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Adhesion molecule Kirrel3/Neph2 is required for the elongated shape of myocytes during skeletal muscle differentiation

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ABSTRACT Kirrel/Neph proteins are evolutionarily conserved members of the immunoglobulin superfamily of adhesion proteins. Kirrel3 is the mouse orthologue of Dumbfounded (Duf), a family member that regulates myoblast pre-fusion events in *Drosophila*. Yet, the role of Kirrel3 in mammalian myogenesis has not been demonstrated. Experiments performed here indicate that the mouse Kirrel3 protein regulates morphological changes of myoblasts that are required for their subsequent fusion into multinucleated myotubes. We show that Kirrel3 is transiently expressed at the tips of myocytes during early myoblast differentiation and that its expression is dependent on the myogenic transcription factor, MyoD. Kirrel3 is transported in vesicles into the plasma membrane and its extracellular domain is cleaved in a proteasome-dependent manner. C-terminal deletion mutant lacking most of the intracellular domain accumulates at cellular extensions, does not undergo extracellular cleavage and induces the formation of large cell aggregates. This result suggests that the processing of the extracellular domain is regulated by the receptor's intracellular region. Knock-down of Kirrel3 in primary muscle progenitor cells (MPCs) prevented spindle shape myocyte formation, and significantly reduced their fusion with other myocytes to form multinucleated myotubes. In addition, migration of Kirrel3-deficient MPCs was randomized relative to the directed migration of control MPCs. We conclude that mouse Kirrel3 is a myobast adhesion molecule which promotes the morphological change of rounded MPC to a spindle shaped myocyte that migrates in a directed fashion and participates in the tight interactions between myocytes prior to their fusion.

KEY WORDS: *Kirrel3/Neph2, Myocyte morphology, muscle fusion*

Introduction

Myoblast fusion is an ordered set of specific cellular events that includes cell morphological changes, migration, recognition, adhesion, alignment and membrane union (Reviewed in Horsley and Pavlath, 2004). Many of the events leading to myoblast fusion are well understood in invertebrates, mostly in *Drosophila*, however our knowledge of the vertebrate process is more limited. The recent finding of the myoblast fusogen, Myomaker, shed light on the final stage of membrane union (Millay *et al*., 2013), but molecules participating in the preceding events like cell-cell recognition and adhesion are waiting for further discovery and investigation.

In *Drosophila* embryos, two types of cells, muscle founder cells (FCs) and fusion competent myoblasts (FCMs) fuse to form the body wall muscles in a segmentary repeating pattern (Dobi *et al*., 2015; Schulman *et al*., 2015). The first step of the process is the recognition between FCs and FCMs. FCs function as "attractants" for surrounding FCMs extending membrane protrusions (filopodia) that attach to the FC membrane. These events are mediated by transmembrane proteins belonging to the immunoglobulin super family (IgSF) including Dumbfounded/Kirre (Duf) and Roughest/Irre (Rst) of the FC, Sticks and Stones (Sns) and Hibris (Hbs) of the FCM (Ruiz-Gomez *et al*., 2000). Duf expression on the FC membrane attracts, in yet an unknown way, the migration of FCM

Abbreviations used in this paper: DM, differentiation medium; EC, extracellular; FL, full length; GM, growth medium; IC, intracellular; KD, knock-down; MPC, muscle progenitor cell.

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towards the FC (Ruiz-Gomez *et al.*, 2000). Subsequently, Duf and the homolog Rst function as heterotypic FC binding partners of FCM proteins, Sns and Hbs (Dworak and Sink, 2002). These specific interactions initiate intracellular signaling events that operate to recruit actin polymerization factors to the distinct membrane sites at which fusion can occur (Gildor *et al.*, 2009; Kesper *et al.*, 2007; Kim *et al.*, 2007; Massarwa *et al.*, 2007). Mammalian homologs of the *Drosophila* proteins from the Kirrel/Neph family were demonstrated to participate in tight interactions between podocytes of mammalian glomeruli filtration apparatus, and more recently in brain functions relating to neural synapses (Helmstadter *et al.*, 2014). Yet the role of Kirrel/Neph family member in skeletal mammalian muscle biology was demonstrated only in one study (Sohn *et al.*, 2009). Myoblasts derived from neonatal Nephrin (homolog of *Drosophila* Sns) knock out mouse displayed defected fusion with myotubes in vitro. That study's conclusion was that Nephrin played a role in secondary myoblasts' fusion into nascent myotubes. Another member of the family, Kirrel3/Neph2, which is expressed in progenitors of fast-twitch muscles in zebrafish, was shown to be essential for their fusion into syncytia (Srinivas *et al.*, 2007). Therefore, it appears that the function of Kirrel/Neph family of adhesion molecules is evolutionary conserved.

The involvement of the Zebrafish homolog of mammalian Kirrel3/Neph2 in fast-twitch myoblast fusion prompted the present investigation of mouse Kirrel3. In the present study we asked whether mouse Kirrel3, a close homolog of *Drosophila* Duf/Rst, is involved in events leading to myoblast fusion. Results presented hereafter, support a role of Kirrel3 in the morphological changes of small rounded MPCs into the spindle shape myocyte; the fusion competent cell.

Results

The expression of Kirrel3 is transiently induced in differentiating C2 myoblasts

Kirrel3 expression was analyzed in differentiating C2 cells. Steady state levels of Kirrel3 mRNA were analyzed in semi quantitative RT PCR reaction (Fig. 1A). Expression is detected in proliferating myoblasts and is significantly induced during the first hours in differentiation medium (DM) with a maximum expression at 5 hours. Expression was later reduced and a second peak was observed at a late differentiation period (72-96 hours). Expression of two other members of the family was also analyzed; Kirrel1 expression was constant at all stages of differentiation while that of Kirrel2 was not detected (data not shown). Next, Kirrel3 expression was analyzed by immunostaining of C2 myoblasts using anti-Kirrel3 antibody (Fig. 1B). Few cells were positively-stained in myoblasts grown in GM (GM; growth medium) or following their 8 hours growth in DM. Much weaker Kirrel3 staining was detected in multinucleated myotubes grown for 24 hours in DM (Fig. 1B). A more detailed analysis of Kirrel3 staining revealed that the protein was concentrated at the tips of spindle shaped myocytes that were positively stained with myosin heavy chain (MyHC) (Fig. 1C). Immunostaining of living myoblasts with an antibody directed to the extracellular region revealed that Kirrel3 was located to the plasma membrane and to cell extensions connecting with adjacent cells (Fig. 1D). Next we inquired whether Kirrel3 mRNA expression is unique to myoblasts and if the myogenic transcription factor, MyoD, was involved in its expression. For that purpose, we used a 3T3 MyoD:ER (ER; estrogen receptor) cell line. These cells expressing a cytoplasmic MyoD:ER chimera protein are gown as fibroblasts. Following the addition of β estradiol to cell medium, the chimera protein translocates into the nuclei at which MyoD induces myoblast cell fate. Kirrel3 expression was significantly induced in DM containing β estradiol but not in DM without the hormone (Fig. 1E). This result indicates that Kirrel3 expression is regulated by MyoD in differentiating myoblasts.

