. The human study was approved by the Ethical Committee of Department of Medicine, Helsinki University Central Hospital and written informed consent was obtained from all participants. 

Lentiviral Prox1 silencing construct (shProx1) and scramble control (shScr) were from the TRCI library for human myoblasts (Sigma-Aldrich)37. For mouse myoblasts we used shProx1 constructs from TRCI and TR2C (TRCN0000070723, TRCN0000070724, TRCN0000420735, TRCN0000425447, TRCN0000432522 from Sigma-Aldrich). The lentivirus-containing supernatant of 293FT cells was concentrated, and cells were transduced overnight with polybrene (Sigma, 8 µg/ml). After transduction, cells were further selected with 3 µg/ml (human myoblasts) or 30 µg/ml (C2C12) puromycin (Sigma). After selection, the cells were cultured for further experiments. Retroviral constructs for Prox1 and GFP (control) were used for transfection of undifferentiated C2C12 cells overnight, followed by recovery for two to three days before the experiments.

Prox1-overexpressing and -silenced cells were transfected with expression plasmids for Prox1 and GFP (full-length Prox1, GFP-NTF1-A-P3.3x luciferase was a gift from Anjana Rao, Addgene plasmid #11783), Notch reporter CSL-luciferase reporter (4xCSL-luciferase was a gift from Raphael Kopan, Addgene plasmid #41726) or Notch1-luciferase reporter22,23 (gift from Tohru Kiyono) by using the jetPEI reagent (Polyplus transfection). TK-Renilla luciferase was used as a transfection control. Promega’s dual luciferase assays were performed according to the manufacturer’s instructions. All experiments were done in quadruplicate and repeated at least 2–4 times.

To analyse the effect of Notch signalling activity on Prox1 expression, we transduced primary human myoblasts with adenoviral constructs enclosing human Notch1 or Notch2 (Ad5 or Ad2, respectively) to inhibit Notch activity, the cells were treated with gamma-secretase inhibitor DAPT (10 µM). Prox expression was analysed after 2–3 days of treatment. To silence Hey1 and Hey2 in human myoblasts, lentiviral vectors were produced from TRCI library constructs (TRCN0000020144 and TRCN000020249, Sigma-Aldrich).

For generation of the Prox1 knockout mutant without DNA binding domain (Prox1-deltaHP), the stop codon was introduced after nucleotides encoding Ser569 (XP_005273251.1) in the construct encoding a MYC-tagged wild type human PROX1 in the lentivector vector pShD44 (ref. 43) using standard site-directed mutagenesis procedures. The presence of mutation was confirmed by sequencing.

RNA extraction and quantitative real-time PCR. Cells were lysed and total RNA was extracted with Nucleospin RNA II Kit (Macherey-Nagel). Muscle tissues were first lysed with Trisure reagent (Bioline) and RNA was further extracted with the Nucleospin RNA II Kit (Macherey-Nagel). cDNA synthesis was performed with the iScript cDNA synthesis kit (Bio-Rad) using 1 µg RNA and random hexamer primers. qRT-PCR was performed with the iQ SYBR green supermix (Bio-Rad). Relative gene expression levels were calculated using the formula 2−ΔΔCt. Primers are listed in Supplementary Table 1.

Whole-genome microarray. The quality of RNA was determined with Bioanalyzer (Agilent Technologies) and analysed on genome-wide Illumina Mouse WG-6 v2 Expression BeadChips (Illumina). Illumina’s GenomeStudio software was used for data quality control and quality control and the further data analysis was performed with the Chipster software (www.chipster.csc.fi). After quantile normalization, statistically significant differences in individual genes between the groups were tested using Empirical Bayes statistics and the Benjamini-Hochberg algorithm controlling FDR. Adjusted FDR values of P<0.05 were considered significant.

Microarray data have been submitted to the GEO database, under the series accession number GSE69199.

Gene set enrichment analysis. The gene expression dataset was transferred to the Gene Set Enrichment Analysis Software (http://www.broadinstitute.org/gsea)15,16 and the analysis was carried out with default parameters except that the ‘exclude smaller sets’ was set to 25 and gene permutation was applied. We used a modified list of 1103 novel gene set (https://www.broadinstitute.org/gsea/gsvgseh) with the addition of slow muscle fibres-specific genes17 and Prox1 target genes in colorectal cancer18. FDR Q value <0.1 was regarded significant.

