Flt3L is a novel regulator of skeletal myogenesis

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SUMMARY

Various cues initiate multiple signaling pathways to regulate the highly coordinated process of skeletal myogenesis. Myoblast differentiation comprises a series of ordered events starting with cell cycle withdrawal and ending with myocyte fusion, each step likely controlled by multiple extracellular signals and intracellular signaling pathways. Here we report the identification of Flt3L (Fms-like tyrosine kinase 3 ligand) signaling as a novel regulator of skeletal myogenesis. Flt3L is a multifunctional cytokine in immune cells, but its involvement in skeletal muscle formation has not been reported. We find that Flt3L is expressed in C2C12 myoblasts, with increasing levels during differentiation. Knockdown of Flt3L, or its receptor Flt3, suppresses myoblast differentiation, which is rescued by recombinant Flt3L or Flt3, respectively. Differentiation is not rescued, however, by recombinant ligand when the receptor is knocked down, or vice versa, suggesting that Flt3L and Flt3 function together. Flt3L knockdown also inhibits differentiation in mouse primary myoblasts. Both Flt3L and Flt3 are highly expressed in nascent myofibers during muscle regeneration in vivo, and Flt3L siRNA impairs muscle regeneration, validating the physiological significance of Flt3L function in myogenesis. Providing a cellular mechanism for the myogenic function of Flt3L, we show that Flt3L promotes cell cycle exit that is necessary for myogenic differentiation. Furthermore, we identify Erk as a relevant target of Flt3L signaling during myogenesis, and demonstrate that Flt3L suppresses Erk signaling through p120RasGAP. In summary, our work reveals an unexpected role for an immunoregulatory cytokine in skeletal myogenesis and a new myogenic pathway.
INTRODUCTION

Skeletal myogenesis is guided by various environmental cues and regulated by distinct signaling pathways, resulting in the activation of myogenic gene expression program via specific transcription factors, in particular those in the MyoD and MEF2 families (Lassar and Munsterberg, 1994; Naya and Olson, 1999; Perry and Rudnicki, 2000; Weintraub, 1993). During embryonic myogenesis, cells in somites undergo myogenic commitment and pass along the myogenic pathway by terminal differentiation and fusion to form multinucleated myofibers (Buckingham, 2001). In adult muscles upon injury, satellite cells become activated and differentiate into myoblasts, which then fuse to form new myofibers or repair existing ones (Montarras et al., 2005). Myogenesis in vivo can be largely recapitulated by differentiation of cultured myoblasts, which follows a series of ordered steps, including cell cycle withdrawal, myogenic protein expression, cell elongation, migration, and fusion to form myotubes.

While satellite cell proliferation is necessary for muscle regeneration and repair, cell cycle withdrawal is a prerequisite for myogenic differentiation. Regulation of the cell cycle is intimately connected to the myogenic gene expression program at the molecular level (Molkentin and Olson, 1996). For instance, MyoD governs both cell cycle withdrawal and myogenic gene expression, partly through its synergistic interaction with Rb (De Falco et al., 2006), a master regulator of the cell cycle progression. MyoD-mediated induction of the G1 cell cycle inhibitor p21 is also necessary for myogenic differentiation (Guo et al., 1995; Halevy et al., 1995). Removal of growth factors induces cell cycle exit in myoblast cultures, but intrinsic proliferation signals, such as those from the insulin-like growth factors (IGFs) that have dual mitogenic and myogenic functions (Florini et al., 1996), need to be suppressed in order for the myogenic program to progress. One of the downstream mediators of mitogenic receptor signaling is the MAP kinase Erk, which promotes G1 cell cycle progression through induction of cyclin D1 expression (Lavoie et al., 1996) among other mechanisms. Consistent with its ability to prevent cell cycle exit, Erk is reported to suppress the early stage of myogenic differentiation (Bennett and Tonks, 1997; Coolican et al., 1997; Wu et al., 2000), and inhibition of Erk signaling enhances differentiation (Coolican et al., 1997). It has been suggested that the engagement of ephrin and the Eph receptors upon cell-cell contact facilitates myogenic differentiation via inhibition of Erk signaling (Minami et al., 2011). It is likely that other signals/pathways also contribute to ensuring cell cycle withdrawal and myogenic differentiation.
Flt3L (Fms-like tyrokinine kinase 3 ligand) is a hematopoietic growth factor with well-established functions in hematopoietic cell development, such as stimulating the proliferation of hematopoietic progenitors (Lyman et al., 1993), promoting lymphoid lineage commitment (Adolfsson et al., 2001), and driving dendritic cell development (Waskow et al., 2008). In hematopoietic cells Flt3L binds to and signals through Flt3, a receptor tyrosine kinase (RTK) structurally related to PDGF receptor, CSF1 receptor, and c-Kit (Lyman and Jacobsen, 1998; Lyman et al., 1993). Downstream signaling activated by Flt3L/Flt3 engagement includes PI3K/Akt and Erk pathways, mediating cell survival and proliferation, respectively (Masson and Ronnstrand, 2009). Flt3 is also one of the most frequently mutated genes in acute myeloid leukemia (AML) and a promising therapeutic target (Kindler et al., 2010).