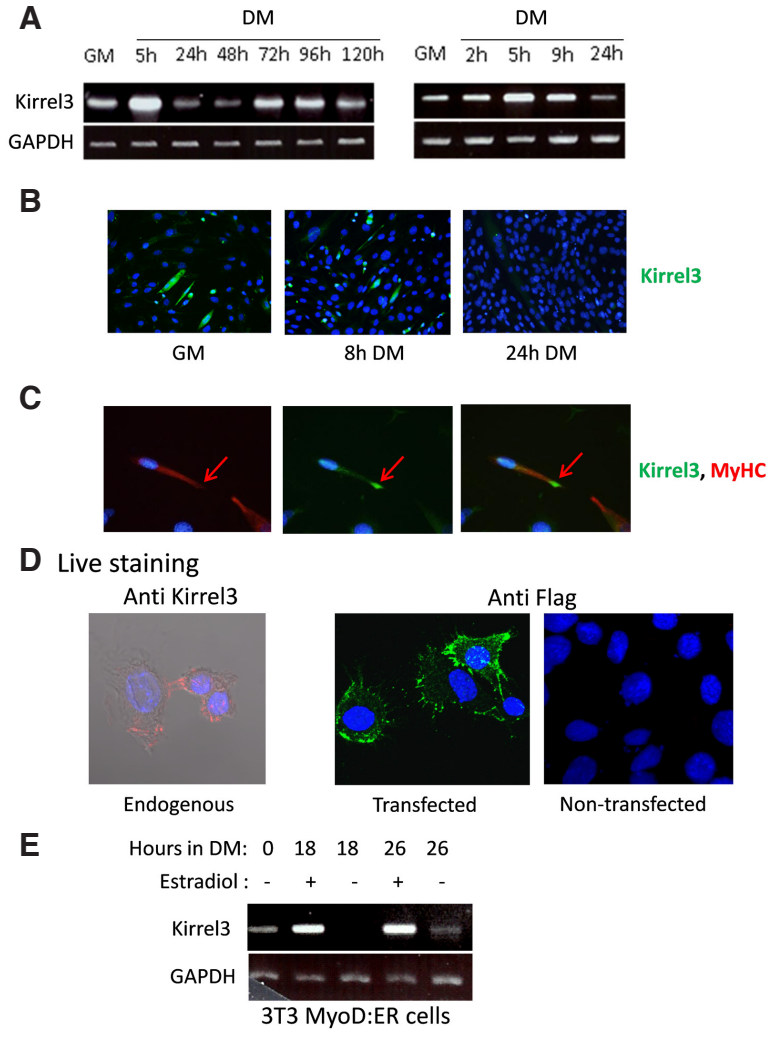

Fig. 1. Kirrel3 expression in myoblasts. (A) *C2 myoblasts were grown in differentiation medium (DM) for the indicated time periods, semi quantitative PCR was performed.* **(B)** *C2 cells were grown in GM and in DM for the indicated time periods. Cells were immunostained with antibody to Neph2 (Santa Cruz, S-14). Nuclei were counterstained with DAPI.* **(C)** *C2 myoblasts were grown in DM for 8 hours. Myocytes were immunostained with antibodies to Myosin heavy chain (MyHC)(red) and Kirrel3 (green). Nuclei were counterstained with DAPI.* **(D)** *C2 cells were grown as in C, and live cells were incubated with anti Kirrel3 antibody followed by fixation and fluorescent staining as described in "Materials and Methods".* **(E)** *3T3-MyoD:ER cells were grown in DM for the indicated time periods in the presence or absence of β estradiol. Kirel3 was analyzed by Semi quantitative RT-PCR.*

The extracellular domain of Kirrel3 is cleaved in the extremities of myocytes

The extracellular domain of Kirrel3 was shown to be cleaved by metalloproteinases in podocyte cells of kidney glomeruli and in bone marrow stroma cells (Gerke *et al.*, 2005; Ueno *et al.*, 2003). Next, we analyzed whether the extracellular domain is cleaved in C2 myoblasts expressing C-terminal epitope-tagged Kirrel3. A band of approximate 30 Kd appeared in a 60 minutes chase period of a pulse-chase experiment (Fig. 2A). This fragment probably represents the intracellular C-terminal domain of the molecule that was observed before (Gerke *et al.*, 2005; Ueno *et al.*, 2003). The inhibitor, MG132, reduced the 30 kd band expression relative to the full length 100 Kd band indicating that cleavage is mediated by the proteasome (Fig. 2B). Since Kirrel3 was involved in tight interactions between cells such as podocytes, we analyzed its levels of expression in confluent and sparsely grown myoblasts (Fig. 2C). Interestingly, myoblasts grown in a confluent culture dramatically reduced the levels of exogenously-expressed Kirrel3, while expression levels of Kirrel3 were relatively stable in sparsely grown cultures. In the latter case, Kirrel3 expression levels were reduced by 50% following 24 hours in culture, probably because cells reached confluency at this stage. This result indicates that Kirrel3's turnover rate is accelerated upon cell-cell interactions. To learn more about Kirrel3 processing, a double tagged receptor was constructed with Flag epitope at the N-terminal extracellular domain and Myc epitope at the C-terminal intracellular domain. The construct was expressed in C2 myoblasts that were stained with antibodies to the tags (Fig. 2D). Surprisingly, both tags were co-stained in vesicle-like structures next to the cell nuclei, while only the intracellular Myc epitope was stained in the periphery and in cellular extensions. This particular staining indicates that Kirrel3 is delivered via the secretory pathway to the plasma membrane and that its extracellular domain is cleaved during this process. Co-staining of Kirrel3 with the Golgi membrane protein, Giantin, indicates that Kirrel3 is indeed transported via the secretory system to the plasma membrane (Supplementary Fig. 1).

