Dual role of delta-like 1 homolog (DLK1) in skeletal muscle development and adult muscle regeneration

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SUMMARY
Muscle development and regeneration is tightly orchestrated by a specific set of myogenic transcription factors. However, factors that regulate these essential myogenic inducers remain poorly described. Here, we show that delta-like 1 homolog (Dlk1), an imprinted gene best known for its ability to inhibit adipogenesis, is a crucial regulator of the myogenic program in skeletal muscle. Dlk1+/− mice were developmentally retarded in their muscle mass and function owing to inhibition of the myogenic program during embryogenesis. Surprisingly however, Dlk1 depletion improves in vitro and in vivo adult skeletal muscle regeneration by substantial enhancement of the myogenic program and muscle function, possibly by means of an increased number of available myogenic precursor cells. By contrast, Dlk1 fails to alter the adipogenic commitment of muscle-derived progenitors in vitro, as well as intramuscular fat deposition during in vivo regeneration. Collectively, our results suggest a novel and surprising dual biological function of Dlk1 as an enhancer of muscle development, but as an inhibitor of adult muscle regeneration.

KEY WORDS: Delta-like 1 homolog, Muscle development, Muscle regeneration, Muscle stem cells, Mouse

INTRODUCTION
Insight into factors that promote or inhibit normal muscle regeneration is crucial to open up new possibilities for treating muscle diseases, such as lethal muscular dystrophies and age-dependent sarcopenia, that cause an enormous economic burden in the increasingly aged population (Saini and Stewart, 2006). Normal muscle develops and regenerates completely from satellite cells (referred to as ‘classical’ muscle stem cells), but the restorative capacity is highly reduced or eradicated in both diseased and atrophied muscles (Evans, 2010). Both skeletal muscle development and regeneration is known to embrace a cascade of temporarily atrophied muscles (Evans, 2010). Both skeletal muscle development and regeneration is known to embrace a cascade of temporarily...

forms of membrane-spanning DLK1 proteins (Smas et al., 1994) with some variants encompassing a protease recognition site that enables processing and shedding (Jensen et al., 1994; Mei et al., 2002). Although DLK1 has been suggested to interact and function through different surface proteins, including NOTCH1, this is still controversial and not well understood. During development, Dlk1 is expressed in developing myofibers and associated satellite cells, whereas its expression is almost completely abolished in adult muscle in both human and mouse (Andersen et al., 2009; Floridon et al., 2000). Likewise, diseased and regenerating muscle express high levels of Dlk1, though the number of DLK1+ satellite cells in these scenarios differ greatly, with only very few appearing during muscle regeneration of healthy muscle (Andersen et al., 2009). By contrast, numerous DLK1+ non-myogenic cells emerge in the interstitium between the myofibers upon muscle damage in human and mouse (Andersen et al., 2009; Waddell et al., 2010). Thus, as Dlk1 can act in a cell autonomous or non-cell autonomous manner, it may affect myogenic precursor cells regardless of which type of cell expresses DLK1 in the muscle. However, the biological function of the muscle-residing DLK1+ cell populations still remains incompletely understood. In this study, we performed a detailed comparison of the skeletal muscle phenotypes of wild-type and Dlk1 knockout mice during embryogenesis and adulthood, as well as during adult muscle regeneration, and thereby unraveled a highly unusual dual role of Dlk1 in skeletal muscle development and regeneration. Our data suggests that inhibitors of Dlk1 function may be useful to facilitate adult muscle regeneration.

MATERIALS AND METHODS

Animals

Dlk1+/− and Dlk1+/+ colonies were maintained by homozygous breeding (Raghunandan et al., 2008) and backcrossed to C57BL/6 mice every third generation in order to ensure maximal genetic diversity and avoid accumulation of spontaneous mutations. Tail or ear DNA was isolated using a DNeasy Kit (Qiagen) and genotype analysis was performed by PCR amplification using the following primers: 5′Dlk1_F, CCAAATT-GCTATAGTCTTCCCT; 5′Dlk1_R, CTGATGAAGAGGACACAGG; 5′Neo_F, TTGAAAAGATGGATTGACGAG; 5′Neo_R, GGCT-
GCGCCGAGCCCTGATGCTCCT, and Taq DNA polymerase (Invitrogen). Animals were housed in plastic cages with a 12/12 hour light/dark cycle, and fed ad libitum with a normal chow (10% fat, 20% protein, 70% carbohydrate). Specific characteristics of the animals have been described previously (Raghunandan et al., 2008) and are in agreement with others (Moon et al., 2002). For all animal experiments we used age- and gender-matched animals, as indicated. All procedures were approved by the Danish Council for Supervision with Experimental Animals (#2010/561-1792).

