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Dissect Kif5b in nuclear positioning during myogenesis: The light chain binding domain and the autoinhibitory peptide are both indispensable

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ABSTRACT

The microtubule motor kinesin-1 is responsible for the nuclear positioning during myogenesis. Here we show that the coiled-coil stalk/tail domain containing the kinesin light chain (KLC) binding sites targets to the perinuclear region like endogenous Kif5b, while the globular tail domain cannot. To investigate which fragments of kinesin heavy chain (Kif5b) is responsible for the myonuclear positioning, we transfect Kif5b expression constructs into Kif5b deficient myoblasts and test their ability to rescue the myonuclear phenotype. We find that the KLC binding domain and the autoinhibitory peptide in the globular tail region are both indispensable for the nuclear membrane localization of Kif5b and the kinesin-1-mediated myonuclear positioning. These results suggest that while the KLC binding domain may directly targets Kif5b to the myonuclear membrane, the autoinhibitory peptide may play an indirect role in regulating the kinesin-1-mediated myonuclear positioning.

1. Introduction

Kinesin-1, the first discovered microtubule-based kinesin motor, consists of two heavy chains (KHCs) and two light chains (KLCs). The two KHCs form a homodimer, and each heavy chain associates with a light chain. In most non-neuronal mammalian cells, a ubiquitous heavy chain isoform Kif5b expressed along with two light chain isoforms KLC1 and KLC2, while in skeletal muscles, KLC2 is known to be the dominant light chain isoform [1]. KHC contains a head domain composed of a microtubule binding site and an ATP-binding/hydrolysis pocket. The following stalk/tail domain is important for interacting with KLC and other holoenzyme subunits or cargos [2–4]. KLCs contain a highly conserved N-terminal heptad repeat (HR) domain that is responsible for binding to KHC, and a following six modular imperfect repeats of 34 amino acids called tetratrico peptide repeats (TPR), which is known to bind to diverse cargos [5].

During myogenesis, the expression of Kif5b is up-regulated, implying significant function for this motor protein [6]. In wild type muscle fibers, the myonuclei redistribute along the elongating myotubes with proper distance between every two of them, and finally move to the periphery of the mature muscle fibers beneath the sarcolemma. However, the nuclei centrally aggregated in Khc/Kif5b deficient *drosophila* and cultured mammalian myotubes [7], as well as in *Kif5b* conditionally knockout mouse muscle fibers [6], which demonstrated an important function of kinesin-1 in myonuclear positioning.

It has been demonstrated that Ensconsin/MAP7 acts as a linker between Khc/Kif5b and the microtubule cytoskeleton in myogenesis. Kif5b motor domain fused with microtubule-binding domain of MAP7 rescues the centrally aggregated nuclei in MAP7 deficient myotubes [7]. But how Kif5b recruits to myonuclear membrane is less studied. KASH domain family members localized at the outer nuclear membranes and SUN domain family members localized at the inner nuclear membranes bridges the nuclear lamina to the cytoskeleton system [8]. For the recruitment of Kif5b to the nuclear membrane, a KASH protein UNC-83 serves as the adaptor for kinesin-1 in *Caenorhabditis elegants* [9]. In mammalian cells, one of the KASH family members, nesprin 2, associates with Kif5b in developing neuronal tissues [10]; and this interaction is mediated by KLC1 in several cell lines tested [11]. In skeletal muscles development, nesprin 1–3 are expressed [12,13]. Nesprin 1 and 2 are important for myonuclear envelope integrity [14]. Nesprin 1 links the nucleus to the actin cytoskeleton and is important for nuclear positioning [15], while nesprin 3 functions in linking the nucleus to the intermediate filaments [13]. Another KASH family member, nesprin 4, has not been studied in skeletal muscles. Nesprin 4 coimmunoprecipitates KHC in several epithelial cell lines. Yeast-two hybrid

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analysis revealed KLCs 1, 2, 3, and 4 to be nesprin 4 binding partners [16].