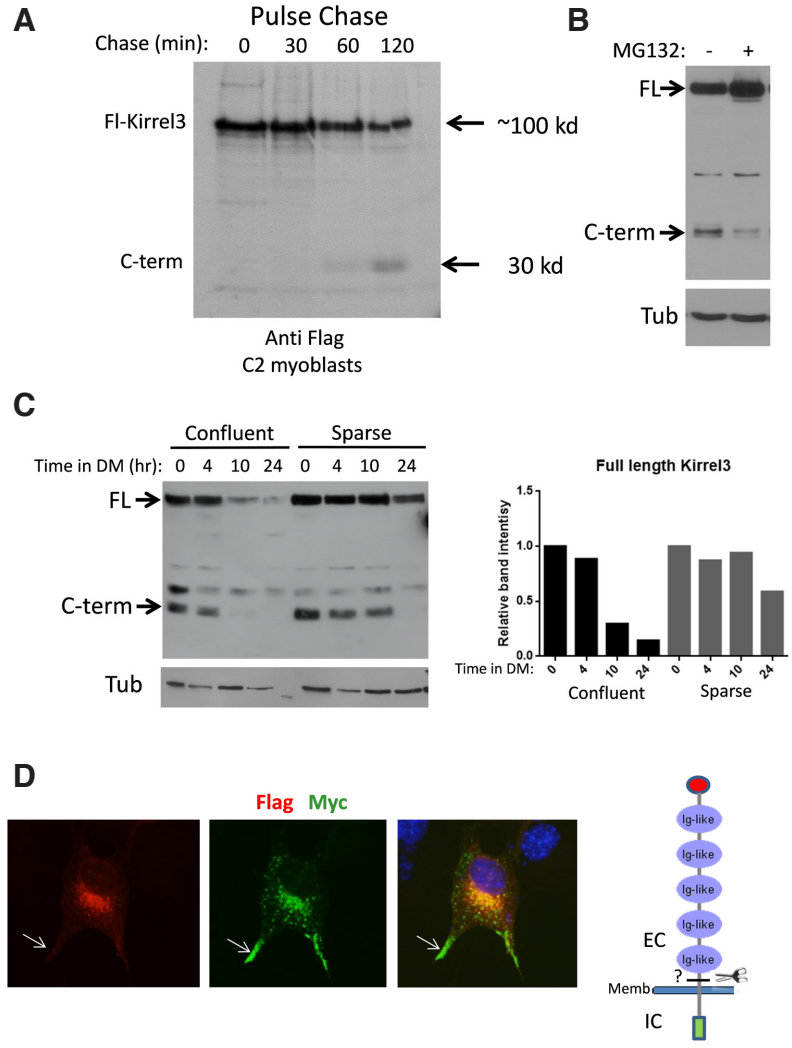

Fig. 2. The extracellular domain of Kirrel3 is cleaved in C2 myoblasts. (A) *C2 myoblasts were transfected with vector encoding the full length Kirrel3 containing a Flag tag at the C-terminal intracellular region. Cells were pulse-labeled and then chased for the indicated time periods. Kirrel3 was immunoprecipitated with anti-Flag antibody.* **(B)** *C2 myoblasts were transfected as is described in A. Cells were grown for 24 hours in DM without or with MG132. Kirrel3 was analyzed by Western blotting with anti-Flag antibody.* **(C)** *C2 myoblasts were transfected as in B and seeded on plates at sub-confluent and confluent concentration. Cells were then grown in DM for the indicated time periods. Kirrel3 was analyzed by Western blotting as in B.* **(D)** *Kirrel3 protein containing one intracellular (Myc) and one extracellular (Flag) tag was expressed in transfected C2 cells. Cells were immunostained with antibodies to the two tags. Nuclei were counterstained with DAPI.*

The intracellular domain of Kirrel3 regulates cleavage of the extracellular domain and cell-cell adhesion

To verify the possible roles of the intra- and extracellular domains of Kirrel3, each domain was deleted and the mutant receptors were expressed in myoblasts. Myoblasts were transfected with full length Kirrel3 (FL), extracellular domain deleted molecule (ΔEC; no extracellular amino acids remained except for the epitope tag) and intracellular region deleted molecule (ΔIC; 16 intracellular amino acids remained and the epitope tag) (Fig. 3A). The FL and ΔEC proteins were concentrated near the nucleus in vesicle-like structures that created a gradient towards the plasma membrane with no particular accumulation at cell-cell contacts. Expression pattern of the ΔIC protein was entirely different with predominant staining at the extremities of the cytoskeletal network and mainly in extensions that were in contact with neighboring cells (Fig. 3A). Some intracellular vesicle staining of the ΔIC was also noticed. The particular staining pattern of the ΔIC protein indicates its possible role in mediating cell-cell interactions. To investigate this possibility, the different mutants were expressed in 293T cells. Transfected cells were grown for 3 hours in bacterial flasks that prevented their adherence to the plastic, and enabled the formation of cell aggregates (Fig. 3B). The FL protein expressing cells formed larger aggregates than non-transfected control cells or cells expressing the ΔEC mutant (lacking the entire extracellular domain). Cells expressing the ΔIC protein, however, formed significantly larger cell aggregates relative to other Kirrel3 expressing cells. We conclude that the ΔIC mutant which is concentrated at cell contact regions, mediates cell-cell interactions. Next, we investigated whether the mutants

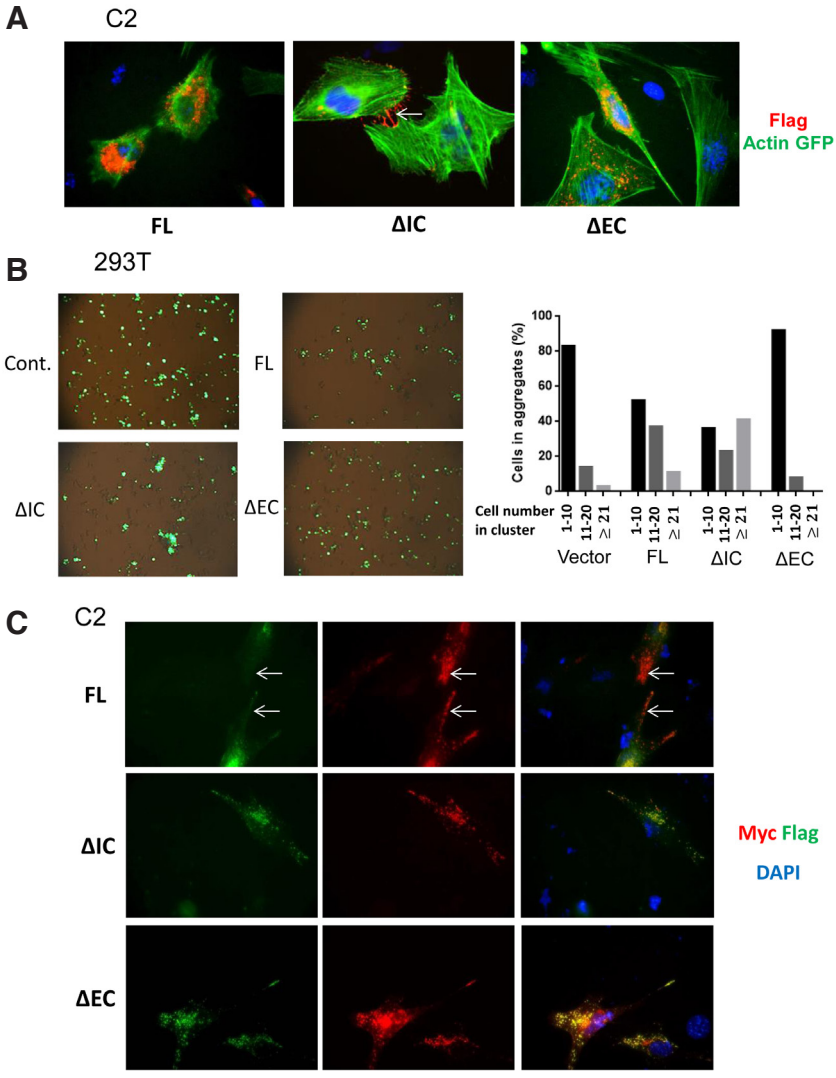

Fig. 3. Analysis of Kirrel3 intracellular and extracellular domains. (A). *Different C-terminal Flag tagged deletion mutants of Kirrel3 lacking the intracellular region (ΔIC) or extracellular Ig-like region (ΔEC) were transfected to C2 cells together with actin-GFP. Cells were immunostained with antibodies to Flag and to GFP. Arrow points at cell extensions expressing ΔIC protein.* **(B)** *293T cells were transfected with the above Kirrel3 mutants and plated on bacterial flasks. GFP positive cells (transfected) were visualized under fluorescent binocular. Cell number in aggregates was quantified and results are presented in the histogram.* **(C)** *Kirrel3 mutants carrying N-terminal and C-terminal epitopes were expressed in C2 cells. Cells were immunostained with antibodies directed to the two tags. Nuclei were counterstained with DAPI. Arrows point at cell extensions stained against Myc epitope only.*