**Skeletal muscle regeneration model**

Skeletal muscle lesion was performed as previously described (Andersen et al., 2009) in 12-week-old female and male Dlk1−/− and Dlk1+/− C57BL/6 mice. Briefly, hindlimb muscles of anesthetized mice were carefully exposed and two wounds were introduced starting from the outer fascia to the widest part of muscles (m). gastrocnemius until reaching the calfbone, then the skin incision was sutured. The m. gastrocnemius was dissected out from mice 0-14 days following muscle damage. For immunohistochemistry, the tissues were snap frozen. For qRT-PCR, the m. gastrocnemius was carefully dissected with removal of fat and tendons, and for each mouse the two m. gastrocnemii were pooled and kept in RNA Lysis Buffer (Applied Biosystems, ABI, Foster City, CA, USA) until further analysis.

**Isolation and culture of muscle-derived cells**

Muscle-derived cells (MDCs) were retrieved (each experiment comprising two m. gastrocnemii from each of two mice) using a previously described protocol (Andersen et al., 2009; Andersen et al., 2008). Briefly, the gastrocnemius muscles were dissected and visible fat, as well as large vessels, were removed. Tissues were subsequently rinsed with Hank’s Balanced Salt Solution (HBSS) + 100 IU/ml penicillin-streptomycin (1% v/v) in tissue culture vessels, were removed. Tissues were subsequently rinsed with Hank’s Balanced Salt Solution (HBSS) + 100 IU/ml penicillin-streptomycin (1% v/v). Cells were released by gentle trituration DMEM + 20% fetal calf serum (FCS) + 1% II collagenase (Worthington, UK) at 37°C for 40 minutes. Cells were pelleted by centrifugation, and the cells were plated on extracellular matrix (ECM; E1270 Sigma-Aldrich Europe)-coated dishes at very low cell density. After 48 hours of culture, 50% confluent cells were passaged, counted by Coulter counting, and plated at 3000 cells/cm². For myogenic differentiation experiments, cells were cultured in DMEM + 20% FCS + 1% PS for 72 hours (used as proliferative samples) and myogenic differentiation was then allowed to occur in DMEM + 2% horse serum + 1% PS for an additional period of 72 hours (used as differentiated samples in experiments). For adipogenic differentiation, cells were cultured as recently described (Andersen et al., 2013). Briefly, adipogenic differentiation was induced in confluent MDCs by the addition of DMEM containing 1% PS, 5% FCS, 1 mM dexamethasone, 0.5 mM 3-isobutyl-1-methylxanthine, 10 mg/mL insulin and 5 μM rosiglitazone for 3 days and further maintained for 7 days in DMEM containing 1% PS, 5% FCS and 10 mg/mL insulin.

**Overexpression of DLK1 in the myogenic cell line C2C12**

The myogenic cell line C2C12 (ATCC) was kept in agreement with general guidelines and maintained in DMEM + 10% FCS + 1% PS at 37°C in humidified 5% CO2. For all experiments, C2C12 cells were seeded at 10,000 cells/cm² and cultured for 48 hours before further treatment. For transfection experiments, cells were equilibrated in serum-free medium for 1 hour prior to transfection for 4 hours with 1 μg/12-well pcDNA2-Dlk1FL (containing full-length mouse Dlk1 cDNA (Baladron et al., 2002) or control pcDNA2 vector using the Lipofectamine 2000 protocol (Life Technologies, Denmark). Immediately following transfection, cells were equilibrated in DMEM + 2% horse serum + 1% PS for 5 days. Conditioned medium from pcDNA2-Dlk1FL or control pcDNA2 vector transfected cells was added to C2C12 cells in order to induce differentiation in the presence/absence of soluble mouse DLK1 for 5 days. The dose-response of C2C12 cells to DLK1 during differentiation was analyzed by addition of purified DLK1 (Jensen et al., 1994) at a concentration of 0, 2.5, 5 or 10 pg/ml for 5 days. Myofiber diameter was measured using Adobe Photoshop on pictures obtained from myosin-stained cultures (n=4).

**Histology, immunofluorescence and immunohistochemistry**

Histology, immunofluorescence was performed as previously described (Andersen et al., 2009; Andersen et al., 2008). Cultured cells were gently rinsed in TBS, fixed in 4% neutral buffered formaldehyde (NBF) (10 minutes), permeabilized for 10 minutes in 0.3% Triton X-100 in TBS, blocked in 2% bovine serum albumin (BSA) in TBS (10 minutes), and incubated for 2 hours with primary antibodies (see below) diluted in 1% BSA in TBS. Secondary antibodies used were Alexa 555- or 488-conjugated donkey anti-mouse IgG (1:200, Molecular Probes), and mounting medium contained DAPI (Vectorshield, Vector Labs).