Several cytokines traditionally involved in inflammatory responses have been found to function in skeletal myogenesis in an autocrine manner that is independent of their immunoregulatory roles, including interleukin-4 (IL-4) (Horsley et al., 2003), IL-6 (Baeza-Raja and Munoz-Canoves, 2004; Okazaki et al., 1996), and others. Neither Flt3L nor Flt3 has been reported to express or function in skeletal muscles. In the current study we have identified a critical role of Flt3L/Flt3 in myoblast differentiation. Our results further reveal that, contrary to its mitogenic function in immune cells, this ligand-receptor pair regulates myogenesis by promoting cell cycle exit through inhibition of Erk, a novel mode of Flt3L/Flt3 signaling.
RESULTS

Flt3L is required for myoblast differentiation.

To identify novel regulators of myoblast differentiation, we performed an RNAi screen in the C2C12 myoblast cell line to examine a selected group of cytokines with previously unknown expression pattern or function in skeletal muscles. Flt3L emerged from that screen as a potential regulator (our unpublished studies). We observed that the expression of Flt3L, at the mRNA levels and more drastically at the secreted protein levels, increased during the course of C2C12 differentiation (Fig. 1A). Two independent shRNAs were delivered by lentiviral transduction into C2C12 cells, and resulted in significant knockdown of Flt3L at both mRNA and protein levels (Fig. 1B). Myotube formation was impaired in Flt3L knockdown cells, with significantly reduced fusion index (Fig. 1C). Furthermore, we found that Flt3L knockdown decreased sarcomeric MHC expression, and delayed the onset of myogenin expression (at day 1 differentiation) (Fig. 1D). This effect on the early myogenic marker myogenin suggested that Flt3L might function at an early step during differentiation.

To ascertain Flt3L’s role in myoblast differentiation, we examined the effect of adding recombinant Flt3L protein to the differentiation medium. Exogenous Flt3L enhanced differentiation of C2C12 cells and also rescued differentiation from the inhibitory effect of Flt3L knockdown, as shown by both myotube formation (Fig. 1E) and myogenic marker expression (Fig. 1F). These results further confirmed the specificity of RNAi targeting. Depletion of Flt3L also impaired differentiation of mouse primary myoblasts (Fig. 1G), validating the physiological relevance of the observations made in C2C12 cultures. Taken together, our observations provide strong evidence for a positive function of Flt3L in myoblast differentiation.

Flt3L is involved in muscle regeneration in vivo.

To gain insight into the function of Flt3L in vivo, we employed a skeletal muscle regeneration model in which muscle necrosis induced by barium chloride (BaCl₂) was followed by a well-defined process of regeneration (Ge et al., 2009). When RNA was extracted from injected tibialis anterior (TA) muscles and subjected to qPCR analysis, Flt3L mRNA level was found to increase modestly on Day 5 post-injury and return to the basal level by Day 7 (Fig. 2A). Because there could be multiple sources of Flt3L mRNA in injured tissues, to better assess Flt3L expression in muscles we performed immunohistochemistry on sections of regenerating TA
muscles. As shown in Fig. 2B, strong Flt3L staining was observed in monocleated cells at the injury site, especially on Day 5 after injury, which might represent proliferating muscle cells or infiltrating immune cells, or both. More importantly, there was pronounced Flt3L expression in newly formed myofibers (identified by their centrally localized nuclei) on Day 5 and 7, which was not in the neighboring uninjured myofibers. This expression pattern would be consistent with a role for Flt3L in an early step of myogenesis. To determine Flt3L function in regeneration, we injected siRNA oligos against Flt3L together with BaCl2 into TA muscles. Knockdown efficiency of the siRNA assessed by mRNA levels was modest (Fig. 2C), perhaps partly due to the fact that the RNA was isolated from total TA muscle and not only the injected portion. Nevertheless, the Flt3L siRNA impaired muscle regeneration (Fig. 2D), resulting in smaller and fewer regenerating myofibers compared to control oligo-injected muscles (Fig. 2E). The collective evidence suggests that Flt3L is a positive regulator of skeletal myogenesis both in vitro and in vivo.

**Flt3, receptor of Flt3L, positively regulates myoblast differentiation.**

In hematopoietic cells, Flt3L is known to signal through its cognate receptor Flt3 (Lyman and Jacobsen, 1998; Lyman et al., 1993). We wondered whether Flt3L functioned in muscle cells via Flt3 in an autocrine fashion. Interestingly, in regenerating muscle, Flt3 mRNA was acutely induced during the early stage of regeneration (Days 3-5 after injury) and returned to low levels by day 14 after injury (Fig. 3A). Immunohistochemistry revealed that the Flt3 protein was highly expressed in regenerating myofibers on Day 5 and Day 7 (Fig. 3B). This expression pattern was suggestive of a functional role for Flt3 during myogenesis, consistent with the function of Flt3L.

To examine Flt3 function in myoblast differentiation, we knocked down Flt3 using two independent lentivirus-delivered shRNAs (Fig. 3C). As shown in Fig. 3D, myotube formation was significantly impaired by Flt3 knockdown. In addition, MHC and myogenin expression were also reduced by the knockdown (Fig. 3E). Conversely, when recombinant Flt3 was overexpressed in C2C12 cells through retroviral delivery, differentiation was enhanced (Fig. 3F&G). The endogenous Flt3 protein was not reliably detected by available antibodies (see Fig. 3G), possibly due to either low expression level or weak antibodies, or the combination of both. Nevertheless, the collective evidence presented here strongly suggests an important role for Flt3 in myogenic differentiation.
Furthermore, we asked whether Flt3L and Flt3 functioned inter-dependently in myogenesis. As shown in Fig. 4A, recombinant Flt3L supplied in the cell medium enhanced differentiation, but this effect was abolished when the receptor was knocked down. Conversely, upon Flt3L depletion, the effect of Flt3 overexpression was completely lost (Fig. 4B). These observations strongly support the notion that Flt3L functions through Flt3 to regulate myogenic differentiation.