Polyacrylamide gel electrophoresis and western blotting. Cells were lysed with cold RIPA buffer (50 mM Tris-HCl at pH 7.6, 150 mM NaCl, 1% NP-40, 0.5% DOC, 0.1% SDS, with protease and phosphatase inhibitors). Muscle tissues were lysed with the same buffer using ceramic beads. The concentration of the lysates was measured with the BCA protein assay kit (Thermo Scientific) following the manufacturer’s instructions. The samples were loaded to SDS-polyacrylamide gel (Bio-Rad Laboratories), proteins were transferred to nitrocellulose membranes and incubated with primary antibody against MyHC I (BA-D5, Developmental Studies Hybridoma Bank, 1:250), Nfatc1 (T5381, Immunoway, 1:1000) or IIB4 (1:1000) antibody using Protein G beads, transferred to the manufacturer’s instructions. All experiments were done in quadruplicate and repeated at least 2–4 times.

To analyse the effect of Notch signalling activity on Prox1 expression, we transduced primary human myoblasts with adenoviral constructs enclosing human Notch1 or Notch2 (Ad5 or Ad2, respectively) to inhibit Notch activity, the cells were treated with gamma-secretase inhibitor DAPT (10 µM). Prox expression was analysed after 2–3 days of treatment. To silence Hey1 and Hey2 in human myoblasts, lentiviral vectors were produced from TRCI library constructs (TRCN0000020144 and TRCN000020249, Sigma-Aldrich).

For generation of the Prox1 knockout mutant without DNA binding domain (Prox1-deltaHP), the stop codon was introduced after nucleotides encoding Ser569 (XP_005273251.1) in the construct encoding a MYC-tagged wild type human PROX1 in the lentivector vector pShD44 (ref. 43) using standard site-directed mutagenesis procedures. The presence of mutation was confirmed by sequencing.

RNA extraction and quantitative real-time PCR. Cells were lysed and total RNA was extracted with Nucleospin RNA II Kit (Macherey-Nagel). Muscle tissues were first lysed with Trisure reagent (Bioline) and RNA was further extracted with the Nucleospin RNA II Kit (Macherey-Nagel). cDNA synthesis was performed with the iScript cDNA synthesis kit (Bio-Rad) using 1 µg RNA and random hexamer primers. qRT-PCR was performed with the iQ SYBR green supermix (Bio-Rad). Relative gene expression levels were calculated using the formula 2−ΔΔCt. Primers are listed in Supplementary Table 1.

Whole-genome microarray. The quality of RNA was determined with Bioanalyzer (Agilent Technologies) and analysed on genome-wide Illumina Mouse WG-6 v2 Expression BeadChips (Illumina). Illumina’s GenomeStudio software was used for data quality control and quality control and the further data analysis was performed with the Chipster software (www.chipster.csc.fi). After quantile normalization, statistically significant differences in individual genes between the groups were tested using Empirical Bayes statistics and the Benjamini-Hochberg algorithm controlling FDR. Adjusted FDR values of P<0.05 were considered significant.

Microarray data have been submitted to the GEO database, under the series accession number GSE69199.

Gene set enrichment analysis. The gene expression dataset was transferred to the Gene Set Enrichment Analysis Software (http://www.broadinstitute.org/gsea)15,16 and the analysis was carried out with default parameters except that the ‘exclude smaller sets’ was set to 25 and gene permutation was applied. We used a modified list of 1103 novel gene set (https://www.broadinstitute.org/gsea/gsvgseh) with the addition of slow muscle fibres-specific genes17 and Prox1 target genes in colorectal cancer18. FDR Q value <0.1 was regarded significant.