undergo cleavage of the extracellular region. Receptor cleavage was analyzed by double epitope-tagged molecules (N-terminal and C-terminal tags) imaging in transfected myoblasts. In accordance to what was observed in Fig. 2D, punctate staining of the intracellular C-terminal tag but not of the extracellular N-terminal tag is observed in the periphery of cells expressing the FL protein, indicating the release of the extracellular domain. Differently, co-staining of both epitopes was predominant in cells expressing the ΔIC and ΔEC proteins (Fig. 3C). Altogether, these results suggest that the ΔIC protein did not undergo cleavage of the extracellular domain. To elucidate the intracellular amino acids that participate in regulating the subcellular localization of Kirrel3, a serial C-terminal deletions were constructed and transfected into myoblasts (Fig. 4). The full length protein and all C-terminal deletions including the one retaining 89 intracellular amino acids showed a similar intracellular vesicular staining. Only the mutant retaining 16 amino acids (the ΔIC mutant, 561 aa) was localized at the actin filaments' extremities and at cell-cell contact regions. Therefore, the amino acid sequence within a stretch of 73 intracellular amino acids (a.a 561-634) regulates the subcellular localization and the extracellular region cleavage. A receptor not containing any intracellular amino acids (545 aa) appeared in large irregular structures.

Knock-down of Kirrel3 dramatically reduces fusion of myoblasts into multinucleated myotubes

The preceding results indicate that Kirrel3 is involved in interactions occurring between differentiating myoblasts. To further elucidate its role in the differentiation of myoblasts into multinucleated myotubes, Kirrel3 was knocked down by infecting C2 cells with lentiviruses expressing shRNA (Fig. 5). Knock down efficiency was verified by PCR analysis of endogenous Kirrel3 mRNA, and by examining exogenously-expressed epitope tagged Kirrel3 (Fig. 5A). In both cases, specific shRNA targeting the coding region of Kirrel3 almost completely abolished the expression of Kirrel3 mRNA or protein, whereas non-specific control shRNA had no effect. To evaluate the possible involvement of Kirrel3 in myoblast differentiation, the expression of skeletal muscle markers was analyzed in shRNA infected C2 myoblasts that were allowed to differentiate for different time periods (Fig. 5B). The expression of the differentiation transcription factor, Myogenin was similarly induced in control as in Kirrle3 KD cells. Likewise, structural myofiber proteins, Troponin and Caveolin3 were similarly induced at 72 hours in DM. An exception was the sarcomeric protein MyHC, whose expression was delayed and reached lower levels in Kirrel3 KD cells relative to control MPCs. Therefore, it appears that Kirrel3 is necessary at a particular stage of differentiation that involves sarcomere formation. To further investigate this possibility, primary muscle progenitor cells (MPCs) infected with lentivirus expressing the above shRNAs were plated and grown in DM for 48 hours before their immunostaining (Fig. 5C). In contrast to control shRNA-infected MPCs that formed elongated myotubes, Kirrel3 shRNA infected MPCs appeared less elongated. Around 50% of MyHC positive control cells were multinucleated whereas only 10% of the Kirrel3 KD cells were multinucleated. We conclude that Kirrel3 affects myocyte's elongation and fusion.

Kirrel3 knock down prevents the formation of spindle shape myocytes

The defected fusion observed, might be a consequence of Kirrel3-depleted myocytes' inability to form the typical elongated,

spindle shape morphology. To analyze the early stage of pre-fusion myocyte elongation, MPCs were analyzed 24 hours following DM addition (Fig. 6). A dramatic morphological difference is noticed between control and Kirrel3-KD myocytes. Control MPCs formed spindle-shape myocytes that were elongated, while most of the Kirrel3-depleted cells failed to elongate and kept their rounded shape structure (Fig. 6A). The average length of the control myocytes was 3 times higher than that of Kirrel3-depleted myocytes. A more detailed analysis of these cells indicated differences in cytoskeletal organization between the different lines (Fig. 6B). Spindle-shape myocytes formed stress actin fibers, whereas rounded myocytes formed a ring of actin filaments adjacent to cell periphery. Similarly, the distribution of microtubules was also different between the spindle shape and rounded myocytes. In a time lapse video taken during the first 24 hours of differentiation, control MPCs that initiate as small rounded cells, gradually elongate to form the typical spindle shape myocytes, while Kirrel3 KD MPCs initiate the elongation process but shrink back into small rounded cells that later detach from the plate (Supplementary Fig. 2). Therefore, matrix adhesion of Kirrel3-deficient cells might be compromised, hence preventing the normal elongation of myocytes.

Directed cell migration is lost in Kirrel3-deficient myocytes

The different morphology of Kirrel3-depleted myocytes could affect their migratory capabilities. We undertook two approaches to compare the migration of the two types of the above mentioned MPCs. The first is a "wound assay" in which the migration of cells into a gap is monitored by capturing images at regular intervals (Fig. 7A). Control MPCs closed the gap faster than Kirrel3-deficient MPCs (Fig. 7B). The second approach took advantage of the "Boyden chamber assay". In this assay, MPCs migrating through an 8 μm pore size membrane was analyzed. A significantly higher amount of control MPCs transversed the membrane relative to the number of Kirrel3-deficient MPCs (Fig. 7C). Together, these results indicate that Kirrel3-deficient MPCs have limited active migratory ability. To gain a better insight at migration differences between control and Kirrel3-depleted MPCs, time lapse imaging of spindle shape MPCs (control) and rounded MPCs (Kirrel3 KD) was performed (Supplementary Fig. 3). The video shows that the control spindle shape MPC moves back and forth in one direction, while rounded Kirrel3 KD MPC moves in a random pattern. The calculated average speed of the latter was 2.7μm/min while that of the former was 0.8μm/min. Yet, since the movement of the rounded cell was random, it translocated to a shorter distance (Supplementary Fig. 4)