**Flt3L and Flt3 support differentiation through promoting cell cycle withdrawal.**

In search of a mechanism by which Flt3L may regulate myogenesis, we noticed a higher cell number by the end of differentiation in Flt3L knockdown cells compared to control cells (e.g., see DAPI staining in Fig. 1C), even though the cell number at the start of differentiation was equal. This could be a result of either an increase in proliferation or a decrease in cell death. Whereas TUNEL assay did not reveal any difference in apoptosis (data not shown), a higher number of BrdU-labeled cells were observed upon Flt3L as well as Flt3 knockdown (Fig. 5A). This suggested that depletion of Flt3L/Flt3 might have prevented cell cycle exit, a prerequisite for differentiation. Indeed, the level of p21, a cell cycle inhibitor known to be required for myogenic differentiation (Guo et al., 1995; Halevy et al., 1995), was decreased in Flt3L and Flt3 knockdown cells (Fig. 5B). Importantly, recombinant Flt3L reduced proliferation (Fig. 5C) and elevated p21 levels (Fig. 5D) in control cells, and restored normal levels of proliferation and p21 expression in Flt3L knockdown cells (Fig. 5C&D). The effect of recombinant Flt3L on cell cycle exit was only observed in differentiating myocytes (in the absence of growth factors) and not in proliferating myoblasts (data not shown).

If promoting cell cycle exit was the main mechanism underlying Flt3L’s myogenic function, one would expect that blocking the cell cycle by an independent means would promote differentiation in Flt3L knockdown cells. To test this, we applied the cell cycle inhibitor Ara-C to differentiating cells. As shown in Fig. 5E, Ara-C treatment rescued differentiation from Flt3L knockdown. Despite toxicity of the drug, which resulted in significantly reduced cell number, the fusion index was brought back to control levels in Flt3L knockdown cells by Ara-C (Fig. 5E). Taken together, our observations suggest that Flt3L most likely regulates myogenic differentiation through promoting cell cycle withdrawal. This anti-proliferative action of
Flt3L/Flt3 during myogenesis is in contrast to its well-known mitogenic effect on immune cells (Lyman et al., 1993; McKenna et al., 2000).

**Flt3L controls cell proliferation through suppressing Erk.**

In search for a molecular pathway that mediates Flt3L’s myogenic and anti-proliferative function, we considered Erk signaling, which had been reported to suppress the early stage of myogenic differentiation (Bennett and Tonks, 1997; Coolican et al., 1997; Wu et al., 2000). Indeed, we observed moderately, but consistently, elevated Erk phosphorylation levels upon Flt3L knockdown in C2C12 cells as well as primary myoblasts (Fig. 6A), and a similar increase in phosphorylation of MEK, the upstream activator of Erk (Fig. 6B). More importantly, inhibition of Erk activation by the MEK inhibitor U0126 was sufficient to suppress proliferation in Flt3L knockdown C2C12 cells as measured by BrdU labeling (Fig. 6C). U0126 also fully restored differentiation from Flt3L knockdown in both C2C12 cells and primary myoblasts (Fig. 6D).

Furthermore, knockdown of either Erk1 or Erk2 faithfully phenocopied U0126 effect and fully rescued differentiation from Flt3L knockdown, as demonstrated by both myogenic marker expression (Fig. 6E) and myotube formation (Fig. 6F). Taken together, these results strongly suggest that Flt3L/Flt3 promotes cell cycle exit, and subsequent myogenic differentiation, by inhibiting Erk signaling.

**p120RasGAP mediates Flt3L suppression of Erk signaling during differentiation.**

Our observation of Flt3L inhibiting Erk was unexpected, as Flt3L activates Erk in immune cells (Masson and Ronnstrand, 2009). However, it has been reported that some RTKs, such as Eph receptor and PDGF receptor, can engage p120RasGAP and subsequently inhibit Ras-Erk signaling (Ekman et al., 1999; Elowe et al., 2001). Incidentally, p120RasGAP has been reported to promote myogenic differentiation (Minami et al., 2011). Hence, we set out to examine the possibility that Flt3L might inhibit Erk through p120RasGAP in skeletal myocytes. First, knockdown of p120RasGAP (Fig. 7A) impaired myotube formation (Fig. 7B), consistent with reported observations (Minami et al., 2011). It was also evident that by the end of 3-day differentiation, the total nuclei number had increased in p120RasGAP knockdown cells compared to control cells (Fig. 7B), even though the cell number was equal at the start of
differentiation. Indeed, BrdU labeling revealed that cell proliferation was increased by p120RasGAP knockdown (Fig. 7C). Both the differentiation and proliferation effects elicited by p120RasGAP knockdown recapitulated the effects of Flt3L or Flt3 knockdown. Significantly, in p120RasGAP knockdown myocytes, recombinant Flt3L no longer had any effect on proliferation (Fig. 7C) or differentiation (Fig. 7B). These results provide strong evidence that Flt3L promotes cell cycle exit and differentiation through p120RasGAP.