For histology and IHC, muscles were snap-frozen in Tissue Tek or fixed overnight in 4% NBF and embedded in paraffin. Paraffin-embedded sections were deparaffinized and rehydrated before antigen demasking for IHC (heating in Tris-EGTA; pH 9). Blocking was performed (10 minutes) in 2% BSA in TBS, and primary antibodies diluted in 1% BSA in TBS were applied overnight at 4°C. Secondary antibodies used were Alexa 555-, 488- or 647-conjugated donkey anti-IgG (1:200, Molecular Probes), and mounting medium contained DAPI (Vectorshield, Vector Labs). For morphological analyses, sections from fixed or snap-frozen gastrocnemius muscle were stained with HE or Sirius Red.

Microscopic examinations were performed using a Leica DMi4000B Cool Fluio Package instrument equipped with a Leica DFC340 FX Digital Camera. In all experiments, exposure (camera settings) and picture processing (brief adjustment of contrast/brightness and color balance by using Photoshop) were applied equally to sample sections and controls (isotype or no primary antibody present). The number of fibers was counted on pictures showing cross-sections of paraffin-embedded muscles at day 14 following lesion (n=5-7). Sections were taken from the site where m. gastrocnemius is broadest. Antibodies used were: mouse anti-myosin (1:200; Developmental Studies Hybridoma Bank), rabbit anti-mouse DLK1 (1:2000; in-house) (Bachmann et al., 1996), rat anti-CD45 (1:50, BD Pharmingen), rabbit anti-collagen I (1:100, Abcam, ab34710); goat anti-desmin (1:50, Santa Cruz Biotechnology), rat anti-laminin-2 (1:50, Abcam), mouse anti-actinin (1:200, Sigma-Aldrich), mouse anti-slow twitch myosin (1:200, Sigma-Aldrich) and mouse anti-myogenin (1:100, DAKO).

**Rotarod**

Twelve-week-old female Dlk1−/− (n=7; one animal died before testing) and Dlk1+/− (n=8) mice were pre-trained on an LE8200 Rotarod system (Panlab, Harvard Apparatus) at days −8 and −6 prior to muscle lesion. Pre-training comprising three sessions during which all animals were successfully trained to stay and run on the rod for one minute at a fixed speed of 24 rpm. Trials were performed during muscle regeneration at day −1, +3, +6 and +14 using a constant speed of 24 rpm and a maximum testing time of 5 minutes. Each trial was performed three times and animals rested for one hour in between. The latency to fall (seconds) from the rod was recorded automatically and all three scores per mouse per day were averaged and recorded as latency to fall (in seconds) for each mouse. Rotarod performance was also tested in adult Dlk1−/− and Dlk1+/− mice to examine muscle phenotype using the rotarod setup described above.

**Oil Red O staining and quantification**

Oil Red O staining was performed as recently described (Andersen et al., 2013). Differentiated cells were rinsed in PBS, fixed (30 minutes) in 10% NBF (VWR, Herlev, Denmark), carefully washed three times in TBS, and stored at 4°C until analysis. TBS was discarded, and cells were briefly exposed to 3% 2-propanol (1 second) and completely air-dried before incubation (1 hour) with filtered Oil Red O solution (Sigma-Aldrich, Europe) prepared according to the manufacturer’s recommendation. Stained cells were rinsed gently with water to remove any precipitates, and examined microscopically with a Leica DMi4000B Cool Fluio Package instrument (Leica Microsystems, Ballerup, Denmark) equipped with a Leica DFC340 FX Digital Camera. In all experiments, camera settings and picture processing were applied equally to all sample sections. Oil Red O
quantification was performed as follows: stained cells were allowed to dry, and Oil Red O was eluted by incubation (10 minutes) with 100% 2-propanol. Eluted staining solution was then transferred to a 96-well microtiter plate and the optical density at 510 nm was read in triplicate using an ELISA plate reader. Empty wells were withdrawn during the entire fixing, staining and quantification procedure, and used as blank controls. Oil Red O staining was performed in four independent biological experiments, each comprising triplicate wells on 12-well plates. Each of these wells was stained with Oil Red O and measured in technical triplicates. The overall mean was used for analysis. For Oil Red O staining of cryo-embedded tissue, sections were air-dried, fixed in 4% NBF, and otherwise processed as described above for cells.

**Coulter cell counting**

Cultured cells were gently detached with 0.25% trypsin-EDTA (Gibco), pelleted and re-suspended in Hank’s Balanced Salt Solution (HBSS, Lonza) + 10% FCS + 1% PS. Cell number and cell diameter were determined using a Beckman Coulter Counter Z2 (Ramcon) fitted with a 100 µm aperture. The size range of particles counted was set at 11–27 µm, and counting was performed in the indicated number of independent experiments each comprising triplicate measurements.