Polyacrylamide gel electrophoresis and western blotting. Cells were lysed with cold RIPA buffer (50 mM Tris-HCl at pH 7.6, 150 mM NaCl, 1% NP-40, 0.5% DOC, 0.1% SDS, with protease and phosphatase inhibitors). Muscle tissues were lysed with the same buffer using ceramic beads. The concentration of the lysates was measured with the BCA protein assay kit (Thermo Scientific) following the manufacturer’s instructions. The samples were loaded to SDS-polyacrylamide gel (Bio-Rad Laboratories), proteins were transferred to nitrocellulose membranes and incubated with primary antibody against MyHC I (BA-D5, Developmental Studies Hybridoma Bank, 1:250), Nfatc1 (T5381, Immunoway, 1:1000) or IIB4 (1:1000) antibody using Protein G beads, transferred to the manufacturer’s instructions. All experiments were done in quadruplicate and repeated at least 2–4 times.

To analyse the effect of Notch signalling activity on Prox1 expression, we transduced primary human myoblasts with adenoviral constructs enclosing human Notch1 or Notch2 (Ad5 or Ad2, respectively) to inhibit Notch activity, the cells were treated with gamma-secretase inhibitor DAPT (10 µM). Prox expression was analysed after 2–3 days of treatment. To silence Hey1 and Hey2 in human myoblasts, lentiviral vectors were produced from TRCI library constructs (TRCN0000020144 and TRCN000020249, Sigma-Aldrich).

For generation of the Prox1 knockout mutant without DNA binding domain (Prox1-deltaHP), the stop codon was introduced after nucleotides encoding Ser569 (XP_005273251.1) in the construct encoding a MYC-tagged wild type human PROX1 in the lentivector vector pShD44 (ref. 43) using standard site-directed mutagenesis procedures. The presence of mutation was confirmed by sequencing.

RNA extraction and quantitative real-time PCR. Cells were lysed and total RNA was extracted with Nucleospin RNA II Kit (Macherey-Nagel). Muscle tissues were first lysed with Trisure reagent (Bioline) and RNA was further extracted with the Nucleospin RNA II Kit (Macherey-Nagel). cDNA synthesis was performed with the iScript cDNA synthesis kit (Bio-Rad) using 1 µg RNA and random hexamer primers. qRT-PCR was performed with the iQ SYBR green supermix (Bio-Rad). Relative gene expression levels were calculated using the formula 2−ΔΔCt. Primers are listed in Supplementary Table 1.

Whole-genome microarray. The quality of RNA was determined with Bioanalyzer (Agilent Technologies) and analysed on genome-wide Illumina Mouse WG-6 v2 Expression BeadChips (Illumina). Illumina’s GenomeStudio software was used for data quality control and quality control and the further data analysis was performed with the Chipster software (www.chipster.csc.fi). After quantile normalization, statistically significant differences in individual genes between the groups were tested using Empirical Bayes statistics and the Benjamini-Hochberg algorithm controlling FDR. Adjusted FDR values of P<0.05 were considered significant.

Microarray data have been submitted to the GEO database, under the series accession number GSE69199.
37. Kaltezioti, V.
38. Bolado-Carrancio, A., Riancho, J. A., Sainz, J. & Rodriguez-Rey, J. C. Activation of nuclear receptor NR5A2 increases Glut4 expression and glucose metabolism in skeletal muscle cells. Mol. Cell. Biol. 27, 3732–3742 (2007).
39. Srinivasan, R. S. et al. PRX1 promotes metabolic adaptation and fuels outgrowth of Wnt(high) metastatic colon cancer cells. Cell Rep. 8, 1957–1973 (2014).
40. Kallio, M. A. et al. Chipster: user-friendly analysis software for microarray and other high-throughput data. BMC Genomics 12, 507 (2011).
41. Mootha, V. K. et al. PGC-1alpha-responsive genes involved in oxidative phosphorylation are coordinately downregulated in human diabetes. Nat. Genet. 34, 267–273 (2003).
42. Yugawa, T. et al. Regulation of Notch1 gene expression by p53 in epithelial cells. Mol. Cell. Biol. 27, 3732–3742 (2007).
43. Ragusa, S. et al. PRX1 regulates the notch1-mediated inhibition of satellite cell differentiation and muscle fibre type regulation. Nat. Commun. 7, 13124 (2016).

