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**Nonmuscle myosin IIA and IIB differentially contribute to intrinsic and directed migration of human embryonic lung fibroblasts**

Masahiro Kuragano<sup>a</sup>, Yota Murakami<sup>a,b</sup>, Masayuki Takahashi<sup>a,b\*</sup>

<sup>a</sup>Graduate School of Chemical Science and Engineering, Hokkaido University, Sapporo, Hokkaido 060-8628, Japan;

<sup>b</sup>Department of Chemistry, Faculty of Science, Hokkaido University, Sapporo, Hokkaido 060-0810, Japan.

\*Address correspondence to: Masayuki Takahashi, Ph.D. (takahash@sci.hokudai.ac.jp)

## **Abstract**

Nonmuscle myosin II (NMII) plays an essential role in directional cell migration. In this study, we investigated the roles of NMII isoforms (NMIIA and NMIIB) in the migration of human embryonic lung fibroblasts, which exhibit directionally persistent migration in an intrinsic manner. NMIIA-knockdown (KD) cells migrated unsteadily, but their direction of migration was approximately maintained. By contrast, NMIIB-KD cells occasionally reversed their direction of migration. Lamellipodium-like protrusions formed in the posterior region of NMIIB-KD cells prior to reversal of the migration direction. Moreover, NMIIB KD led to elongation of the posterior region in migrating cells, probably due to the lack of load-bearing stress fibers in this area. These results suggest that NMIIA plays a role in steering migration by maintaining stable protrusions in the anterior region, whereas NMIIB plays a role in maintenance of front-rear polarity by preventing aberrant protrusion formation in the posterior region. These distinct functions of NMIIA and NMIIB might promote intrinsic and directed migration of normal human fibroblasts.

## **Keywords:**

Cytoskeleton, nonmuscle myosin II, cell migration, migratory cell polarity

Abbreviations:

1P-RLC, monophosphorylated regulatory light chain at Ser19; 2P-RLC, diphosphorylated regulatory light chain at Thr18 and Ser19; BBS, blebbistatin; IDM, intrinsic and directed migration; KD, knockdown; mAb, mouse monoclonal antibody; MTOC, microtubule-organizing center; NLS, nuclear localization signal; NMHC, nonmuscle myosin heavy chain; NMII, nonmuscle myosin II; pAb, rabbit polyclonal antibody; RLC, regulatory light chain; S1, subfragment-1; siRNA, small interfering RNA.

## 1. Introduction

Cell migration is essential for embryogenesis, the immune response, and wound healing [1]. Cells can exhibit directionally persistent migration in response to various external cues, such as gradients of chemoattractants in chemotaxis and substrates with stiffness gradients in durotaxis. Certain cells with a high level of intrinsic directionality display directionally persistent migration even in the presence of uniform concentrations of chemoattractants [2]. Reorganization of the actin cytoskeleton and microtubules is reportedly important for establishment of front-rear polarity and proper advancement of the leading edge during directional cell migration [2–4]. However, the molecular mechanism via which intrinsic cell directionality is maintained remains unclear.

Nonmuscle myosin II (NMII), an actin-based motor protein, plays important roles in directional cell migration via actin cytoskeleton organization [5,6]. NMII is composed of two heavy chains (NMHC-IIs) and two pairs of light chains. This motor protein has two globular heads, which are involved in motor activity, and a long rod-like tail, which is involved in filament formation. In mammalian cells, there are three isoforms of NMII, namely, NMIIA, NMIIIB, and NMIIIC. *In vitro* studies suggest that NMIIA and NMIIIB, which are the two major isoforms, mainly function in the translocation (a motor property) and crosslinking (a structural property) of actin filaments, respectively [5,7]. NMIIA is indicated to play a role in the

formation of a stable protrusion in the anterior region of fibroblasts through the proper regulation of Rac1 signaling [8]. On the other hand, NMIIB is suggested to promote directional migration by mediating the formation of contractile actomyosin structures in the posterior region [9]. However, there remain unanswered questions regarding the roles of NMII isoforms in intrinsic and directed migration (IDM) of cells.