**Relative quantitative real-time PCR (qRT-PCR)**

Total RNA was extracted from cell cultures or muscle tissue using the semi-automated 6100 Nucleic Acid Prep Station system, according to manufacturer’s instructions (Applied Biosystems). For cDNA synthesis, 0.3–0.4 µg of total RNA was reverse transcribed with a High Capacity cDNA RT Kit (#4368813, Applied Biosystems), and qRT-PCR reactions were performed in technical triplicates using commercially available Taqman assays. The PCR was run on a 7900HT Fast Real-time PCR system (Applied Biosystems) and robust and valid qRT-PCR data were obtained by normalizing the raw data against multiple stably expressed endogenous control genes as determined by the qBase Plus platform (Hellemans et al., 2007; Vandesompele et al., 2002).

**Statistical analyses**

All analyses comprised at least three independent experiments. One- or two-way ANOVA (with or without repeated measure), and two-tailed t-tests were performed as indicated using GraphPad Prism to test significance (P<0.05).

**RESULTS**

**Dlk1-null mice are developmentally impaired in their muscle mass and function owing to inhibition of the myogenic program**

Our previous results have demonstrated DLK1 expression in skeletal muscle during development, regeneration and disease (Andersen et al., 2009; Floridon et al., 2000). However, the functional significance of DLK1 in skeletal muscle is not well understood. Herein, we therefore performed a detailed assessment of the muscle phenotype in Dlk1−/− adult mice. As previously reported by others (Moon et al., 2002), adult Dlk1-null mice were clearly growth retarded compared with gender- and age-matched wild-type controls. The body weights of adult Dlk1−/− female and male mice were significantly (P<0.0001) reduced to a similar extent (15.8% and 14.0%, respectively; mean; n=38–43) (Fig. 1A). Surprisingly, we found an even greater (34.8% and 31.9%; mean; n=30–34) decrease in the m. gastrocnemius mass in Dlk1−/− females and males, respectively, compared with their corresponding wild types (Fig. 1B). Thus, the m. gastrocnemius-to-body mass ratio was dramatically reduced (P<0.0001) in female (average 21.3%; n=30–34) and male (average 21.2%; n=30) Dlk1-null versus wild-type mice (Fig. 1C), suggesting that lack of DLK1 causes a dramatic inhibitory effect on muscle mass in adult mice. This diminished muscle mass was mirrored by a poor functional
performance on the rotarod apparatus; Dlk1<sup>+/−</sup> mice performed significantly better (24%) than Dlk1-null mice (Fig. 1D). As a higher body mass usually correlates with a shorter active rotation time (McFadyen et al., 2003), this figure might be an underestimation. No apparent difference was observed in muscle morphology between Dlk1<sup>−/−</sup> and Dlk1<sup>+/−</sup> mice, and no centrally located nuclei were observed, suggesting normal maturation of the myofibers (data not shown). It is known that Dlk1 is absent in normal adult skeletal muscle (Andersen et al., 2009). Thus, it seems likely that Dlk1 does not exert any effect at this adult stage, at least in a cell-autonomous manner, considering that soluble DLK1 protein variants produced and secreted by other cells may be present. Even so, we hypothesized that the presence of high Dlk1 levels described in embryonic mouse skeletal muscle (Andersen et al., 2009; Floridon et al., 2000; Yevtodiyenko and Schmidt, 2006) could account for the functional muscle phenotype we observed in Dlk1<sup>−/−</sup> adult muscle (Fig. 1A-E). To evaluate this, we first identified DLK1 expression in embryonic muscle anlage at the protein level using DLK1-PAX7 double immunofluorescence staining (Fig. 1E). We next performed a detailed molecular analysis of the myogenic gene expression program in dissected hindlimb buds from E11.5 Dlk1<sup>−/−</sup> and Dlk1<sup>+/−</sup> mice (Fig. 1F). Whereas Pax3, Pax7 and Myf5 levels were slightly higher in Dlk1<sup>−/−</sup> mice compared with wild type, we found a clear and significant suppression of the Mef2c, Meis1 and Myod1 genes (Fig. 1F). These three latter transcription factors are known to cooperate in order to activate the myogenic transcriptional program (Black and Olson, 1998). Our data thus support the conclusion that the observed muscle-retarded phenotype in Dlk1<sup>−/−</sup> mice is likely to be due to a restrained myogenic differentiation program in muscle progenitors during skeletal muscle development. Notably, we also observed a lower expression in Dlk1-null limb buds of genes associated with mesenchyme, whereas a tendency for higher amounts of some vascular transcripts was seen (Fig. 1F), which is in agreement with the known involvement of Dlk1 in bone and vascular morphogenesis (Abdallah et al., 2011; Chen et al., 2011; Rodríguez et al., 2012).