Consistent with the notion that p120RasGAP mediates Flt3L inhibition of Erk, we found elevated pErk levels in p120RasGAP knockdown myocytes (Fig. 8A). To further confirm that p120RasGAP regulates differentiation through Erk, we knocked down p120RasGAP and treated the cells with U0126. As shown in Fig. 8B, U0126 rescued differentiation from the negative impact of p120RasGAP knockdown, suggesting that Erk mediates p120RasGAP function during differentiation.

Taken together, we propose that at the onset of myoblast differentiation, Flt3L stimulates its receptor Flt3 in an autocrine fashion, activates p120RasGAP, and subsequently suppresses Ras-Erk signaling, which leads to cell cycle withdrawal that is essential for myogenic differentiation (Fig. 8C).
DISCUSSION

We have identified Flt3L as a myocyte-secreted cytokine necessary for myoblast differentiation and muscle regeneration. Previous reports of Flt3L function are exclusively in the immune system, where Flt3L and its receptor Flt3 are well-established regulators of survival and proliferation of hematopoietic stem cells and progenitor cells (Lyman and Jacobsen, 1998). Our findings of Flt3L and Flt3 expression in differentiating myoblasts and regenerating muscles, and their roles in myogenic differentiation, reveal unexpected functionality of this ligand-receptor signaling. The cellular mechanism by which Flt3L/Flt3 supports differentiation is also surprising. In contrast to their mitogenic functions in immune cells (Lyman et al., 1993; McKenna et al., 2000), Flt3L and Flt3 promote cell cycle exit that is necessary for myogenic differentiation. We have further demonstrated that Flt3L/Flt3 inhibits proliferation in differentiating myoblasts by suppressing Erk signaling, again in stark contrast to the reported activation of Erk by Flt3L in hematopoietic cells (Masson and Ronnstrand, 2009). While Flt3L/Flt3 activation of Erk signaling in immune cells is through SHP2 recruitment to the receptor (Heiss et al., 2006), inhibition of Erk in muscle cells by the same ligand-receptor pair is mediated by p120RasGAP, a mechanism similar to that employed by several other RTKs, such as the Eph receptor and PDGF receptor (Ekman et al., 1999; Elowe et al., 2001).

Suppression of mitogenic Erk signaling is necessary for the initial progression of the myogenic differentiation program (Bennett and Tonks, 1997; Coolican et al., 1997; Wu et al., 2000). Although growth factor deprivation at the induction of differentiation removes mitogenic signals, autocrine factors such as insulin-like growth factors (IGFs) have dual myogenic and mitogenic functions in myoblasts (Florini et al., 1996). We propose that autocrine Flt3L signaling plays an essential role in antagonizing the mitogenic signals specifically during myogenic differentiation. Consistent with this stage-specific role of Flt3L, the anti-mitogenic effects of Flt3L, revealed by both gene depletion and addition of exogenous Flt3L, are only observed upon serum withdrawal and not in myoblasts cultured in growth medium (our unpublished observation). Induction of Flt3L secretion upon differentiation partially explains this phenomenon, but it is likely that other yet-to-be-identified mechanisms contribute to the specificity of Flt3L action during myogenesis. Recently, the membrane-bound ligand EphrinA and its receptor EphA have been proposed to transduce cell-cell contact signals to suppress Erk signaling at the initiation of myoblast differentiation (Minami et al., 2011). Flt3L/Flt3 and
EphrinA/EphA signaling may cooperate to ensure suppression of mitogenic signals to allow the full extent of myogenic differentiation.

Little is known about the molecular mechanisms underlying regulation of Flt3L or Flt3 gene expression in hematopoietic cells, other than transcriptional control of Flt3 by the homeodomain-containing transcription factor MEIS1 in AML (Wang et al., 2005). Our observations suggest that the expression of Flt3L and Flt3 is tightly regulated in skeletal muscle. Both proteins are expressed transiently in newly formed myofibers during muscle regeneration, consistent with their function of promoting cell cycle exit and supporting an early step of myogenesis. Deciphering the regulation of Flt3L/Flt3 expression in skeletal muscle in future studies may lead to new insights into the myogenic regulatory network.

Other immunoregulatory cytokines have been reported to express in skeletal myocytes and play key roles in myogenesis. For instance, interleukin-4 (IL-4) regulates myoblast recruitment and late-stage fusion to allow growth of myotubes/myofibers (Horsley et al., 2003). The chemokine Cxcl12 also regulates myoblast migration and myocyte fusion (Bae et al., 2008; Chong et al., 2007; Griffin et al., 2011; Melchionna et al., 2010). IL-6 promotes myogenic differentiation possibly by modulating the transcriptional program at the initiation of differentiation (Baeza-Raja and Munoz-Canoves, 2004; Okazaki et al., 1996). Several other cytokines are known to impact myogenic differentiation, but they may function in the inflammatory response of muscle injury, rather than exerting cell-autonomous roles in muscle cells. Examples include Cardiotrophin-1 (CT-1) and Oncostatin M (OSM), both of which inhibit myogenic differentiation and muscle regeneration (Miyake et al., 2009; Xiao et al., 2011). It is noteworthy that Flt3L is unique among this collection of myogenic cytokines, in its control of cell cycle exit. Many more cytokines, especially chemokines, have been found to be expressed in skeletal myocytes (Griffin et al., 2011) and regenerating muscles (Hirata et al., 2003; Sachidanandan et al., 2002). Future investigations will likely reveal the extensiveness of myogenic regulation by traditional immunoregulatory cytokines.
MATERIALS AND METHODS