This study examined the effect of knockdown (KD) of specific NMII isoforms on the directional migration of normal human fibroblasts, and suggested that distinct functions of NMIIA and NMIIB promote the intrinsic directionality.

## **2. Materials and methods**

### *2.1. Cell culture*

TIG-1 cells (JCRB0501, TIG-1-20; human embryonic lung fibroblasts) were obtained from the Health Science Research Resources Bank and maintained in MEM alpha (GIBCO/Life Technologies) supplemented with 10% fetal bovine serum, 50 U/mL penicillin, and 50 µg/mL streptomycin. Cells were cultured at 37°C in humidified air containing 5% CO<sub>2</sub>.

### *2.2. Plasmids*

pEGFP-NMHC-IIB was a kind gift from Dr. Robert S. Adelstein (NIH). pmCherry-NMHC-IIA was constructed as previously described [10]. pmCherry-actin was a kind gift from Dr. Keiju Kamijo (Tohoku Medical and Pharmaceutical University). pmCherry-tagged nuclear localization signal (NLS) was a kind gift from Dr. Toshiaki Imagawa (Hokkaido University). Construction of pNMIIB-S1-R709C-EGFP was described in a recent paper [11]. The nucleotide sequences of the DNA fragments amplified by PCR were verified by sequencing using an ABI PRISM 310 DNA sequencer (Applied Biosystems).

### *2.3. Transfection*

Cells were transfected with plasmids using Xfect Transfection Reagent (Takara Bio USA) in fetal bovine serum- and antibiotic-free OPTI-MEM (GIBCO/Life Technologies), according to the manufacturer's protocol. Transfected cells were replated onto coverslips (Matsunami) or glass-bottom dishes (IWAKI, ASAHI GLASS) precoated with 10 µg/mL fibronectin (Roche Diagnostics) for immunofluorescence or time-lapse observation, respectively.

### *2.4. KD analysis*

Human NMHC-IIA-specific (GGCCAAAGAGAACGAGAAGUU), human NMHC-IIB-specific (GGAUCGCUACUAUUCAGGAUU), and nonsense (GCGCGCUUUGUAGGAUUCGUU) small interfering RNAs (siRNAs) were purchased from Thermo Scientific Dharmacon. siRNAs were transfected using Lipofectamine RNAiMAX Transfection Reagent (Invitrogen), according to the manufacturer's protocol. After transfection for 72 h, immunofluorescence or time-lapse observation was performed. To express exogenous proteins, cells were transfected with the respective expression vector at 24 h after siRNA transfection.

### 2.5. Immunofluorescence

Indirect immunofluorescence was performed as described by Kiboku *et al.* [10]. The following primary antibodies were used: anti- $\alpha$ -actinin rabbit polyclonal antibody (pAb) (a kind gift from Dr. Keiju Kamijo), anti-diphosphorylated regulatory light chain at Thr18 and Ser19 (2P-RLC) pAb (Cell Signaling Technology), anti-monophosphorylated regulatory light chain at Ser19 (1P-RLC) mouse monoclonal antibody (mAb) (a kind gift from Dr. Yasuharu Sasaki, Kitasato University). Cy3- or FITC-conjugated goat anti-rabbit IgG (H+L) and FITC-conjugated goat anti-mouse IgG (H+L) (Jackson ImmunoResearch Laboratories) was used as the secondary antibody. For direct immunofluorescence, anti-NMHC-IIB and anti-NMHC-IIA

pAbs [12,13] were labeled with Alexa Fluor 488 and Alexa Fluor 596, respectively, using the Zenon Antibody Labeling Kit (Molecular Probes), according to the manufacturer's protocol. Actin filaments were stained with TRITC-phalloidin (30 ng/mL; Sigma-Aldrich) or Alexa Fluor 350-conjugated phalloidin (0.5 µg/mL; Molecular Probes). Images were captured using a conventional fluorescence microscope (BX50WI; Olympus) equipped with a single-chip color CCD camera (DP70; Olympus) and an objective lens (UPlanApo 20×/0.70 NA; UPlanApo 60×/0.90 NA; UPlanFl 100×/1.30 NA Oil; Olympus) together with DP Controller software (Olympus). All procedures were performed at room temperature. Immunofluorescence images were analyzed using ImageJ software (NIH).