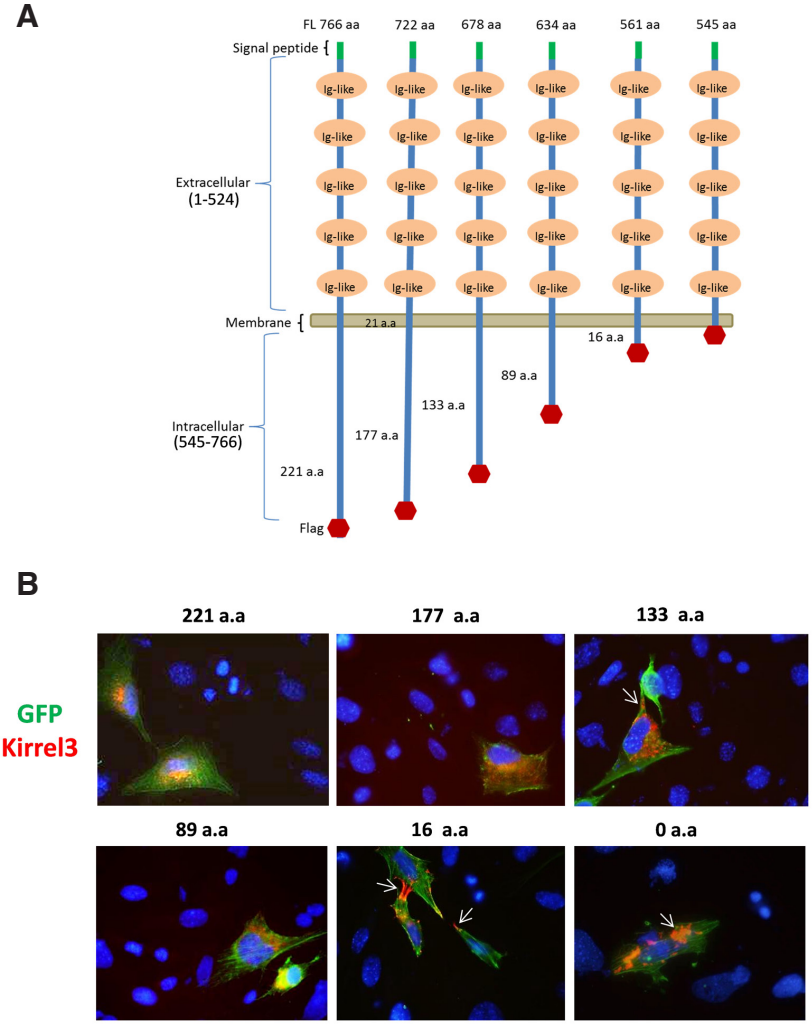

Fig. 4. Expression pattern of Kirrel3 C-terminal deletion mutants. (A) *Mutants are presented in the scheme.* **(B)** *C2 myoblasts were transfected with vectors encoding the different mutants together with actin-GFP. Cells were immunostained with antibodies to Flag and to GFP. Arrows point at filopodia and contact regions expressing Kirrel3 proteins. Nuclei were counterstained with DAPI.*

Discussion

The involvement of cell adhesion molecules in mammalian muscle fusion was mostly studied in mouse myoblasts cell culture models and in regenerating adult muscles. Unlike in flies where members of IgSF (Kirre/Duf and Rst/Irre-C) are the only identified adhesion molecules, there is a wide variety of molecules involved in the recognition and adhesion of mouse myoblasts (reviewed in (Horsley and Pavlath, 2004)). Nephrin, the vertebrate homolog of Sns was the only adhesion molecule of the IgSF demonstrated in mouse myoblast fusion before the present study that reveals the role of yet another member, Kirrel3/Neph2, a homolog of Kirre/Duf and Rst/Irre-C. Kirrel3 is expressed in C2 myoblasts at two phases, early and late, that could correspond with the events leading to primary and secondary fusion. In the early phase, myoblast-myoblast fusion (primary fusion) results in the formation of nascent myotubes, while in the second phase, myoblasts fuse with nascent myotubes (secondary fusion) (Zhang and McLennan, 1995). Kirrel3 expression is dramatically reduced between these phases. This type of expression pattern indicates that Kirrel3 is necessary at a specific stage of the fusion process and its down regulation between stages may be necessary to allow the continuation of the process. This notion is supported by another experiment in which we observed that cell confluency affected protein levels of Kirrel3. Epitope tagged Kirrel3 protein was highly and stably expressed in sub-confluent

myoblast culture, whereas its expression was dramatically reduced in confluent myoblast culture, indicating that Kirrel3 may be dispensable following the formation of myoblast-myoblast interactions. Assuming that Kirrel3 is a pre-fusion molecule functioning at early recognition phase between myoblasts, its removal from the site of subsequent fusion may be necessary.

Extracellular Ig-like region shedding in Kirrel3 was described in kidney podocyte cells (Gerke *et al.*, 2005) and in stroma cells that support the growth of bone marrow hematopoietic stem cells (Ueno *et al.*, 2003). In podocytes, extracellular cleavage was mediated by metalloproteinases in a tyrosine phosphorylation-dependent manner, though the importance of the process remains unknown. The present study's findings indicate that processing of Kirrel3 also occurs in myoblasts. The protein is processed and the entire or majority of its extracellular domain is released. The remaining C-terminal fragment likely includes the intracellular and membrane regions. The involvement of the proteasome and intracellular sequences of Kirrel3 in the extracellular cleavage indicates that processing is regulated within the cell. Even more interesting is the observation suggesting that the cleaved receptor is mainly localized to cell extremities and extensions (i.e., filopodia and lamellopodia). Moreover, distribution of the processed receptor is asymmetric with a tendency to concentrate at one side of the cell. We can suggest at least two hypotheses for this type of expression; A: the receptor is recycled and signaling is terminated following interactions with neighboring cells. This process is necessary for the cells to advance to the next stage of the fusion process, B: the secreted extracellular region functions to attract neighboring myoblasts expressing similar domains on their plasma membrane. This type of mechanism was suggested to regulate myoblast recognition in the fly (Ruiz-Gomez *et al.*, 2000; Strunkelnberg *et al.*, 2001). In *Drosophila*, FCMs migrate towards FCs in a Duf/Rst and Sns dependent manner (Kocherlakota *et al.*, 2008). The mechanism is unknown, though the possibility that FCMs move along a gradient of secreted Duf is indirectly supported by the presence of truncated form of Duf in the media of S2 cells transfected with full-length Duf (Chen and Olson, 2001). The hypotheses concerning the extracellular domain's role should be the subject of future research.

The expression of Kirrel3 on myoblasts' membranes raises another interesting question that relates to myoblasts interactions prior to the fusion event: Are Kirrel3 interactions homotypic or heterotypic as in the fly? In flies, fusion occurs between two distinct cell populations expressing different IgSF members (reviewed in (Abmayr and Pavlath, 2012)). There is a clear preference of heterotypic interactions between Duf/Rst expressed in FCs and Sns expressed in FCM (Bour *et al.*, 2000). In mammals, there is no evidence to suggest the existence of different MPCs populations expressing unique IgSF members. However, several indirect observations may point at possible heterotypic interactions between MPCs. First, heterotypic interactions of Kirrel3 with Nephrin were demonstrated in the mouse kidney (Gerke *et al.*, 2005). Second, Nephrin was shown to be expressed on myoblast membranes and is required for their fusion (Sohn *et al.*, 2009). Thus, it is possible that Nephrin and Kirrel3 are either co-expressed in homogeneous myoblast population, or that each one is expressed on a distinct population of myoblasts that fuse together. Furthermore, secondary fusion between myocytes and nascent myotubes may represent the mammal equivalent of heterotypic interactions in *Drosophila*. It should be noted that the *in vitro* system may not represent heterogeneous populations of fusion competent cells that may exist in the context of the whole animal.