Antibodies and other reagents. Anti-MHC (MF20) and anti-myogenin (F5D) were obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD, National Institutes of Health and maintained by The University of Iowa, Department of Biological Sciences. Anti-Flt3 (S-18) for both Western and immunohistochemistry was from Santa Cruz Biotechnology (Dallas, TX). Anti-Flt3L antibody for immunohistochemistry, mouse Flt3L recombinant protein, and mouse Flt3L ELISA kit were from R&D Systems (Minneapolis, MN). Anti-p120RasGAP was from BD Biosciences (San Jose, CA). Anti-tubulin was from Abcam (Cambridge, MA). All other primary antibodies were from Cell Signaling Technology (Danvers, MA). All secondary antibodies were from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). Gelatin, bromodeoxyuridine (BrdU), U0126 and cytosine β-D-arabinofuranoside hydrochloride (Ara-C) were from Sigma-Aldrich (St. Louis, MO).

Cell culture. C2C12 myoblasts were maintained in DME containing 1 g/L glucose with 10% fetal bovine serum at 37°C with 7.5% CO2. To induce differentiation, cells were plated on tissue culture plates coated with 0.2% gelatin and grown to 100% confluence before switching to differentiation medium (DME containing 2% horse serum). The cells were replenished with fresh differentiation medium daily for 3 days.

Mouse primary myoblast isolation and differentiation. All animal experiments in this study followed protocols approved by the Animal Care and Use Committee at the University of Illinois at Urbana-Champaign. Primary myoblasts were isolated from 2- to 5-day-old FVB neonates as described previously (Ge et al., 2011), and maintained at low density on 1% gelatin-coated tissue culture plates. Differentiation was induced at 50-70% cell density in differentiation medium (see above) for 2 days with the medium changed daily.

Immunofluorescence microscopy and quantitative analysis of myocytes. C2C12 cells and primary myoblasts differentiated in 12-well plates were fixed and stained for MHC and DAPI as previously described (Park and Chen, 2005). The stained cells were examined with a Leica DMI 4000B fluorescence microscope. The fluorescent images were captured using a RETIGA EXi camera, and analyzed with Q-capture Pro51 software (Q-ImagingTM). Fusion index was calculated as the percentage of nuclei in myotubes with ≥2 nuclei. Each data point was generated from scoring 5 randomly chosen microscopic fields.
**Lentivirus-mediated RNAi.** shRNAs in the pLKO.1-puro vector were purchased from Sigma-Aldrich (MISSION® TRC). Clone IDs are: Flt3L #1, TRCN0000025059; Flt3L #2, TRCN0000025060; Flt3 #1, TRCN0000023739; Flt3 #2, TRCN0000378645; p120RasGAP, TRCN0000322311; Erk1, TRCN0000234920; Erk2, TRCN0000360489. Lentivirus packaging was performed as previously described (Yoon and Chen, 2008). Virally transduced C2C12 cells were selected in 3 μg/mL puromycin for 2 days, followed by 3-day differentiation. Virally transduced primary myoblasts were induced to differentiate without puromycin selection.

**Quantitative RT-PCR.** C2C12 cells or regenerating muscles were lysed in Trizol (Invitrogen). RNA was isolated following the manufacturer’s protocol. cDNA was synthesized from 1 μg RNA using qScript cDNA synthesis kit (Quanta Biosciences) following the manufacturer’s protocol, followed by quantitative PCR with StepOne Plus (Applied Biosystems) using gene specific primers. β-actin was used as a reference to obtain the relative fold change for target samples using the comparative C_T method. Mouse Flt3L primers: forward 5’-gctggatagagcaactgaag-3’; reverse 5’-gatgttggtctggacgaatc-3’. Mouse β-actin primers: forward 5’-ttgctgacaggatgcagaatc-3’; reverse 5’-atccacatctgctggaaggt-3’. Pre-validated mouse Flt3 primers were purchased from Qiagen (QuantiTect Primer Assays).

**Measurement of secreted Flt3L.** Media were collected from differentiating C2C12 cultures, and the Flt3L amount was measured using the Quantikine™ ELISA system for mouse Flt3L (R&D Systems) following the manufacturer’s protocol.

**Western blotting.** Cells were lysed directly in 1xSDS sample buffer with 10% β-mercaptoethanol. Proteins were resolved on SDS-PAGE and transferred onto polyvinylidene fluoride membrane (Millipore) and incubated with various antibodies following the manufacturer’s recommendations. Detection of horseradish peroxidase–conjugated secondary antibodies was performed with Western Lightning Chemiluminescence Reagent Plus (PErkinElmer), and images were developed on x-ray films. Quantification of Western blot band intensities was performed by densitometry of x-ray images using the software ImageJ.