## *2.6. Time-lapse observation*

Time-lapse images were captured with an inverted microscope (IX71; Olympus) equipped with a single-chip color CCD camera (DP70; Olympus) and an objective lens (LCPlanFl 20×/0.40 NA; UPlanApo 60×/0.90 NA; UPlanFl 100×/1.30 NA Oil; Olympus). During observation, cells were warmed on a thermoplate set to 37°C (MATS-U55R30; Tokai Hit). Images were captured every 5 min and analyzed using Lumina Vision version 2.4.2 software (Mitani Corporation). Images of cells expressing mCherry-NMHC-IIA and EGFP-NMHC-IIB were captured using an inverted microscope (Ti-E; Nikon) equipped with an oil-

immersion objective lens (Plan Apo-VC 60×/1.40 NA; Nikon). During observation, cells were warmed in an incubation chamber heated to 37°C (INUBG2H-TIZB; Tokai Hit). Images were captured and analyzed using NIS-Elements C software (Nikon).

### **3. Results**

#### *3.1. NMIIA and NMIIB contribute to IDM of fibroblasts by steering migration and maintaining front-rear polarity, respectively*

While studying the migration of several types of fibroblasts, we noticed that human embryonic lung fibroblasts (TIG-1, MRC-5, and WI-38 cells) exhibited IDM (e.g., see Supplementary Movie S1 and Fig. S1) for a long duration (more than 6 hours). To elucidate the underlying molecular mechanism, we investigated the involvement of NMII using TIG-1 cells. Upon treatment with blebbistatin (BBS), a NMII ATPase inhibitor, aberrant protrusions formed throughout TIG-1 cells and a long tail remained in the posterior region. BBS-treated cells moved unsteadily, resulting in a decrease in directional persistence and an increase in migration speed (Supplementary Movie S2 and Fig. S1).

Next, to elucidate how NMII isoforms contribute to this directional persistence, we knocked down each isoform individually (Fig. 1, Supplementary Fig. S2, and Movies S3–5).

The level of NMHC-IIC was below the detection limit of immunoblotting in TIG-1 cells and other types of human embryonic lung fibroblasts (MRC-5 and WI-38 cells) (Supplementary Fig. S2); therefore, we examined the effects of KD of NMIIA and NMIIB. Based on the immunoblotting results, we roughly estimated that expression of NMHC-IIA was about 2.6-fold higher than that of NMHC-IIB in TIG-1 cells (Supplementary Fig. S2). Control siRNA-treated cells exhibited IDM for a long duration (Supplementary Movie S3, and Fig. 1B). NMIIA-KD cells migrated unsteadily, but their direction of migration was approximately maintained (Supplementary Movie S4, and Fig. 1A and B). Unstable protrusions formed in the anterior region of NMIIA-KD cells. On the other hand, NMIIB-KD cells showed IDM, at least for a short duration, but they suddenly reversed their direction of migration (Supplementary Movie S5, and Fig. 1A and B), resulting in a decrease in directional persistence compared with control and NMIIA-KD cells (Fig. 1C). These results suggest that NMIIA and NMIIB contribute to IDM of fibroblasts by steering migration and maintaining front-rear polarity, respectively. NMIIB-KD cells migrated about 2-fold faster than control cells (Fig. 1D). This increase is comparable to that observed for embryonic fibroblasts derived from NMIIB-knockout mice [14]. The increased migration speed of TIG-1 cells upon BBS treatment (Supplementary Fig. S1D) is likely due to the inhibition of NMIIB activity. NMIIB might play a role in deceleration of cell migration.