In our experiment, we found that both endogenous Kif5b and the transfected Kif5b full-length tail strongly localize at the outer nuclear membrane in differentiating myoblasts. Functional analysis demonstrated that in addition to the KLC binding domain, the C-terminal globular tail domain, especially the autoinhibitory peptide, is indispensable for the Kif5b-mediated myonuclear positioning. The coiled-coil stalk/tail domain of Kif5b is necessary for the myonuclear membrane targeting, while the globular tail domain cannot target to nuclear membranes alone, suggesting other regulatory mechanisms involved.

2. Materials and methods

2.1. DNA constructs

HA-tagged and GST-tagged Kif5b constructs were described previously [6]. For Flag-tagged Kif5bs, Kif5b fragments were subcloned with a 3× Flag tag at N-terminus into pcDNA3.1. Mouse KLC2 and nesprin 4 cDNA was amplified by RT-PCR from differentiated wild type myoblast cells and sequenced correct. For Flag-tagged KLC2 constructs, KLC2 1–187 aa or 146–619 aa was subcloned with a 3×Flag tag at N-terminus into pcDNA3.1 to generate pKLC2-HR or pKLC2-TPR, respectively. For HA-tagged nesprin 4, full length nesprin 4 was subcloned with an HA tag at C-terminus into pcDNA3. For His-tagged nesprin 4, nesprin 4 was subcloned into pET28a to generate pET-nesprin 4.

2.2. Antibodies

Kif5b antibody was generated in rabbits against synthesized peptide: FDKEKANLEAFTADKDIA, and purified IgG was used in this study [17]. KLC antibody 63–90 was kindly provided by Dr. S.T. Brady (University of Illinois at Chicago, USA). Other antibodies were as follows: anti-lamin A/C, anti-His and anti-GST were all from Santa Cruz. FITC-Phalloidin (Sigma) was used for F-actin labeling.

2.3. Primary culture of myoblast cells

The primary myoblast cells from wild type ($kif5b^{+/fl}$) and Kif5b conditionally knockout mice ($Pax2\text{-}Cre: Kif5b^{-/fl}$) were isolated as described [6]. The myoblast cells were cultured in DMEM growth medium supplemented with 15% FBS and 2.5 ng/ml bFGF. Differentiation was promoted by changing medium to DMEM supplemented with 3% horse serum [18].