**Muscle injury, regeneration and Flt3L knockdown in mice.** Ten-week-old male FVB mice were used in all the regeneration experiments. Muscle injury was induced by injection of BaCl_2 (50 μl of 1.2% w/v in saline) into TA muscles as previously described (Ge et al., 2009). On various days after injury, the mice were euthanized, and the TA muscles were collected, followed by RNA extraction, or crossection and staining as described in the next section. To
knock down Flt3L in vivo, 0.5 nmol Silencer Select siRNA targeting mouse Flt3L (ID# s66155, Ambion) or the non-targeting negative control #1 were co-injected with 1.2% w/v BaCl₂ into mouse hind limb TA muscles. Five days after injury, the injected muscles were collected and subjected to RNA isolation or cryosection.

**Muscle tissue cryosection, H&E staining, and immunohistochemistry.** TA muscles were isolated, frozen in liquid nitrogen-cooled 2-methylbutane, and embedded in TBS tissue freezing medium (Thermo Fisher Scientific). Sections of 10-μm thickness were obtained with a cryostat (Microm HM550; Thermo Fisher Scientific) at -20 °C, placed on uncoated slides, and stained with hematoxylin and eosin (H&E). Separately, the sections were fixed by 1.5% paraformaldehyde, incubated with anti-Flt3L or anti-Flt3 antibody, followed by incubation with FITC- or Texas Red-conjugated secondary antibody. The stained slides were examined with a microscope (DMI 4000B; Leica) with a 10× dry objective (Fluotar, numerical aperture 0.4; Leica). The bright-field and fluorescence images were captured at 24 bit and 8 bit, respectively, at room temperature using a camera (RETIGA EXi; Q-Imaging) equipped with Q-Capture Pro51 software (Q-Imaging). The images were then processed in Photoshop CS5 (Adobe), where brightness and contrast were adjusted. Fluorescence images were pseudo-colored and adjusted, when necessary, by identical parameters for all samples in the same experiment. An area of 614,400 μm² at the center of degenerated region of each TA muscle was selected for scoring centrally nucleated regenerating myofiber numbers and their cross section area.

**Retrovirus-mediated Flt3 overexpression.** pLXSN and pLXSN-Flt3 (Fukuda et al., 2005) (kind gifts from Dr. Seiji Fukuda of Shimane University, Japan, and Dr. Louis Pelus of Indiana University) were packaged into retroviruses and used to infect C2C12 cells. Briefly, 10 μg of DNA was transfected into phoenix cells using calcium phosphate, with 25 μM chloroquine added to the growth medium. After overnight incubation, cells were replenished with fresh growth medium and incubated at 32 °C. At 48 hr post-transfection, the medium was collected and cleared by centrifugation at 350 g for 5 min, and used directly to infect C2C12 myoblasts, with 8 μg/mL polybrene. After overnight infection, fresh medium was added to the cells, followed by 1 mg/mL G418 selection one day later for a total of 7-10 days. C2C12 myoblasts were maintained at sub-confluent condition throughout the infection and selection process.

**BrdU labeling.** C2C12 myoblasts or differentiating myocytes were incubated with BrdU (final concentration 10 μM) for 2 hrs and then fixed with 3.7% formaldehyde. After treatment by
4 N HCl for 30 min, immunostaining with anti-BrdU antibody followed by FITC-labeled anti–mouse IgG was carried out, and nuclei were stained by DAPI. Fluorescence microscopy and quantification were performed in a similar manner as that described under “Immunofluorescence microscopy and quantitative analysis of myocytes” above.

**Statistical analysis.** All data are presented as mean ± s.d.. Whenever necessary, statistical significance of the data comparison was analyzed by performing one-sample or paired $t$ test. The specific types of tests and the p-values, when applicable, are indicated in the figure legends.
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FIGURES LEGENDS

Fig. 1. Flt3L is a positive regulator of myoblast differentiation. (A) C2C12 myoblasts were induced to differentiate. Cell lysates and media were collected over differentiation time course for RNA isolation followed by qRT-PCR (left panel), or for ELISA (right panel), respectively. (B) C2C12 myoblasts were transduced overnight with lentiviruses expressing shRNAs for Flt3L (“shFlt3L”) or a scrambled hairpin sequence with no homology to any human and mouse sequence (“Control” or “C”). After two days of puromycin selection, cell lysates and media were collected and examined as described in A. (C) Flt3L knockdown cells were induced to differentiate for 3 days, followed by staining for MHC (green) and DAPI (red), and quantification of fusion index. (D) Western analysis of lysates for cells described in C throughout the time course. (E) Flt3L knockdown cells were differentiated in the absence or presence of 100 ng/mL recombinant Flt3L (“rec. Flt3L”) for 3 days, followed by staining for MHC (green) and DAPI (red), and quantification of fusion index. (F) Western analysis of lysates for cells described in E. (G) Flt3L knockdown primary myoblasts were induced to differentiate for 2 days, followed by staining for MHC (green) and DAPI (red), and quantification of fusion index. All data shown are mean ± s.d. (n = 3 for each experiment). For A&B, one sample t test was performed to compare each data to control. For C, E, &G, paired t test was performed to compare each data to control. *P <0.05, **P <0.01. Scale bars: 100 μm.