### *3.2. NMIIB prevents formation of lamellipodium-like protrusions in the posterior region*

Focusing on the tail region of migrating cells, we found that the tail could retract in NMIIB-KD cells, but not in BBS-treated cells (Supplementary Movie S7 and Fig. 2A). This is probably because adhesion to the substrate is weakened in the tail region [8] and active NMIIB is present in this region, as mentioned later. Lamellipodium-like protrusions formed in the posterior region of NMIIB-KD cells prior to reversal of the migration direction (Supplementary Movie S8, and Fig. 2A and B). Positioning of the microtubule-organizing center (MTOC) at the anterior side of the nucleus establishes front-rear polarity in migrating fibroblasts on a 2D surface [4]. The positioning of the MTOC in migrating TIG-1 cells was consistent with this finding (Supplementary Movie S9 and Fig. S3). Even during the unsteady migration of NMIIB-KD cells, the MTOC reoriented to the anterior side of the nucleus (Supplementary Movie S10 and Fig. S3). However, when the migration direction of NMIIB-KD cells was reversed, the MTOC did not reorient toward the new anterior side (Supplementary Movie S11 and Fig. S3). These results imply that formation of lamellipodium-like protrusions in the posterior region, rather than MTOC repositioning, triggers the reversal of front-rear polarity in NMIIB-KD cells. NMIIB can likely maintain front-rear polarity by preventing formation of lamellipodium-like protrusions in the posterior region.

### *3.3. NMIIA and NMIIB maintain the shapes of the anterior and posterior regions, respectively*

Migrating TIG-1 cells had a highly polarized shape reminiscent of a broom (e.g., see control siRNA-treated cells in Fig. 3A). This polarized shape could be important for IDM of fibroblasts. Next, we examined the involvement of the localization of each NMII isoform in the shape of TIG-1 cells. In control cells, NMIIA was mostly localized throughout the cell and NMIIB colocalized with NMIIA, except in the anterior region (Fig. 3A), as previously demonstrated in other cell types [12,15–18]. Both isoforms were particularly enriched at the lateral sides of the cell body, and the level of NMIIA was very low in the thin tail, except at the very end. Similar findings were made in living cells expressing fluorescent NMII isoforms (Supplementary Movie S12 and Fig. 3B). NMIIA KD led to the formation of multiple protrusions in the anterior region, resulting in an increase in cell area (Fig. 3C). This morphological phenotype is similar to that of NMIIA-null mouse embryonic stem cells [8]. On the other hand, NMIIB KD led to elongation of the posterior cell body and tail, even though NMIIA was localized here, resulting in an increase in the axis ratio (Fig. 3D). These morphological defects were mostly rescued by exogenous expression of the knocked down NMII isoform, but not by exogenous expression of the other isoform (Supplementary Fig. S4).

To identify the region of NMII isoforms responsible for this rescue of morphological defects, we examined the effects of exogenous chimeric NMIIs on cell shape (Supplementary Fig. S4). Localization of NMII isoforms in migrating cells is directed by the C-terminal tail region of their heavy chain subunits [16], which contain essential domains for filament formation, such as assembly competence domains [19] and nonhelical tailpieces [20]. NMIIIB/IIA-tail, but not NMIIA/IIB-tail, localized in the anterior region and rescued the morphological defect of NMIIA-KD cells (i.e., formation of aberrant protrusions) (Supplementary Fig. S4), indicating that the anterior region has a normal shape if either of the NMII isoforms can localize there. Meanwhile, the morphological defect of the posterior region (i.e., elongation of the cell body) in NMIIIB-KD cells was not completely rescued by either chimeric NMII, indicating that intact NMIIIB is required to maintain the shape of the posterior region.