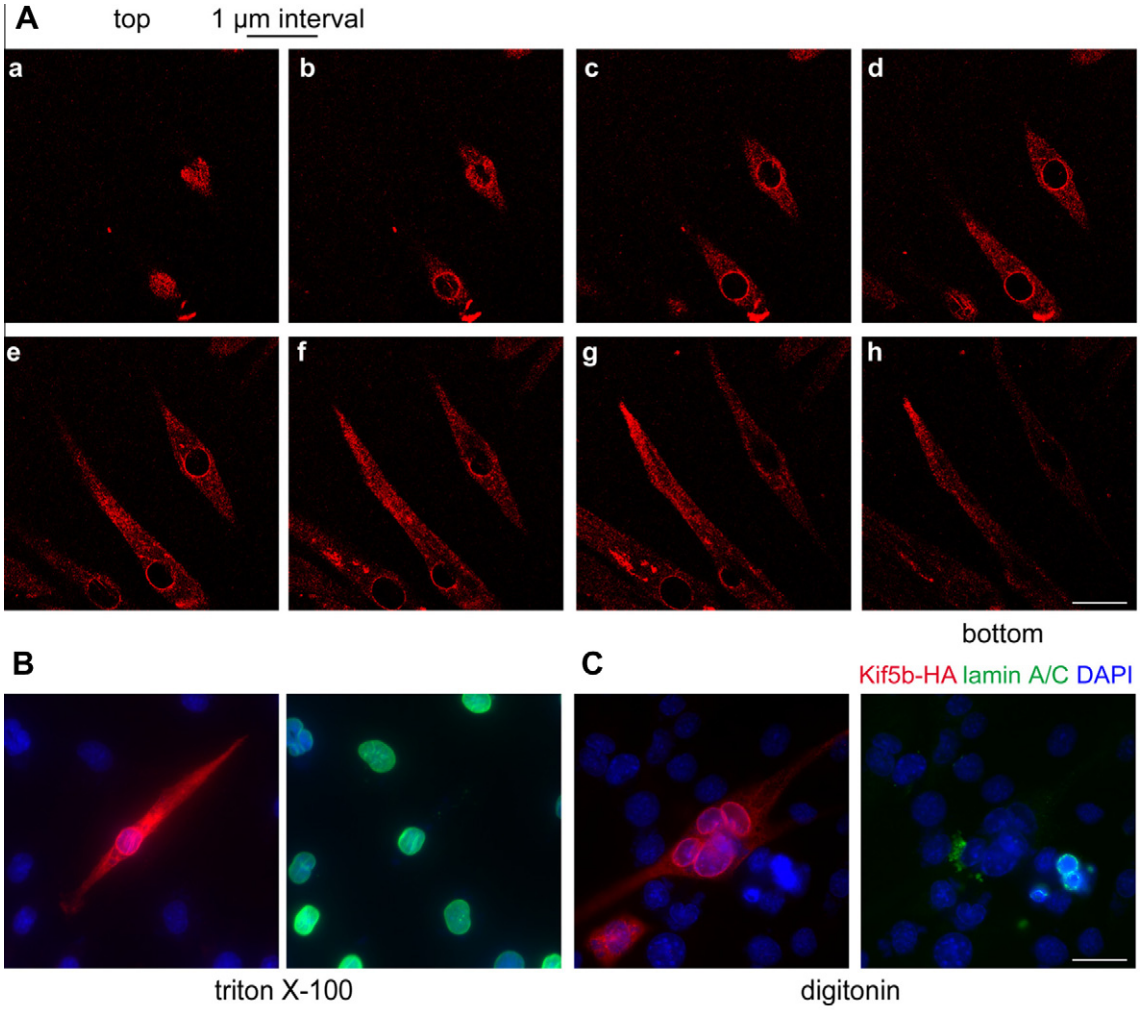

Fig. 1. Kif5b localizes at the outer nuclear membranes. (A) Wild type myoblast cells differentiated for 2 days were immunostained against endogenous Kif5b. The cells were scanned in Z-stack at 1 μm interval. Note that Kif5b formed a ring-like pattern at the nuclear membrane region (b–g). (B and C) Myoblast cells were permeabilized with 0.2% Triton X-100/PBS (B) or 30 μg/ml digitonin (C) before immunostaining. Kif5b staining at the nuclear membrane region was observed with only plasma membrane permeabilization (C), while lamin A/C as a nuclear protein control could only be stained with nuclear membrane permeabilization (B). Scale bar: 20 μm.

For transfection of plasmid DNA into the myoblast cells, 3 μl Fugene 6 (Roche) was diluted with 50 μl opti-MEM (Invitrogen), and then 1 μg plasmid DNA was added to the transfection reagents. The complex was incubated for 20 min at RT. Myoblast cells for transfection were digested with trypsin/EDTA and resuspended in differentiation medium. 3×10^4 cells were mixed with the transfectant and seeded in 8-well chamber slides (Nunc). The chamber slide was pre-coated with 100 μg/ml poly-L-ornithine (Sigma) at RT overnight, followed by 10 μg/ml laminin (Sigma) at 37 °C overnight in a humidified incubator.