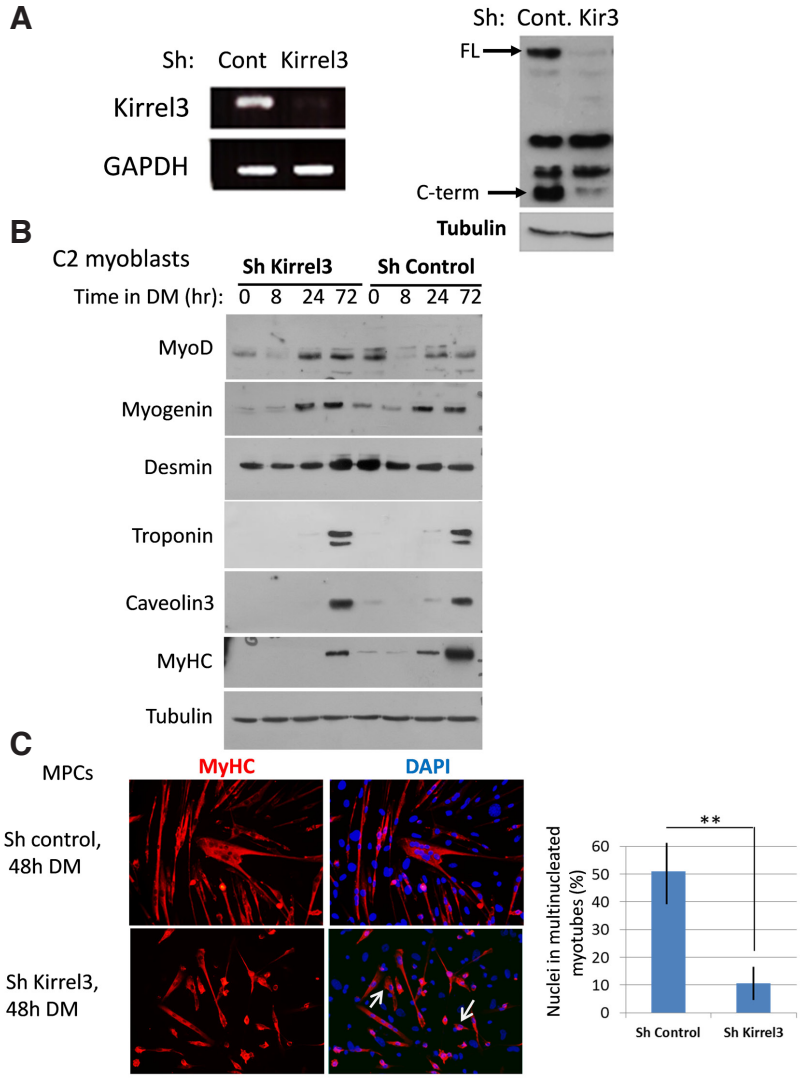

Fig. 5. Knock down of Kirrel3 prevents fusion of mononucleated myocytes into multinucleated myotubes. (A) *C2 myoblasts were infected with lentivirus expressing shRNA to Kirrel3. Expression of endogenous Kirrel3 was analyzed by semi quantitative PCR and of transfected kirrle3 by Western analysis with anti-Flag antibody. Expression of full size Kirrel3 and the cleaved c-terminal product were markedly reduced by shKirrel3.* **(B)** *ShRNA-infected myoblasts were allowed to differentiate in DM for different time periods. Expression of several differentiation markers was analyzed by Western blotting.* **(C)** *Freshly-isolated muscle progenitor cells (MPCs) were infected with shRNA expressing lentiviruses as indicated. MPCs were allowed to differentiate for 48 hours in DM, and immunostained with antibody to MyHC. Nuclei were counterstained with DAPI. The percentage of nuclei in MyHC-positive myotubes with two or more nuclei out of the total number of nuclei in MyHC-positive cells was calculated (n=200). Two independent experiments were performed. *, $p \leq 0.05$.*