**Dlk1-depleted heterogeneous muscle-derived cell cultures exhibit an enhanced myogenic phenotype**

Although Dlk1 is downregulated after birth and its expression is almost abolished in adult skeletal muscle, it is re-expressed during muscle regeneration (Fig. 2A) (Andersen et al., 2009). We have shown that Dlk1 expression peaks at day 7 following a knife-cut lesion in m. gastrocnemius (Andersen et al., 2009), yet the role of this high Dlk1 induction during muscle regeneration remains to be determined. In agreement with our previous results and those of others (Waddell et al., 2010), we found that DLK1 localizes mainly to interstitial cells (outside laminin<sup>+</sup> myofibers) (Fig. 2A) of a non-myogenic cell lineage (Andersen et al., 2009) during muscle regeneration. To elucidate biological effects of Dlk1 in muscle regeneration, we established heterogeneous cultures of proliferating and differentiating muscle-derived mononuclear cells (MDCs; containing muscle progenitors and interstitial cells), isolated from day 7 post-lesion Dlk1<sup>−/−</sup> and Dlk1<sup>+/−</sup> m. gastrocnemius (Fig. 2B). Flow cytometry of proliferating Dlk1<sup>+/−</sup> MDC cultures revealed that 16.6±2.1% (mean±s.d.; n=3) of the MDCs expressed DLK1 (Fig. 2C) but, as shown by a 52% reduction in Dlk1 mRNA levels and supported at the protein level by immunocytochemistry, Dlk1 expression decreased with *in vitro* myogenic differentiation, as expected (Fig. 2B,D). Neither Dlk1 mRNA nor protein was detected in Dlk1<sup>−/−</sup> muscle tissue and MDC cultures (Fig. 2A-D), thus verifying our experimental setup. As seen during muscle embryogenesis, we found several important myogenic transcription factors, as well as structural proteins, to be regulated by Dlk1 (Fig. 2E). However, most unexpectedly, the expression pattern was inverse to that observed during muscle development (Fig. 1). Thus, Dlk1<sup>−/−</sup> MDCs, compared with Dlk1<sup>+/−</sup> MDCs, expressed significantly higher levels of the myogenic transcription factors Pax3, Pax7 and Myf5, and showed a tendency to increased expression of Mef2c, Myod1 and Myog (Fig. 2E). A similar pattern of enhanced myogenesis in Dlk1<sup>−/−</sup> MDC cultures was seen for the major structural muscle proteins Myh7, Tnnt2 and Des. By contrast, Ncam1 and Cdx3, two genes also often associated with myogenic precursors (Zammit et al., 2006), but also with other cells, seemed not to be affected by Dlk1 (Fig. 2E). These results thus suggested that Dlk1 exerts an unexpected inhibitory role on the myogenic differentiation program of adult muscle progenitors *in vitro*. We also noticed an increased level of proliferating cell nuclear antigen (Pena) in Dlk1-depleted MDCs (Fig. 2F), which is in agreement with a recent study showing growth inhibition of DLK1-overexpressing myogenic cells (Waddell et al., 2010). Visualization of myofibers (Fig. 2G) in the Dlk1<sup>−/−</sup> and Dlk1<sup>+/−</sup> MDCs supported this conclusion, as Dlk1<sup>+/−</sup> myofibers appeared more mature and larger than those present in Dlk1<sup>−/−</sup> cultures; this was quantitatively confirmed by an increased expression level of the structural muscle protein myosin (Fig. 2H). These results thus indicate that Dlk1, at least *in vitro*, plays a regulatory role during adult muscle remodeling in which depletion of Dlk1, unexpectedly, promotes a myogenic program.

Additionally, we found a clear and significant downregulation of vascular genes (supplementary material Fig. S1B) as well as a modest increase in some mesenchymal genes, whereas others remained unchanged (supplementary material Fig. S1C). Dlk1 may thus play a dual regulatory role, increasing vascular but decreasing myogenic commitment during adult muscle regeneration, whereas mesenchymal remodeling seems to be unaffected.

**In vivo muscle regeneration is improved in Dlk1-deficient animals**

To elucidate whether our findings demonstrate a biological mechanism during *in vivo* adult muscle regeneration, we performed a detailed series of knife-cut lesions in Dlk1<sup>−/−</sup> and Dlk1<sup>+/−</sup> gastrocnemius muscles. Because no difference was observed in the muscle phenotype between genders (Fig. 1), we designed our experimental setup to comprise high biological diversity with two females and two males (*n=4*) of each strain and analyzed muscle regeneration during 14 days.