2.4. Immunofluorescence

For staining on cultured cells, myoblast cells differentiated for 2–3 days in 8-well chamber slides were fixed with 4% PFA/PBS at 4 °C for 10 min, and permeabilized with 0.2% Triton X-100/PBS or 30 μg/ml digitonin for 10 min at 4 °C as indicated. The cells were then incubated with primary antibodies at 4 °C overnight, followed by FITC- or Cy3-conjugated secondary antibodies at RT for 1 h. Slides were mounted with *SlowFade*® Gold antifade reagent with DAPI (Invitrogen). Images were captured by fluorescence

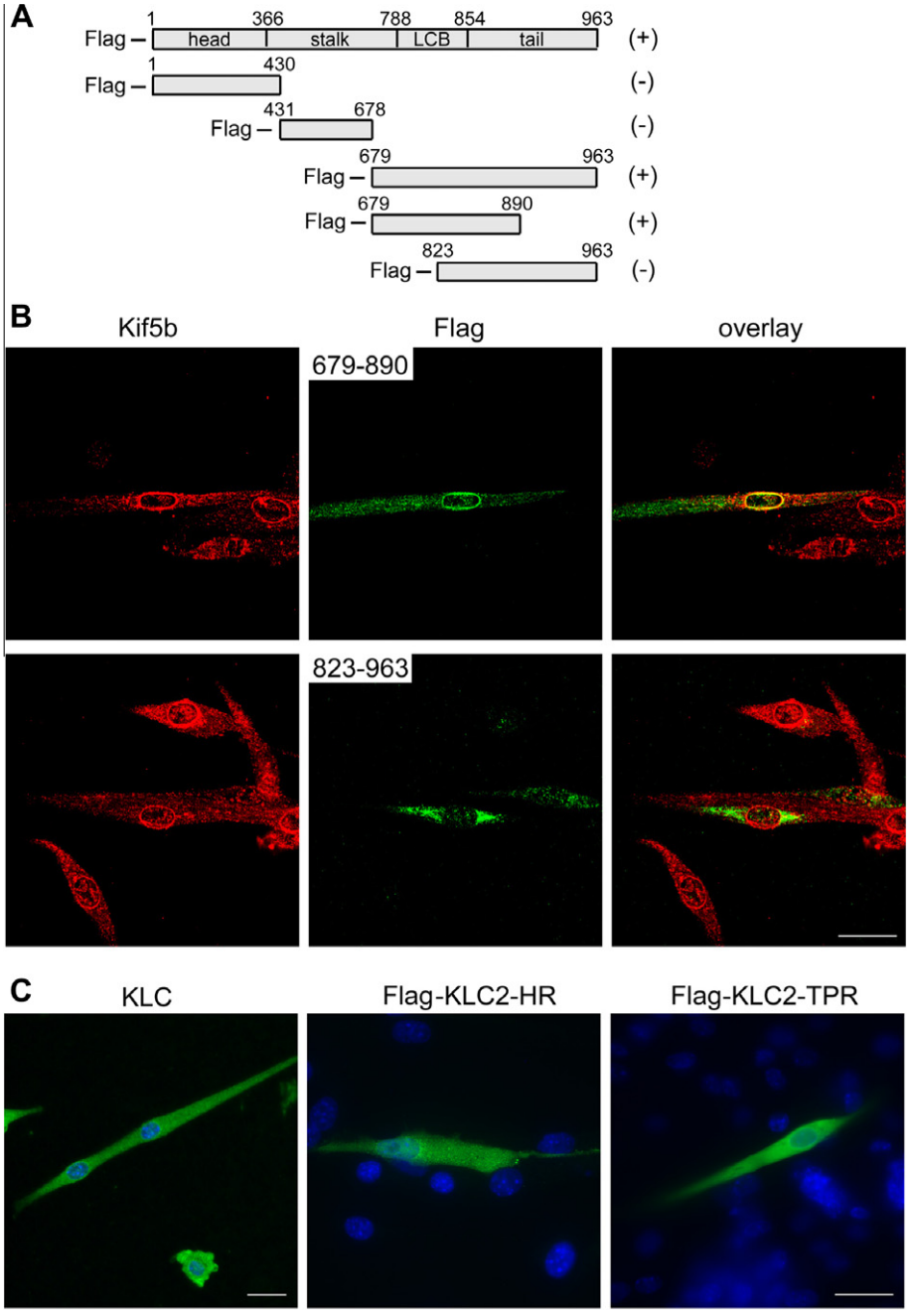

Fig. 2. Kif5b 679–890 aa targets to the nuclear membranes. (A) Flag-tagged Kif5b fragments were transfected into myoblasts upon changing into differentiation medium. The cells were immunostained against the Flag tag in myoblast cells differentiated for 2–3 days. + and − indicate the capability and incapability in nuclear membrane targeting. LCB, light chain binding domain. (B) Representative results of Kif5b 679–890 aa (upper panel) or 823–963 aa (lower panel) transfected cells. The cells were double stained with anti-Kif5b and anti-Flag antibodies. (C) Distribution of endogenous KLC (left panel), Flag-tagged KLC2 1– 187 aa (middle panel) and KLC2 146–599 aa (right panel). Scale bar: 20 μm.

microscope Olympus BX51 or confocal microscope Carl Zeiss LSM700.

2.5. Pull-down assays

His-tagged and GST-tagged proteins were expressed in BL21 *Escherichia. coli* cells overnight at 16 °C with 0.5 mM IPTG induction. The bacterial cells were then collected and resuspended in lysis buffer as indicated below and sonicated for 3 min (5 s on/9 s off) to release the soluble fusion proteins. The *E. coli* cell debris were removed by centrifugation at 12,000*g* for 30 min at 4 °C, and the crude fusion protein solution were used in experiments. For His pull-down assay, His-tagged EGFP or nesprin 4 were lyzed in PBS with protease inhibitor cocktail (Roche) and incubated with 40 μl His-Select® Nickel Affinity Gel (Sigma) for 1 h at 4 °C. GST-tagged Kif 679–890 proteins were lyzed in PBS, and the soluble fraction was incubated with His-tagged proteins pre-bound to Nickel-loaded agarose beads for 1 h at 4 °C. For GST pull-down assay, GST-Kif5b fragments were lyzed in PBS with protease inhibitors and incubated with 40 μl glutathione-Sepharose 4B beads (Amersham Pharmacia Biotech) for 1 h at 4 °C, and then incubated with His-tagged nesprin 4 lyzed in TNET buffer (50 mM Tris–Cl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1 mM DTT and protease inhibitors) for 1 h at 4 °C.