Fig. 2. Flt3L is necessary for complete muscle regeneration. (A) Regeneration of mouse TA muscles was induced by BaCl2 injury. On various days after injury (AI), total RNA was isolated from the TA muscles and subjected to analysis by qRT-PCR to determine the relative levels of Flt3L mRNA. Saline injection into contra-lateral TA muscles served as no injury control (“C”). (B) Regenerating muscles on various days AI were cryosectioned and subjected to H&E staining and immunostaining with anti-Flt3L antibody. Each pair of images (anti-Flt3L and H&E) was from sections of the same muscle. The majority of myofibers shown on the AI images were newly regenerated (with central nuclei). Each star (*) marks an uninjured myofiber. Results shown are representative of three independent experiments. Scale bar: 50 μm. (C) Control or Flt3L siRNA was co-injected with BaCl2 into TA muscles, followed by 5-day regeneration. The muscles were then isolated, and subjected to RNA isolation and qRT-PCR. (D) TA muscles as described in C were cryosectioned and H&E stained. The majority of myofibers in the enlarged
images were newly regenerated as evident from their central nuclei. (E) Sections shown in D were quantified for regenerating myofiber cross-section area (CSA) and fiber number. Scale bar: 50 μm. All data shown are mean ± s.d. (n = 3 for each condition, except for A: n=3-6). For A&C, one sample t test was performed to compare each data point with control. For E, paired t test was performed to compare each data to control. *P < 0.05.

**Fig. 3. Flt3 is required for myoblast differentiation.** (A) Regeneration of mouse TA muscles was induced by BaCl2 injury. RNA was isolated from non-injured control (“C”) and regenerating muscles over regeneration time course and subjected to qRT-PCR. (B) Regenerating muscles on various days AI were cryosectioned and subjected to H&E staining and immunostaining with anti-Flt3 antibody. Each pair of images (anti-Flt3 and H&E) was from sections of the same muscle. All myofibers shown on the AI images were newly regenerated (with central nuclei). Results shown are representative of three independent experiments. Scale bar: 50 μm. (C) C2C12 myoblasts were transduced overnight with lentiviruses expressing shRNAs for Flt3 (“shFlt3”) or control as described in Fig. 1B, followed by isolation of RNA and qRT-PCR. (D-E) Knockdown cells were induced to differentiate for 3 days and stained for MHC (green) and DAPI (red), and quantified for fusion index (D), or lysed over the differentiation time course for Western analysis (E). (F-G) C2C12 myoblasts were transduced with retroviruses expressing Flt3 (rec. Flt3) or empty vector (“vector”) as a control, selected with G418, and then induced to differentiate for 3 days, followed by staining for MHC (green) and DAPI (red), and quantification (F), or Western analysis (G). All data shown are mean ± s.d. (n = 3-4). For A, C, and G, one sample t test was performed to compare each data point to control. For D&F, paired t test was performed to compare each data point to control. *P <0.05, **P <0.01. Scale bars in D & F: 100 μm.

**Fig. 4. Flt3L promotes differentiation through Flt3.** (A) C2C12 myoblasts were transduced overnight with lentiviruses expressing shRNAs for Flt3 as described in Fig. 3C, followed by differentiation for 3 days with or without 100 ng/mL recombinant Flt3L (“rec. Flt3L”). Cells were stained for MHC (green) and DAPI (red), and quantified for fusion index. (B) C2C12 myoblasts were transduced with retroviruses expressing recombinant Flt3 (“rec. Flt3”) or empty vector (“vector”) as a control, as described in Fig. 3F, followed by overnight Flt3L lentivirus
transduction, 2-day puromycin selection and differentiation for 3 days. Cells were stained for MHC (green) and DAPI (red), and quantified for fusion index. All data shown are mean ± s.d. or representative images from 3 independent experiments. Paired t test was performed to compare each data point to control. *P <0.05. Scale bars: 100 μm.

Fig. 5. Flt3L and Flt3 inhibit myocyte proliferation. (A) C2C12 myoblasts were transduced overnight with lentiviruses expressing shRNAs for Flt3L and Flt3 as described in Figs. 1B and 3C, and differentiated for one day. During the last 2 hrs the cells were labeled with BrdU and fixed for staining of BrdU and DAPI. Quantification of percentage of DAPI-stained cells positive for BrdU staining is shown. (B) Cells were transduced as in A, followed by differentiation for 3 days, and cell lysates were analyzed by Western blotting at indicated times of differentiation. (C-D) Cells were transduced with lentiviruses expressing shRNAs for Flt3L or control as described in A, and differentiated with or without 100ng/ml recombinant Flt3L (“rec. Flt3L”) for one day, followed by BrdU labeling, staining, and quantification as described in A (C), or Western analysis (D). (E) Cells were transduced overnight with lentiviruses expressing shRNAs for Flt3L as described in Fig. 1B, followed by 3-day differentiation with or without 2 µg/mL Ara-C during the first day of differentiation. Cells were then stained for MHC (green) and DAPI (red), and quantified for fusion index. All data shown are mean ± s.d. (n = 3 for each experiment). Paired t test was performed to compare each data point to control. *P <0.05, **P <0.01. Scale bar: 100 μm.