Acknowledgements
We thank Jenny Hogstrom, Seppo Kajalainen and Jianpin Cheng for their help with the vector constructs, Dr Guillermo Oliver for kindly providing the Prox1CreERT2 mice and Dr Karyn Esser for the HSA-MerCreMer mice. Dr Juhani Helman kindly provided human skeletal muscle samples for histochemistry, Drs Tohru Kiyoumi and Panagiotis Politi the Notch1-luciferase construct and Dr Jan Kitajewski the Ad-hNotch1 construct. Kiri Lintula, Maria Arrano de Kivikko, Tanja Laakonen and Tapio Tainola are acknowledged for their excellent technical help. We also thank the Biomedical Imaging Unit for microscope support and the AAV Gene Transfer and Cell Therapy Core Facility of Biocentrum Finland for the AAV-vectors. The monoclonal antibodies for Pax3, Pax7, MF-20 and Myh7 were obtained from the Developmental Studies Hybridoma Bank, created by the NICHD of the NIH and maintained at The University of Iowa, Department of Biology, Iowa City, IA 52242. The Jenny and Antti Wihuri Foundation, the Academy of Finland (Centre of Excellence Program (271845, K.A.), Marie Curie Actions of European Union’s Seventh Framework Programme FP7/2007–2013 (Grant 317250 VESSEL), the Leducq Foundation (11CVD03, K.A.), the Sigrid Juselius Foundation (Z.W., K.A.) and the Cancer Society of Finland (K.A. and Z.W.) are acknowledged for funding. H.A.K. acknowledges funding from the Academy of Finland (grant 258753) Liv och Hälsa Foundation and Finska Läkaressällskapet. Z.W. currently receives funding from the János Bolyai Fellowship (Hungarian Academy of Sciences, BO/0006815/8), the Semmelweis University Starting Grant (40148 11659) and the National Research, Development and Innovation Office (Hungary, OTKA-NN 118018).

Author contributions
R.K., I.S. and Z.W. planned and conducted the experiments, Y.H.N. and H.A.K. isolated the vector constructs, Dr Guillermo Oliver for kindly providing the Prox1CreERT2 mice and Dr Karyn Esser for the HSA-MerCreMer mice. Dr Juhani Helman kindly provided human skeletal muscle samples for histochemistry, Drs Tohru Kiyoumi and Panagiotis Politi the Notch1-luciferase construct and Dr Jan Kitajewski the Ad-hNotch1 construct. Kiri Lintula, Maria Arrano de Kivikko, Tanja Laakonen and Tapio Tainola are acknowledged for their excellent technical help. We also thank the Biomedical Imaging Unit for microscope support and the AAV Gene Transfer and Cell Therapy Core Facility of Biocentrum Finland for the AAV-vectors. The monoclonal antibodies for Pax3, Pax7, MF-20 and Myh7 were obtained from the Developmental Studies Hybridoma Bank, created by the NICHD of the NIH and maintained at The University of Iowa, Department of Biology, Iowa City, IA 52242. The Jenny and Antti Wihuri Foundation, the Academy of Finland (Centre of Excellence Program (271845, K.A.), Marie Curie Actions of European Union’s Seventh Framework Programme FP7/2007–2013 (Grant 317250 VESSEL), the Leducq Foundation (11CVD03, K.A.), the Sigrid Juselius Foundation (Z.W., K.A.) and the Cancer Society of Finland (K.A. and Z.W.) are acknowledged for funding. H.A.K. acknowledges funding from the Academy of Finland (grant 258753) Liv och Hälsa Foundation and Finska Läkaressällskapet. Z.W. currently receives funding from the János Bolyai Fellowship (Hungarian Academy of Sciences, BO/0006815/8), the Semmelweis University Starting Grant (40148 11659) and the National Research, Development and Innovation Office (Hungary, OTKA-NN 118018).

Additional information
Supplementary Information accompanies this paper at http://www.nature.com/naturecommunications
Competing financial interests: The authors declare no competing financial interests.
Reprints and permission information is available online at http://www.nature.com/reprintsandpermissions/
How to cite this article: Kivelä, R. et al. The transcription factor Prox1 is essential for satellite cell differentiation and muscle fibre type regulation. Nat. Commun. 7, 13124 doi: 10.1038/ncomms13124 (2016).

This work is licensed under a Creative Commons Attribution 4.0 International License. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in the credit line; if the material is not included under the Creative Commons license, users will need to obtain permission from the license holder to reproduce the material. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/