3. Results and discussion

3.1. Kif5b localizes at the outer nuclear membranes in differentiating myoblast cells

Our previous study showed that the cell nuclei centrally aggregated in the *Kif5b* conditionally knocked out skeletal muscle fibers, as well as in *in vitro* differentiated *Kif5b* knockout myoblasts, demonstrating an important role of Kif5b in myonuclear positioning [6]. Immunofluorescence using anti-Kif5b antibodies showed that Kif5b exhibited strong fluorescence staining signal at the nuclear membrane region as well as in the cytoplasm; especially the Z-stack scanned images clearly showed that Kif5b formed an empty sphere around the nuclei (Fig. 1A). To ensure that Kif5b was localized at the outer but not the inner nuclear membranes, we used digitonin to permeabilize plasma membrane while retaining the nuclear membrane intact before applying the antibody to the cells. As a control, nuclear lamin A/C could not be stained without nuclear membrane permeabilization, whilst Kif5b still show staining signals around the nuclei, confirming that the signals were from the outer nuclear membranes (Fig. 1B and C).

3.2. Kif5b 679–890 aa contains signal for nuclear membrane targeting

To determine whether the coiled-coil stalk/tail domain or the globular tail domain targets to the nuclear membrane, we dissected Kif5b into several Flag-tagged fragments and transfected them into the wild type or mutant myoblast cells to examine their localization. We found that the full length stalk/tail domain (679–963 aa) and coiled-coil stalk/tail domain (679–890 aa), but not the head domain (1–430 aa), the stalk domain (431–678 aa), or the partial tail domain (823–963 aa), strongly target to the nuclear membrane (Fig. 2A and B), indicating that the coiled-coil stalk/tail domain 679–890 aa is important for nuclear membrane targeting. In addition, this domain is not overlapped with the MAP7 binding domain (327–536 aa), which links Kif5b to the microtubules through MAP7 [7], suggesting that Kif5b may simultaneously interact with the nuclear membrane adaptors and the microtubule adaptors, and mediate myonuclear dispersion on microtubule networks.