Similarly to previous observations (Andersen et al., 2009), we observed that Dlk1 mRNA increased in Dlk1<sup>−/−</sup> gastrocnemius at day 4 post-lesion, peaked at day 7 and, except for a small peak at day 10, it thereafter declined to reach basal levels at day 14 (Fig. 3A). To evaluate the overall regeneration process in Dlk1<sup>−/−</sup> and Dlk1<sup>+/−</sup> lesioned muscle, we examined Hematoxylin and Eosin (HE), collagen/CAD45-, DLK1/myosin- and Sirius Red-stained sections and performed various qRT-PCRs. In both strains, numerous degrading myofibers (supplementary material Fig. S2A), as well as an influx of inflammatory CD45<sup>+</sup> cells (Fig. 3B), could be seen at day 2 post-lesion, reflecting an initial phase of muscle degeneration and inflammation, a result that was confirmed quantitatively by qRT-PCR for caspase 3 (pre-apoptotic marker) and Cad45 (Ptpcr), Thy1, Cd14 and Cd68 (hematopoietic markers) (Fig. 3C). In parallel, a significant increase was found for...
proliferating cell nuclear antigen (Pcna) (Fig. 3C), suggesting activation of muscle-residing cells, which is in agreement with observations by others (Yan et al., 2003). At day 7 following lesion, numerous interstitial DLK1+/CD31– (Fig. 3A), DLK1+/Myosin– and DLK1+/actinin–/laminin– cells (supplementary material Fig. S2B) were observed exclusively in the regenerating zone of Dlk1+/+ muscle, though many interstitial cells were seen in Dlk1−/− sections as well (Fig. 2A; supplementary material Fig. S2B). The regenerative phase was confirmed by the presence of many newly formed actinin+ and myosin+ myofibers with centrally localized nuclei (supplementary material Fig. S2B). At day 14, the overall muscle architecture was re-established in Dlk1−/− and Dlk1+/+ muscles (Fig. 3A,D; supplementary material Fig. S2A). Total collagen deposition as shown by Sirius Red staining at days 0-14 was equally distributed in samples from both strains (Fig. 3D; supplementary material Fig. S2C), and except for a modest increase (days 2-4) in collagen I mRNA levels in Dlk1−/− muscles, testing of 13 genes associated with mesenchyme and fibrosis did not reveal any difference between Dlk1−/− and Dlk1+/+ animals (data not shown). These findings thus revealed that Dlk1−/− animals fully accomplish skeletal muscle regeneration with no overall change in inflammatory response and fibrotic scarring.

To investigate quantitatively whether Dlk1 depletion altered the myogenic capacity during skeletal muscle regeneration, we performed a detailed qRT-PCR study of myogenic genes in Dlk1−/− and Dlk1+/+ muscles at days 0-14 post-lesion (Fig. 3E). Similar to the in vitro situation described above (Fig. 2), we found important myogenic transcription factors, as well as structural muscle proteins,
to be regulated in vivo by Dlk1, in an inverse pattern to that observed during development (Fig. 1). As such, there were substantially increased amounts of the myogenic transcriptional regulators Mef2c and Meis1, but also of Pax7, in Dlk1-depleted animals, whereas no overall difference was seen for Myod1, Myf5, myogenin or myostatin (Fig. 3E). Likewise, mRNA corresponding to the structural myogenic proteins Des, Myh1 and Myh7 were all highly increased in Dlk1−/− animals compared with Dlk1+/+ controls, whereas genes corresponding to Myh2, Tnnt2, Ncam1 and Cd34 were unaffected (Fig. 3E). It has recently been shown that lack of Dlk1 promotes the self-renewing state of PAX7+ myogenic precursor cells (Waddell et al., 2010). We therefore determined whether Dlk1-null animals had a larger reservoir of myogenic precursors available for postnatal muscle formation. Indeed, we did find higher basal levels of Pax3, Pax7 and Myf5 in non-lesioned Dlk1−/− adult gastrocnemius muscles (Fig. 3F), indicating increased
satellite cell numbers, which might explain the enhanced myogenic differentiation seen in DlkI-null muscles. Taken together, these results point to a novel, and surprising, biological function of DlkI as an inhibitor of adult skeletal muscle regeneration. To test this result functionally, we examined rotarod performance during the regeneration process, and found that DlkI−/− animals increased their ability to run, whereas DlkI+/− animals performed constantly (Fig. 3G). Furthermore, we quantified the number of myofibers 14 days after regeneration, and found DlkI−/− animals to comprise fewer myofibers per area compared with DlkI+/− controls (Fig. 3H), suggesting hypertrophy of DlkI-depleted muscles. This result clearly contradicts our previous data showing muscle hypertrophy in the Callipyge sheep and in transgenic mice that overexpress membrane-bound DLK1 (Davis et al., 2004). We did not, however, find any centrally located nuclei in neither DlkI−/− nor DlkI−/−-isoform cultures, and this result was confirmed in paraffin-embedded DlkI−/− and DlkI+/− muscle sections (Fig. 3H) and in DlkI−/− animals showing degenerative fibers (Davis et al., 2004). We did not, however, find any centrally located nuclei in either DlkI−/− or DlkI−/−-isoform cultures, and this result was confirmed in paraffin-embedded DlkI−/− and DlkI+/− muscle sections (Fig. 3H) and in DlkI−/− animals showing degenerative fibers (Davis et al., 2004). We did not, however, find any centrally located nuclei in either DlkI−/− or DlkI−/−-isoform cultures, and this result was confirmed in paraffin-embedded DlkI−/− and DlkI+/− muscle sections (Fig. 3H) and in DlkI−/− animals showing degenerative fibers (Davis et al., 2004). We did not, however, find any centrally located nuclei in either DlkI−/− or DlkI−/−-isoform cultures, and this result was confirmed in paraffin-embedded DlkI−/− and DlkI+/− muscle sections (Fig. 3H) and in DlkI−/− animals showing degenerative fibers (Davis et al., 2004).