#### *3.4. NMIIIB is required for load-bearing stress fibers in the posterior region of migrating cells*

To determine why the posterior region is elongated in NMIIIB-KD cells, we investigated the extent of NMII activation (i.e., phosphorylation status of the regulatory light chain (RLC)). Phosphorylation of RLC at Ser19 promotes actin-activated  $Mg^{2+}$ -ATPase activity and filament formation, and this is further enhanced by diphosphorylation at Thr18 and Ser19

[5,6]. Immunoprecipitation analysis revealed that both NMIIA and NMIIB contained 1P-RLC and 2P-RLC (Supplementary Fig. S5). Immunofluorescence showed that both 1P-RLC and 2P-RLC were present at the lateral sides of the posterior region in control cells, and that 2P-RLC, but not 1P-RLC, was absent from this region in NMIIB-KD cells (Fig. 4A and B). This suggests that NMIIB is required to maintain 2P-RLC in this region.

We suspected that elongation of the posterior region might be due to a lack of load-bearing stress fibers. The motor domain of NMII, subfragment-1 (S1), preferentially binds to stretched actin filaments in *Dictyostelium* cells [21]. Moreover, we recently found that the human NMIIB-S1 mutant NMIIB-S1-R709C localizes to stretched stress fibers in immortalized fibroblasts [11]. Thus, we utilized NMIIB-S1-R709C as a probe to detect load-bearing stress fibers, which contain stretched actin filaments, in migrating cells. NMIIB-S1-R709C-EGFP accumulated at lateral stress fibers in the posterior region of control cells (Supplementary Movie S13, and Fig. 4C and D), indicating that actin filaments in these stress fibers are stretched. Similar findings were made in NMIIA-KD cells; however, NMIIB-S1-R709C-EGFP was diffusely distributed in NMIIB-KD cells (Supplementary Movies S14 and S15, and Fig. 4C and D), suggesting that actin filaments in the posterior region are no longer stretched in the absence of NMIIB. Taken together, these results suggest that NMIIB generates tension needed to bear

load in posterior stress fibers of migrating cells. These load-bearing stress fibers may prevent formation of aberrant protrusions in the posterior region, resulting in IDM.

#### **4. Discussion**

This study examined the roles of NMII isoforms in IDM of normal fibroblasts. KD experiments revealed that NMIIA and NMIIB differentially contribute to IDM by controlling the shapes of the anterior and posterior regions, respectively.

Cell migration is steered by the proper formation of protrusions at the leading edge [3]. NMII in lamellae is responsible for proper advancement of the leading edge [22]. NMIIA contributes to form proper protrusion in the anterior region by decreasing Rac1 activity [8]. Maintenance of low Rac1 activity induces IDM in a variety of cells [23]. The morphological defect (i.e., aberrant protrusion formation) of NMIIA-KD cells was restricted to the anterior region (Supplementary Movie S4), whereas aberrant protrusions formed throughout BBS-treated cells (Supplementary Movie S2). Consequently, directional persistence was decreased less in NMIIA-KD cells than in BBS-treated cells (Supplementary Fig. 1C and Fig. S1C). In NMIIA-KD cells, active NMIIB remaining in the posterior region could block the formation of membrane protrusions.

The main morphological defect of NMIIB-KD cells was elongation of the posterior region (Fig. 3). Actin filaments in lateral stress fibers located in the posterior region would be in a stretched conformation judging based on the binding of the S1 probe (Fig. 4), indicating that these stress fibers can bear a high mechanical load and become more elastic upon NMIIB KD. This change might lead to elongation of cell shape in the posterior region. Moreover, this defect was rescued by exogenous expression of NMIIB (Supplementary Fig. S4). NMIIB has a higher duty ratio than NMIIA (i.e., the NMIIB motor head spends longer in the strong actin-binding states during the ATPase cycle), indicating that NMIIB can function in crosslinking (structural property) of actin filaments [5]. This behavior of NMIIB may be required for resistance against the high mechanical load applied to stress fibers in the posterior region. Uyeda *et al.* proposed that myosin II preferentially binds to stretched actin filaments in a mechanical positive feedback manner in *Dictyostelium* cells [21]. NMIIB might be essential for this positive feedback loop in mammalian cells.