As 679–890 aa covers the major KLC binding site (788–813 aa) and minor binding site (814–854 aa) [19], we also tested whether it is possible that KLC mediated its targeting to the nuclear membranes. We performed immunofluorescence against endogenous KLC, or against the overexpressed Flag-tagged KLC HR (1–187 aa) domain which binds to heavy chain or TPR (146–619 aa) domain which binds to most of its cargos [5]. Endogenous KLC and KLC fragments showed mainly cytoplasmic staining, while slight perinuclear signal can also be observed (Fig. 2C). To exclude the interference of endogenous Kif5b in wild type cells, we also transfected these KHC and KLC expression constructs in mutant myoblast cells and got similar staining pattern (data not shown). As KLC might bind to diverse cargos in a cell, it is reasonable that most of KLC proteins localize in the cytoplasmic region. Meanwhile, the slight perinuclear localization of KLC suggests that it may also participate in the nuclear membrane targeting of kinesin-1.

3.3. Kif5b interacts with nesprin 4 in vitro

As overexpressed Kif5b 679–890 aa strongly targeted to the myonuclear membrane, we hypothesized that Kif5b may directly interact with the presumptive nuclear membrane adaptors. The KASH family membrane nesprin 2 and 4 have been reported to interact with kinesin-1 [10,11,16]. As nesprin 2 is a giant protein

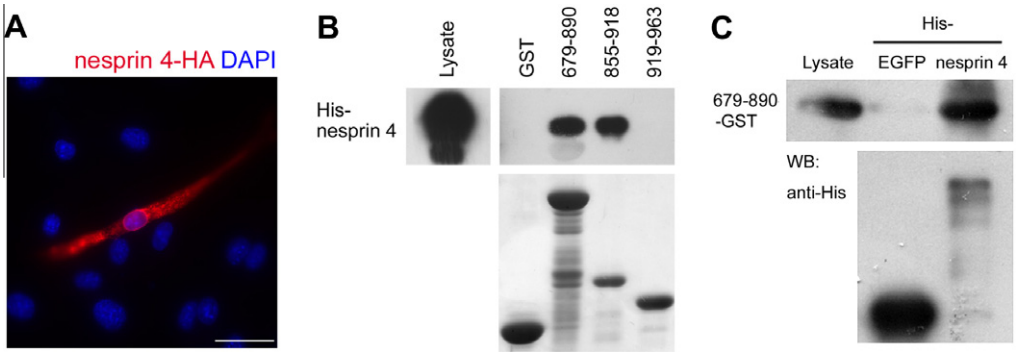

Fig. 3. Kif5b 679–890 directly interacts with nesprin 4. (A) Immunofluorescence against HA-tagged nesprin 4 in differentiated myoblast cells. Scale bar: 20 μm. (B) GST-tagged Kif5b fragments 679–890 aa and 855–918 aa, but not 919–963 aa or GST alone could pull down His-nesprin 4. Upper panel shows the Western blot result of the pull-down elutes using anti-His antibody. Lower panel shows the coomassie blue staining result of the GST-tagged bait protein lysates. (C) His-nesprin 4, but not His-EGFP, was able to pull down GST-tagged Kif5b 679–890 aa. Upper panel shows the Western blot result of the pull-down elutes using anti-GST antibody. Lower panel shows the Western blot result of the His-tagged bait proteins using anti-His antibody. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