Fig. 6. Flt3L inhibits proliferation and promotes differentiation through inhibiting Erk signaling. (A) C2C12 myoblasts and primary myoblasts were transduced overnight with lentiviruses expressing shRNA for Flt3L, followed by differentiation. Cell lysates were subjected to Western analysis. Representative blots are shown. Three independent experiments were quantified to yield the ratio between phospho-Erk and total Erk. (B) C2C12 cells with Flt3L knockdown were treated and analyzed as in A for pMEK levels. (C) C2C12 cells were transduced as in A, followed by differentiation with or without 3 µM U0126 for one day. During the last 2 hrs the cells were labeled with BrdU and fixed for staining of BrdU and DAPI. Quantification of percentage of DAPI-stained cells positive for BrdU staining is shown. (D) C2C12 and primary myoblasts were transduced as in A, followed by differentiation with or
without 3 µM U0126 during the first day. At the end of differentiation the cells were stained for MHC (green) and DAPI (red), and quantified for fusion index. (E-F) Cells were transduced overnight with lentiviruses expressing shRNAs for Flt3L, Erk1, Erk2, or in combination as indicated, followed by puromycin selection and differentiation. Cells were then subjected to Western analysis (E), or staining for MHC (green) and DAPI (red), and quantification of fusion index (F). All data shown are mean ± s.d. (n = 3 for each experiment). For A&B, one sample t test was performed to compare Flt3L knockdown to control cells; for all other data paired t test was performed to compare each data point to control. *P <0.05, **P <0.01. Scale bars: 100 µm.

Fig. 7. Flt3L promotes differentiation through p120RasGAP. (A) C2C12 myoblasts were transduced overnight with lentiviruses expressing shRNAs for p120RasGAP (“shp120”) or control, followed by puromycin selection. Cell lysates were subjected to Western analysis. (B) Cells were transduced as in A, followed by differentiation with or without 100ng/ml recombinant Flt3L. The cells were then stained for MHC (green) and DAPI (red), and quantified for fusion index. (C) Cells were transduced as in A, followed by differentiate with or without 100ng/ml recombinant Flt3L for one day. During the last 2 hrs the cells were labeled with BrdU and stained for BrdU and DAPI. Quantification of percentage of DAPI-stained cells positive for BrdU staining is shown. All data shown are mean ± s.d. (n = 3 for each condition). Paired t test was performed to compare each data to control. *P <0.05. Scale bar: 100 µm.

Fig. 8. Flt3L regulate skeletal myogenesis through p120RasGAP-Erk signaling. (A) C2C12 myoblasts were transduced overnight with lentiviruses as described in Fig. 7A, followed by differentiation. Cell lysates were subjected to Western analysis. Representative blots are shown. Three independent experiments were quantified to yield the ratio between phospho-Erk and total Erk. (B) Cells were transduced as in A, followed by differentiation with or without 3 µM U0126 during the first day. At the end of differentiation the cells were stained for MHC (green) and DAPI (red), and quantified for fusion index. All data shown are mean ± s.d. (n = 3 for each experiment). For A, one sample t test was performed to compare p120RasGAP knockdown to control cells; for B, paired t test was performed to compare each data point to control. *P <0.05. Scale bar: 100 µm. (C) A proposed model: Flt3L/Flt3 regulates myogenesis by promoting cell cycle withdrawal through the p120RasGAP-Ras-Erk pathway.
Fig. 1 Ge et al.

A

Flt3L mRNA levels

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C

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MHC Myogenin Tubulin

E

Control shFlt3L Control shFlt3L

F

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MHC Myogenin Tubulin

G

Control shFlt3L

fusion index

Control shFlt3L
Fig. 2  Ge et al.

A) Flt3L mRNA levels over time (C135714)

B) Images showing the expression of anti-Flt3L and H&E staining for different time points (unjured, Day5 AI, Day7 AI, Day14 AI).

C) Comparison of Control and siFlt3L on Flt3L mRNA levels.

D) Images of Control and siFlt3L conditions under H&E staining.

E) Graph showing CSA (m²) and myofiber number with statistical significance (*).
Fig. 3 Ge et al.

A. Bar graph showing Flt3 mRNA levels over days AI: C 1 3 5 7 14.

B. Images showing unjured, Day5 AI, Day7 AI, and Day14 AI.

C. Graph showing Flt3 mRNA levels for Control and shFlt3 #1, #2.

D. Graph showing fusion index for Control, shFlt3 #1, #2.

E. Table showing MHC, Myogenin, and Tubulin expression in Control, shFlt3 #1, #2.

F. Images showing vector and rec. Flt3.

G. Graph showing MHC and Myogenin expression in vector and rec. Flt3.
Fig. 4 Ge et al.

A

Control  shFlt3  Control  shFlt3

rec. Flt3L  rec. Flt3L

B

Control  shFlt3L  Control  shFlt3L

vector  rec. Flt3  vector  rec. Flt3
Fig. 6 Ge et al.

A

B

C

D

E

F

G

H

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K

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Q

R

S

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V

W

X

Y

Z

[Graphs and images showing experimental results related to cell fusion and expression levels of various proteins and gene knockdown effects.]
Fig. 7 Ge et al.

**Figure B**: Western Blot analysis showing the expression levels of p120RasGAP and Tubulin in Control and shp120 conditions.

**Graph B**: Bar graph representing the fusion index under different conditions of p120RasGAP and Tubulin.

**Figure C**: BrdU positive cell count in Control and shp120 conditions with and without Flt3L treatment.
Cell cycle withdrawal, myogenic differentiation

Fig. 8 Ge et al.