**Dlk1-null mice display substantial levels of the adipokine adiponectin during skeletal muscle regeneration without concomitant regulation of adipogenic conversion**

Whereas the function of DLK1 in muscle is under intense investigation, its role as an important regulator of adipogenic differentiation is well established (Moon et al., 2002). Because adipose deposition can be seen in skeletal muscle disease, probably as a result of pathological differentiation of myogenic-residing precursors into adipocytes (Vettor et al., 2009), we speculated whether DlkI regulates adipogenic commitment of muscle-residing progenitors. Indeed, we did observe a substantial increase in adipokine adiponectin during skeletal muscle regeneration (Garcès et al., 1999), the exact local level of DlkI appears to be of great importance for the specific myogenic outcome and this might explain why several contradictions exist within others’ and our own data on DlkI in muscle.
remodeling. Although Bmp7 levels were increased in Dlk1−/− compared with Dlk1+/− animals more or less throughout the regeneration period, no overall changes were observed in Pparg, Cehpd, Cidea and Ppargc1a gene expression (Fig. 5G). However, Prdm16, a factor recently shown to promote brown adipogenesis over myogenesis from a common (brown adipogenic/myogenic) progenitor (Yin et al., 2013), was lower in over myogenesis from a common (brown adipogenic/myogenic) stage is, so far, known to recapitulate its fetal myogenic program upon damage (Zammit et al., 2006). Indeed, we did find a temporary re-expression of the myogenic gene program during muscle regeneration, but this was substantially enhanced in Dlk1−/− mice, suggesting that Dlk1 might act as a brake on adult muscle regeneration. This result is contrary to some of the results reported by Waddell et al. (Waddell et al., 2010). However, as Dlk1 is foremost expressed in non-myogenic cells, the study by Waddell et al. only achieved a modest 33% reduction in muscle Dlk1 using their Myf5-dependent Dlk1 knockdown strategy, whereas we used mice with complete absence of Dlk1. It is known that changes in the levels of Dlk1 might affect the differentiation outcome (Bauer et al., 1998; Nueda et al., 2007) and the relative large amount of muscle-residing Dlk1 remaining in the Waddell et al. study is thus likely to have a rather large effect. In support of that, we found that low amounts of DLK1 enhanced the levels of the early myogenic transcription factor Myod1, whereas higher DLK1 levels had the opposite effect. Furthermore, we used a stab lesion model in m. gastrocnemius whereas Waddell et al. (Waddell et al., 2010) performed cardiotoxin-induced lesion of the tibialis anterior muscle, two parameters (type of muscle and lesion) that are known to have a large impact on the regeneration scheme and might explain some of the differences observed for the role of Dlk1 in muscle regeneration. Alternatively, different functions of Dlk1 protein variants have been described (Ferrón et al., 2011; Garcés et al.,