about 800 kDa which is difficult for cloning and expression, we chose nesprin 4 (~42 kDa) to study the protein–protein interaction. We found that nesprin 4 was expressed in myoblasts differentiated for 2 days by RT-PCR (data not shown). We then cloned the coding sequence of nesprin 4 from the cDNA of the differentiated myoblasts. HA-tagged nesprin 4 targeted to the nuclear membrane in the myotubes (Fig. 3A), showing that it might be one of the nuclear membrane adaptors for cytoskeleton network during myogenesis. To test whether Kif5b has direct interaction with nesprin 4, we expressed GST-tagged Kif5b 679–890 aa, 855–918 aa and 919–963 aa to pull down His-tagged nesprin 4 expressed in BL21 *E. coli* cells. The globular tail domain 919–963 aa could not pull down nesprin 4. In contrast, 679–890 aa and 855–918 aa could pull down nesprin 4 (Fig. 3B). Alternatively, we performed His pull-down assay. His-tagged nesprin 4 could efficiently pull-down GST-Kif 679–890, while His-tagged EGFP could not (Fig. 3C). These data together verify that Kif5b coiled-coil stalk/tail domain 679–890 aa has the capacity to directly interact with nesprin 4, which might have potential function in anchoring Kif5b at nuclear membranes. However, as shown above, 823–963 aa covering the nesprin 4 binding site 855–918 aa could not target to the nuclear membrane, suggesting that the 679–854 aa α-helix domain in the stalk region, which covers the KLC binding domain, is indispensable for the nuclear membrane targeting of Kif5b, and this process may be nesprin 4-independent.

3.4. The KLC binding domain and the globular tail domain of Kif5b are both indispensable for myonuclear dispersion

In previous report, we have shown that transfection of Kif5b expression vector into the mutant myoblast cells rescued the localization of several potential cargo proteins [6]. To confirm that Kif5b was responsible for the myonuclear positioning, we performed a similar rescue assay by overexpressing Kif5b in the mutant cells. As previous demonstrated [6], the wild type cells fused into multi-nuclear myotubes with the nuclei distributed along the shaft after differentiation (Fig. 4A, left panel). However, the nuclei aggregated in the centre of the mutant cell-derived myotubes (Fig. 4A, right panel). We transfected a series of Kif5b expression constructs and monitored the nuclear dispersion in multinuclear myotubes (Fig. 4B). We noticed that the exogenous expressed Kif5b 1–918 aa and Kif5b lack the KLC binding domain (dLCB) tended to accumulate at the bipolar tips of the transfected myoblasts, but did not localize at the nuclear membrane region; while Kif5b 1–929 aa showed a similar localization pattern like the full-length Kif5b (Fig. 4C). The exogenous expression of full-length Kif5b and Kif5b 1–929 aa promoted the nuclei dispersion in more than 50% transfected elongating mutant myotubes with more than two nuclei. In contrast, Kif5b 1–918 aa or Kif5b lack the KLC binding domain failed to rescue the nuclei positioning in 30 transfected multinuclear myotubes (Fig. 4C). These data indicated that the KLC binding domain which may function in the nuclear membrane targeting, together with the globular tail domain, especially 919–929 aa, are both indispensable for the perinuclear localization of Kif5b and nuclei dispersion in fused myotubes.

The 919–929 aa contains the autoinhibitory peptide QIAKPIRP [20], which can bind to the motor domain of Kif5b and inhibit the initial step of microtubule-stimulated ADP release when Kif5b is first loaded on the microtubule [21]. It is interesting that Kif5b fragment (823–963 aa) covering this peptide cannot target to the myonuclear membranes but plays an important role in regulating the localization of full length Kif5b and myonuclear trafficking. One explanation is that the autoinhibitory conformation of Kif5b is a prerequisite for its binding to the nuclear membrane anchors. The mechanism for the significance of the inhibitory peptide and the role of KLC and other KASH domain family members in kinesin-1-meidated myonuclear positioning need further investigation.