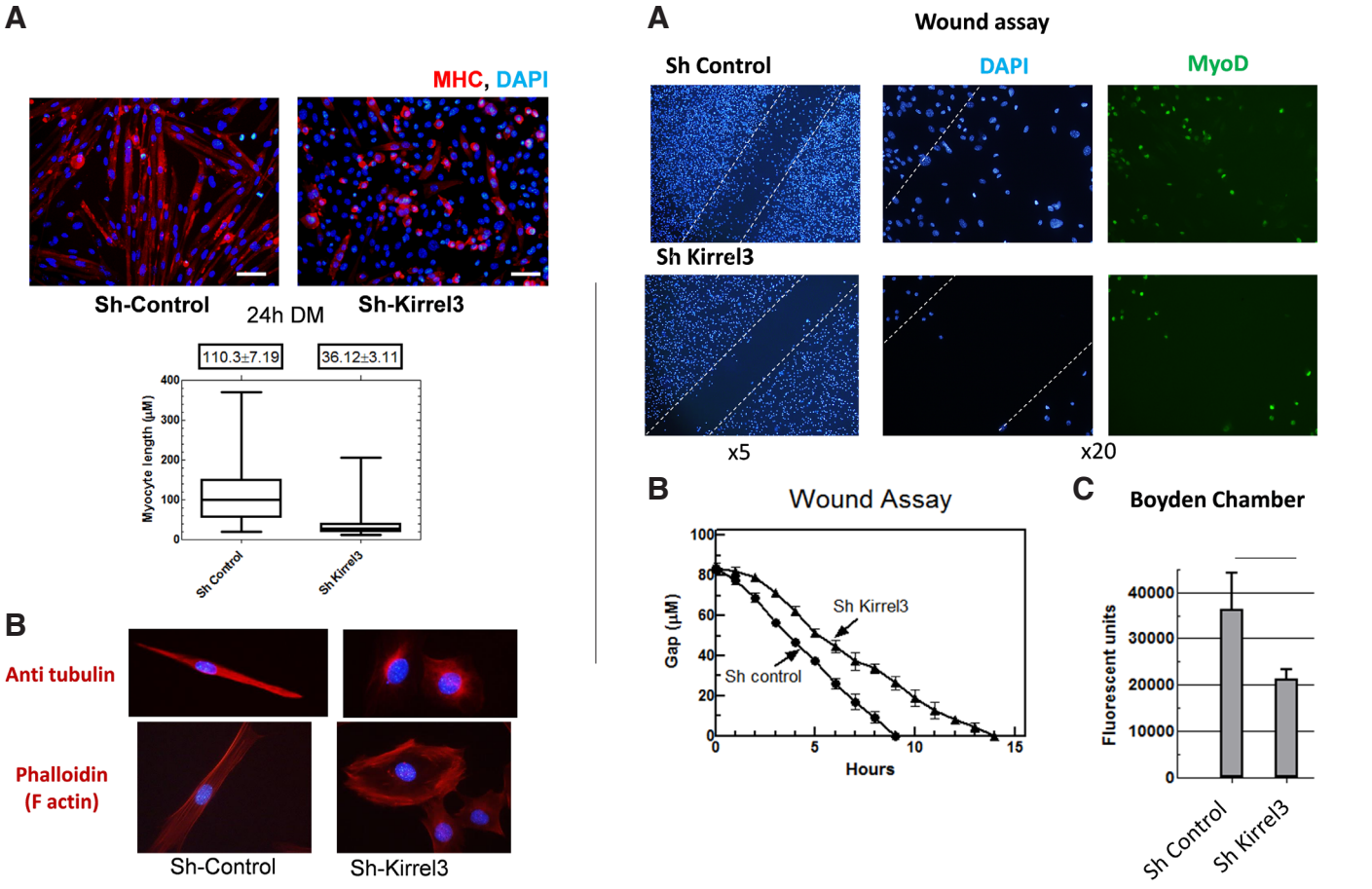

Fig. 6 (Left). Kirrel3 deficient MPCs do not elongate. (A) *Primary MPCs infected with lentivirus expressing shRNA were grown in DM for 24 hours. Cells were immunostained using anti MyHC (MF20). Nuclei were counterstained with DAPI. Three independent experiments were performed. The length of mononucleated myocytes (MyHC positive) was measured in several microscopic fields (n=200). Scale bar: 50μm.* **(B)** *MPCs were grown as above and were incubated with anti α tubulin to visualize microtubules or with phalloidin to visualize actin filaments. Nuclei were counterstained with DAPI. Representative cells are shown.*

Fig. 7 (Right). Kirrel3 is required for directed migration of MPCs. (A) *Wound assay: Primary MPCs infected with lentivirus expressing shRNA were plated and grown for 8 hours in DM. A gap was introduced and movement of cells into the gap was analyzed 5 hours later. Activated MPCs were detected with anti MyoD antibody, and nuclei were stained using DAPI.* **(B)** *Wound assay was performed as is described in A. Cells were analyzed using time lapse imaging in one hour intervals. Sh Control MPCs closed the gap after 9 hours, while sh Kirrel3 MPCs closed the gap after 14 hours.* **(C)** *"Boyden chamber" assay. MPCs were infected as described above. MPCs were seeded in the top chamber in serum-free media, while 15% FBS was added to the bottom chamber. Cells were left for 5 hours to migrate through 8μm membrane. Three independent experiments were performed and stained cells were quantified.*

The most interesting finding of the present study is the absolute requirement of Kirrel3 expression for the morphological change from the small rounded cell to the elongated spindle shape myocyte. This structural change is likely mediated by adhesive function of Kirrel3 and/or recruitment of the actin polymerization machinery to cell extremities. In the absence of Kirrel3 expression, myoblasts appear to initiate differentiation normally, expressing myogenic differentiation transcription factors and structural proteins of the myotube. Still, these cells do not undergo elongation and remain rounded. In a time lapse video, Kirrel3 deficient MPCs do elongate and retract back to the small rounded shape, as if they cannot sustain their adhesion to the substratum. Another observation that indicates a role for Kirrel3 in myocyte elongation is its unique immunostaining at the tips of elongating myocytes. The Bar domain protein, Graf1 was similarly stained at the tips of elongating myocytes (Doherty *et al.*, 2011). This protein which is a Rho-GTPase activating protein was suggested to cause dissolution of actin foci at the myocyte tips that is required for the elongation of the pre-fused myocyte. Kirrel3 might be the molecule which recruits the Rho GTPase complex to the tips at which actin filaments are being remodeled to elongate the myocyte. Myocyte elongation is critical for the parallel apposition of their membranes with other myocytes or myotubes enabling the subsequent pore formation (Wakelam, 1985).

During early differentiation, Kirrel3 deficient MPCs migrate differently than control MPCs. These cells largely lose their ability to fill the gap in a "wound assay" and migrate through a membrane in a trans-well assay. This loss is likely a consequence of Kirrel3-depleted MPCs inability to change their small rounded morphology, unlike control MPCs that undergo elongation. Control elongated myocytes migrate back and forth in an approximate straight line

continuing the long axis of the cell, while rounded Kirrel3-deficient myocytes move randomly. Although movement of the latter cells is three times faster than that of control myocytes, they move to shorter distances. Interestingly, satellite cells (MPCs) employ blebbing or amoeboid-base movement which is believed to be advantageous for their "rolling" along myofibers after emerging from the basal lamina (Griffin *et al.*, 2010). Indeed, rounded Kirrel3-deficient myocytes have cortical actin meshwork that is typical to cells displaying blebbing type of movement (Fackler and Grosse, 2008). This actin architecture is completely different from that of control myocyte cells containing stress actin fibers and filopodial structures. It was previously demonstrated that cell mobility dramatically changes during differentiation, with myocytes exhibiting less motility than myobalsts (Griffin *et al.*, 2010; Powell, 1973). The decreased motility could increase the probability of cell-cell contacts necessary for differentiation (Krauss *et al.*, 2005) and facilitating myocytes to fuse with each other (Nowak *et al.*, 2009). Thus, defective fusion of Kirrel3-deficient myoblasts is likely due to MPCs arrest in the rounded morphology which prevents both directional movement and the formation of tight cell-cell contacts necessary for fusion.

In conclusion, processes leading to myoblast fusion in flies and mammals may be more conserved than previously thought. Although mammalian fusion involves a complex network of adhesion molecules that does not exist in flies, the involvement of Nephrin (Sohn *et al.*, 2009) and Kirrel3/Neph2 (this study) in mouse pre-fusion events may indicate that downstream events of actin filament remodeling and membrane fusion are also evolutionary conserved. Future studies should focus at the role of these IgSF receptors in mammalian embryonic development and in adult muscle regeneration.

Materials and Methods

Cell culture

C2 cells were a gift from Dr. David Yaffe (Yaffe and Saxel, 1977). 3T3 cells that expressed the MyoD-estrogen receptor (ER) chimera protein were prepared by retroviral infection and puromycin selection. Cell lines were maintained in Dulbecco's modified Eagle's medium supplemented with 15% calf serum (Hyclone), penicillin, and streptomycin (growth medium). To induce differentiation, we used Dulbecco's modified Eagle's medium supplemented with 2% horse serum (differentiation medium, DM). Differentiation of 3T3 MyoD:ER cells was induced by the addition of DM that contained 10^{-7}M β estradiol. Muscle progenitor cells (MPCs) were isolated from the hind legs of 3- to 4-week-old mice. Muscle tissues were separated from bones and cartilage, dissected and minced, followed by enzymatic dissociation at 37°C with 0.25% trypsin-EDTA for 30 min. Cells were filtered through 100 μm membrane (Cell strainer, BD Falcon) and were cultured in rich medium (BIO-AMF-2, Biological Industries, Ltd.). To separate satellite cells from fibroblasts, pre-plating procedure was performed twice. MPCs were grown on gelatin or collagen coated plates. Differentiation was induced in DMEM containing 5% horse serum (Biological Industries). Transfections to C2 cells were performed with a Turbofect *in vitro* transfection reagent, (Fermentas), according to the suggested protocol.