The most surprising result of this study was the reversal of migration direction in NMIIB-KD cells (Supplementary Movie S5 and Fig. 1). The first recognizable event in this process was the formation of lamellipodium-like protrusions in the posterior region. Vicente-Manzanares *et al.* reported that NMIIB creates a rear in migrating cells [18] and that exogenous diphosphomimetic RLC (T18D, S19D) prevents Rac1 activation in the area corresponding to

the posterior region [24]. Based on these results, they proposed that NMII containing 2P-RLC can prevent formation of abnormal protrusions in the posterior region. In this study, we directly demonstrated that NMIIB KD reduced the endogenous level of 2P-RLC in the posterior region and induced the formation of lamellipodium-like protrusions, which supports their hypothesis. Guo and Wang demonstrated that local application of cytochalasin D (an inhibitor of actin polymerization) to the posterior region induces the reversal of migration direction in immortalized fibroblasts (NIH-3T3) on micropatterned strips (i.e., a 1D surface) [25]. This finding strengthens the hypothesis that the posterior actin cytoskeleton containing NMIIB helps to prevent protrusion formation in the tail. The direction of migration was not reversed in BBS-treated cells (Supplementary Movie S2 and Fig. S1B), suggesting that active NMIIA remaining in the posterior region is partly responsible for this phenomenon. Indeed, NMIIA containing 1P-RLC remained localized throughout NMIIB-KD cells (Fig. 4B). In NMIIB-KD cells, NMIIA may promote formation of protrusions in the posterior region in the same manner as in the anterior region [8], and this likely triggers the reversal of migration direction. In other words, NMIIB may play a critical role in IDM by preventing formation of aberrant protrusions in the posterior region. The MTOC was positioned to the anterior side of the nucleus in migrating TIG-1 cells (Supplementary Movie S9), consistent with findings in other types of fibroblasts [4]. However, the MTOC did not reorient to the anterior region at the initial stage of the reversal

of migration direction in NMIIB-KD cells (Supplementary Movie S11). Recently, Zhang *et al.* proposed that the localization of the MTOC relative to the cell centroid, rather than to the nucleus, is important for the migration direction of mesenchymal cells and that positioning of the MTOC in the posterior region establishes front-rear polarity [26]. Similar events may occur during the reversal of the migration direction in NMIIB-KD cells.

This study clarified the roles of each NMII isoform in the anterior and posterior regions of normal fibroblasts during IDM. We propose that NMIIA controls the proper formation of protrusions in the anterior region to steer migration, whereas NMIIB prevents the formation of protrusions in the posterior region to maintain front-rear polarity.

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## Conflict of Interests

The authors declare no competing financial interests.