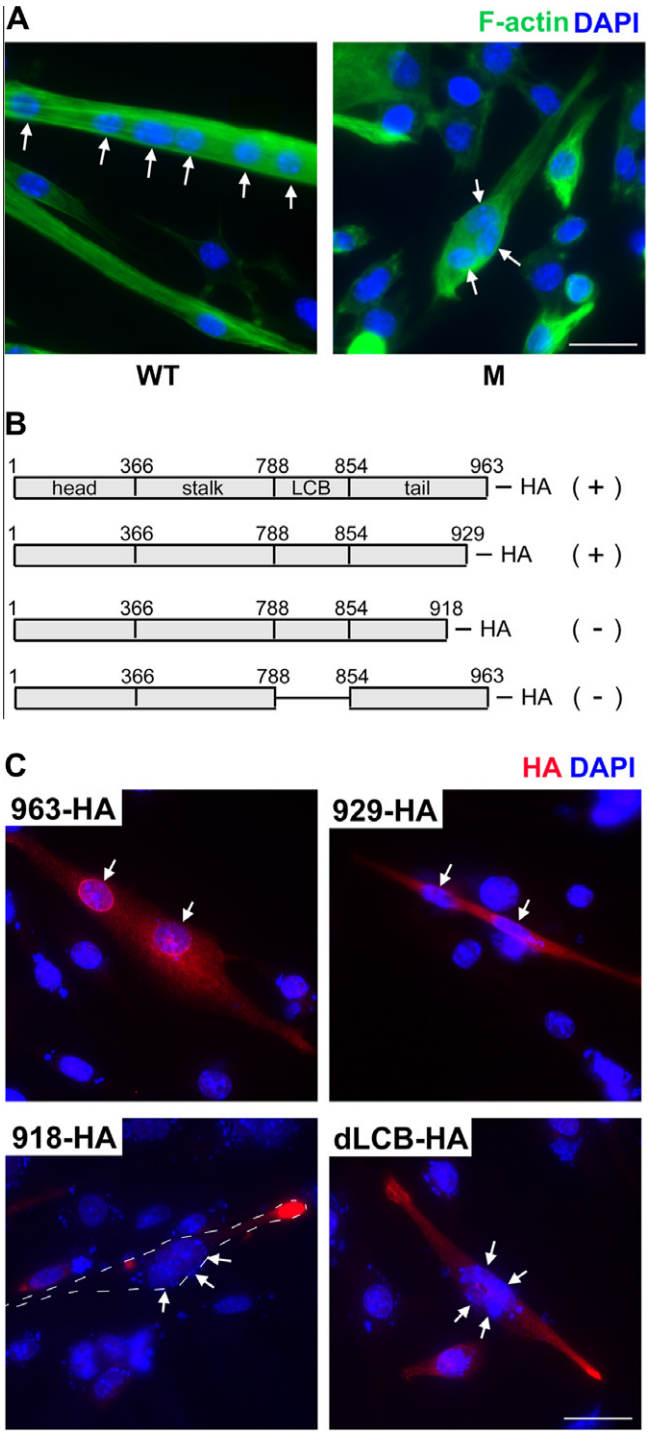

Fig. 4. Kif5b rescues the myonuclear positioning in *Kif5b* knockout mutant myotubes. (A) Wild type or *Kif5b* knockout mutant myoblast cells differentiated for 2 days were labeled with FITC-Phalloidin and DAPI. The nuclei dispersed throughout the long axis of the wild type cells, but aggregated in the centre of the mutant cells. (B) HA-tagged Kif5b constructs were transfected into mutant cells when changing into differentiation medium to perform the rescue assays of nuclei dispersion. + and – indicate the capability and incapability in promoting nuclear dispersion. LCB, light chain binding domain. (C) Representative results of Kif5b constructs transfected myotubes. The cells were stained with anti-HA antibody and DAPI. The nuclei in transfected cells are marked with arrows. Dashed line shows the cell boundary. Scale bar: 20 μm.

Acknowledgments

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