Viral infection

To prepare lentiviruses, 293T cells were transfected with expression vectors CMV-VSVG, ΔNRF and lentiviral vector. The medium was collected 48 h post transfection and transferred onto MPCs or C2 cells. To prepare retroviruses, 293gp (gag, pol) cells were transfected with CMV-VSVG expression vector and retroviral vector. Virus particles collection and infections were performed as described.

Wound assay and Boyden chamber

MPCs were plated onto pre-gelatinized dishes for 48 hr. Cells were grown overnight without any serum (DM). Damage was incurred via scraping in unidirectional manner to create a gap between cells. After 8 hours in DM with cell cycle inhibitor, the cells were immunostained with D and an anti MyoD antibody and were viewed at x20 or x100 magnification under a fluorescence microscope (Olympus, model BX50). Boyden chamber assay was performed with CytoSelect™ Cell Migration Assay, 8 μm of Cell Biolab inc, according to the manufacture protocol.

Immunocytochemistry

Immunocytochemistry was performed by fixing with 4% PFA in PBS, permeabilizing with 0.1% Triton X-100 in PBS, blocking with 5% serum/PBS, and incubating with primary antibody for at least 2 h. Cells were then incubated with secondary antibodies Alexa-Fluor 488-conjugated anti-mouse and Alexa-Fluor 594-conjugated anti-rabbit (Invitrogen) for 1 h. The following primary antibodies were used: Neph2 (Santa Cruz S-14, 1:10), MyHC (MF-20 1:10), M2 Flag (Sigma 1:500), Polyclonal Flag (Sigma, 1:500) GFP (1:500), c-Myc (Santa Cruz 1:500), α tubulin (1:2000). F-actin was detected with Phalloidin-Tritc at 1:200. Nuclei were stained with 4′, 6-diamidino-2-phenylindole (DAPI, Sigma.) To detect extracellular epitopes, living cells were washed with PBS and incubated in blocking buffer (3% BSA in PBS) for 15 min. Primary antibody incubation (Kirrel3 (Sigma, 36-50, 1:200) or anti Flag (1:200) was then performed on ice for 2 hours, followed by fixation with 4%PFA in PBS and incubation with secondary antibody. These cultures were visualized and photographed in fluorescence microscope (Olympus, model BX50) or in the Zeiss LSM 700 upright confocal microscope (Thornwood, NY) equipped with an X63/1.3 NA oil objective, and solid-state lasers.

RT-PCR (Reverse Transcriptase PCR)

Total RNA extraction was performed using a commercial kit, TRI REAGENT™ (MRC). The procedure was conducted according to the manufacturer's protocol. RT reactions were performed following DNase I treatment, phenol-chloroform extraction, and ammonium acetate precipitation. RNA was incubated with 1 μl of a random hexamer (0.1 mg/ml), 4 min at 65 °C. The RT reaction mixture was added including pairs of primers, which were incubated for 45 min at 42 °C. The following pairs of primers were used: for the identification of Kirrel3 expression in C2 cells: F 5' ACTGCACACCCAAGTTGCCCG 3' R 5' TCAGGTGATGCTCTCCTGAGAG 3'. Mouse Kirrel3 mRNA sequence (NM_026324) was used to clone full length (FL) Kirrel3- by RT-PCR from C2 cells. The fragment was initially cloned into CS2+ plasmid and then sub-cloned into the viral pCLNCx v.2 vector used for retroviral infection using a reverse primer containing the Flag sequence. Kirrel3 deletion mutants were constructed by PCR amplification with specific primers that contained sequences of Flag epitope as described. All mutants were verified by DNA sequencing. The following primers were used in PCR: Fl-Kirrel3-Flag: F 5' CGGGATCCATGAGACCTTTCCAGCT GGAT 3' R 5' GCTCTAGATTATTTATCGTCATCGTCTTTGTAGTCGACGTGAGTCTGCATCCGCCG 3'; ΔIC Kirrel3-Flag: F 5' CGGGATCCATGAGACCTTTCCAGCTGGAT 3' R 5' GCTCTAGATTATTTATCG TCATCGTCTTTGTAGTCTTTGGCTGATACAACACCTTT 3';ΔEC Kirrel3-Flag: F 5'GCGGATCCCCGTCATCATCGGGGTGGCCGTA 3' R 5' GCTCTAGATTATTTATCGTCATCGTCTTTGTAGTCGACGTGAG TCTGCATCCGCCG 3'; Kirrel3 mutants with 2 tags: Flag at the N-terminus and Myc at the C-terminus were constructed by cloning PCR products to pSecTag2A plasmid (Invitrogen), using a forward primer with 5' Flag sequence and a reverse primer. The PCR fragments were ligated to the vector 3' to the signal peptide sequences and 5' to Myc epitope in the vector obtaining: N-signal peptide-Flag-Kirrel3-myc-C. Knockdown of Kirrel3 protein in satellite cells was achieved by lentiviral infection using 2 shRNA expression vectors; Kirrel3-specific Sh 5'CCGGCTTCAGGATGATGCCGTGTATCTCGAGATACACGGCATCATCCTGAAGTTTTTG 3', Non-specific Sh 5'CCGGTCCAGACCATATACAACTGTACTCGAGTACAGTTGTATATGGTCTGGATTTTTG 3' (Sigma, Mission shRNA bacterial glycerol stock).

Western Blotting

C2 cells were lysed directly in the RIPA buffer containing inhibitors. Forty micrograms of protein cell lysate were separated by SDS-PAGE, transferred onto nitrocellulose membrane, and processed for Western analysis by standard procedures. Primary antibodies used were: anti-α-tubulin (Sigma 1:10000), anti-Myosin Heavy Chain (MF-20, 1:10), anti Myogenin, anti-MyoD (Santa cruze), anti Desmin, anti Caveolin 3 (BD Biosciences). Primary antibodies were incubated overnight at 4 °C. Membranes were washed and then incubated with the appropriate horseradish peroxidase-conjugated secondary antibodies. Proteins were visualized using the enhanced chemiluminescence kit by Pierce.

Pulse Chase experiment

C2 Kirrel3-Flag myoblasts (3 h in DM) were incubated in methionine free medium for 1hr and then labeled with 150 μCi/ml S^{35}-methionine for 1hr. The proteins of one plate were extracted (pulse; time 0) and the medium in three other plates was replaced with DM enriched with non-labeled methionine. Proteins were extracted at various intervals (30, 60, 120 min) in lysis buffer (RIPA) and Kirrel3-Flag was immunoprecipitated with anti-Flag antibody (1hr at 4^0C). Proteins were eluted from Protein A/G agarose beads in Laemmli sample buffer and resolved on SDS-polyacrylamide gel.

Statistical analysis

Most experiments were repeated three times except those specifically mentioned. Data are reported as the mean ± SE. Statistical significance was analyzed using the Student's *t* test. $P < 0.05$ (*), $P < 0.01$ (**) were considered statistically significant.

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