DISCUSSION
We, and others, have shown numerous data consolidating a role for Dlk1 in developing, regenerating and diseased muscle, yet the function of this gene in muscle biology remains to be elucidated. One recent study (Waddell et al., 2010) in which Dlk1 was depleted in the myogenic cell lineage (using a Myf5-Cre mouse) suggests that Dlk1 is necessary for and promotes proper muscle regeneration. However, our results imply a far more complex role for Dlk1 in skeletal muscle, as a novel myogenic factor that, on the one hand, promotes muscle development but, on the other hand, inhibits muscle regeneration. To our knowledge, such a dual role of a protein has never been reported for skeletal muscle, a tissue that in its adult stage is, so far, known to recapitulate its fetal myogenic program upon damage (Zammit et al., 2006). Indeed, we did find a temporary re-expression of the myogenic gene program during muscle regeneration, but this was substantially enhanced in Dlk1−/− mice, suggesting that Dlk1 might act as a brake on adult muscle regeneration. This result is contrary to some of the results reported by Waddell et al. (Waddell et al., 2010). However, as Dlk1 is foremost expressed in non-myogenic cells, the study by Waddell et al. only achieved a modest 33% reduction in muscle Dlk1 using their Myf5-dependent Dlk1 knockdown strategy, whereas we used mice with complete absence of Dlk1. It is known that changes in the levels of Dlk1 might affect the differentiation outcome (Bauer et al., 1998; Nueda et al., 2007) and the relative large amount of muscle-residing Dlk1 remaining in the Waddell et al. study is thus likely to have a rather large effect. In support of that, we found that low amounts of DLK1 enhanced the levels of the early myogenic transcription factor Myod1, whereas higher DLK1 levels had the opposite effect. Furthermore, we used a stab lesion model in m. gastrocnemius whereas Waddell et al. (Waddell et al., 2010) performed cardiotoxin-induced lesion of the tibialis anterior muscle, two parameters (type of muscle and lesion) that are known to have a large impact on the regeneration scheme and might explain some of the differences observed for the role of Dlk1 in muscle regeneration. Alternatively, different functions of Dlk1 protein variants have been described (Ferrón et al., 2011; Garcés et al.,
1999) and the proper equilibrium of expression among them might affect the outcome. In this sense, it is unclear how the knockdown strategy used by Waddell et al. affected this equilibrium. In relation to this, we found that only the soluble form of DLK1 was sufficient for myosin enhancement, whereas Myod1 levels depended on the exact level of DLK1. This clearly points to a complex system in which fine-tuning of DLK1 may have a large impact on the myogenic outcome, and which might explain the contradictions regarding Dlk1 in muscle that have been reported herein and by others (Waddell et al., 2010). Finally, Dlk1 is expressed by both the satellite-/muscle cell itself and interstitial cells depending on the developmental stage, and secreted protein variants are also expressed by other more distant cells. Thus, DLK1 might act upon the muscle cell by direct cell-cell contact or indirectly by a paracrine or exocrine mechanism. In agreement with that hypothesis, our data show that during development Dlk1 is expressed in the muscle cells where it seems to promote myogenesis, a scenario that is similar to that observed by Waddell et al. This is further supported by previous studies showing that overexpression of the ovine Dlk1 in mouse muscle cells dramatically stimulates myogenesis (Davis et al., 2004). Collectively, the combined data indicate dual functions for cell-autonomous and non-cell-autonomous DLK1 activity in skeletal muscle, a scenario also demonstrated for NOTCH1 (Becam et al., 2010). In skeletal muscle, NOTCH1 activation is necessary for the specification of myogenic stem cells and maintenance of the transit-amplifying state whereas the decay of NOTCH signaling is

Fig. 5. Dlk1 exerts no effect on the in vitro and in vivo adipogenic potential of muscle-residing cells. (A,B) Relative qRT-PCR revealed that the expression of adiponectin was significantly higher in Dlk1−/− MDCs (both proliferating (P) and differentiating (D)) compared with Dlk1+/+ controls (A) as well as in Dlk1−/− versus Dlk1+/+ regenerating adult mouse gastrocnemius muscle (B). (C) Phases microscopic of cultured Dlk1−/− and Dlk1+/+ MDCs during a 10-day adipogenic induction scheme. (D) qRT-PCR of several white and brown adipose tissue-related genes in Dlk1−/− and Dlk1+/+ MDCs at day 0 and 10 of in vitro adipogenesis. (E,F) Oil Red O staining (E) and quantification (F) of Dlk1−/− and Dlk1+/+ MDCs at confluence (day 0) and after 10 days of adipogenic induction. (G) qRT-PCR of white and brown adipose tissue-related genes during in vivo regeneration in Dlk1−/− and Dlk1+/+ animals. (H) Oil Red O staining of muscle at days 2, 7 and 14 of regeneration. In agreement with the expression profiles shown in G, deposition of lipids did not differ between genotypes. Error bars indicate s.d. Statistical significance was tested by a two-way ANOVA (*P<0.05, **P<0.01, ***P<0.001, ****P<0.0001). For qRT-PCR, raw data were normalized against (A) 18s and Tbp (qBase-M: 0.258; CV 0.089), (D) Gapdh, Actb and Rpl13a (qBase-M: 1.453; CV 0.467), and (G) Gapdh, Pgk1 and Tbp (qBase-M: 0.354; CV 0.149). Scale bar: 500 μm.
a prerequisite for fusion and terminal differentiation of myoblasts (Gildor et al., 2012; Mourikis et al., 2012). As such, constitutive NOTCH1 activation in Myf5-derived cells leads to a complete absence of skeletal muscle in Myf5{\textsuperscript{Cre-}:R26R{\textsuperscript{loxP-Notch-DLK1-floxed}} embryos and a dramatic increase in Pax7\textsuperscript{-}/Myf5\textsuperscript{-}/Myog myogenic stem/progenitor cells (Mourikis et al., 2012). Strikingly in this regard, we observed that 

**References**


DLK1, a regulator of muscle