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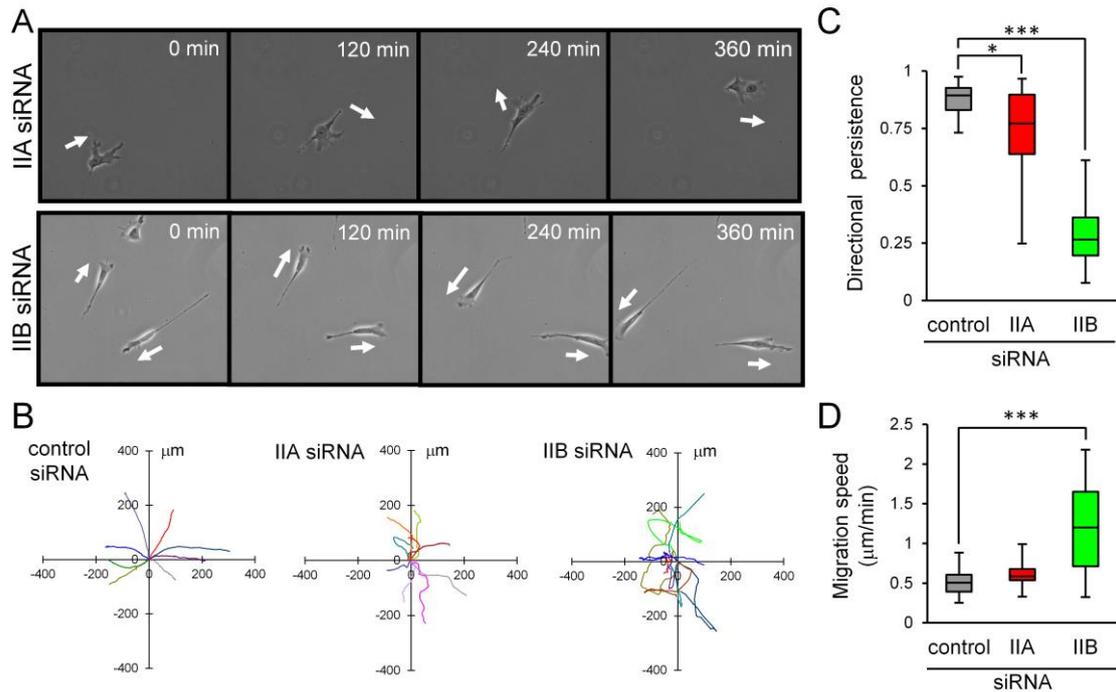
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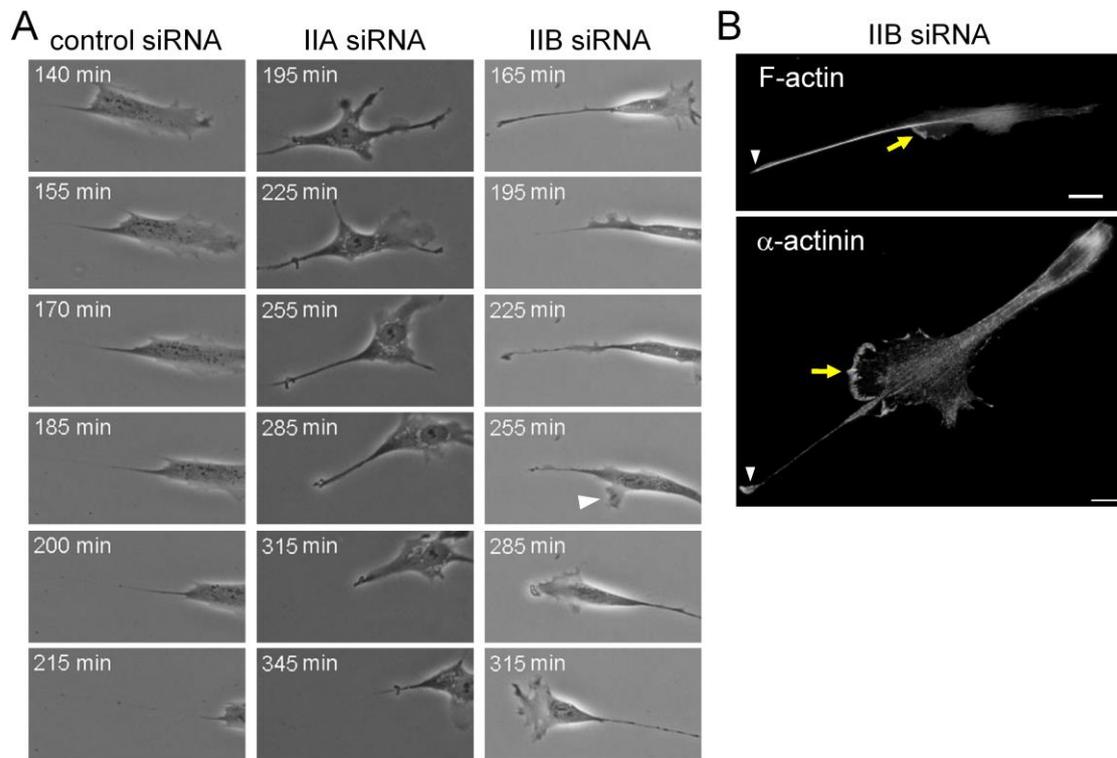
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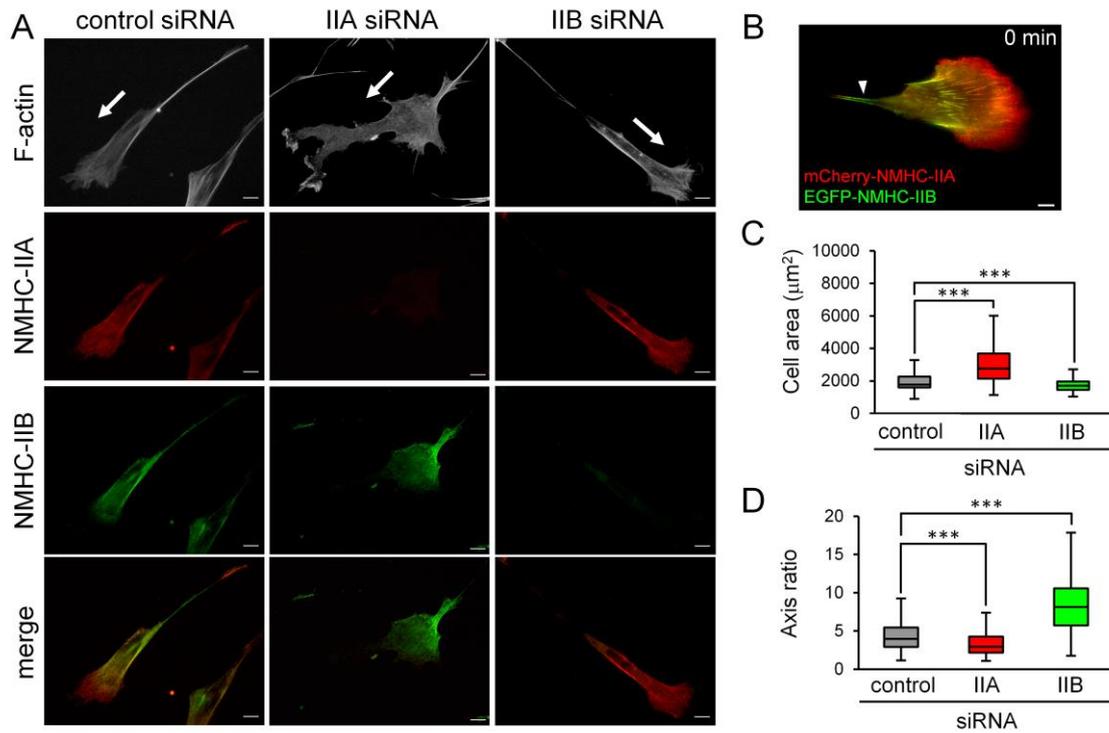
**Fig. 1.** Effects of KD of each NMII isoform on directional migration.

(A) Time series of images showing the migration of the indicated siRNA-treated TIG-1 cells from Supplementary Movies S4 and S5. White arrows indicate the direction of migration. (B–D) Migration paths (B), directional persistence (C), and migration speed (D) of cells treated with each siRNA ( $n > 15$  cells/condition). The migration path was determined by tracking the nucleus visualized with mCherry-NLS. The paths of eight representative cells are shown by different colors (B). Directional persistence was calculated as the direct distance from the start point to the end point divided by the total path distance (C).  $*P < 0.05$ ,  $***P < 0.0005$ .



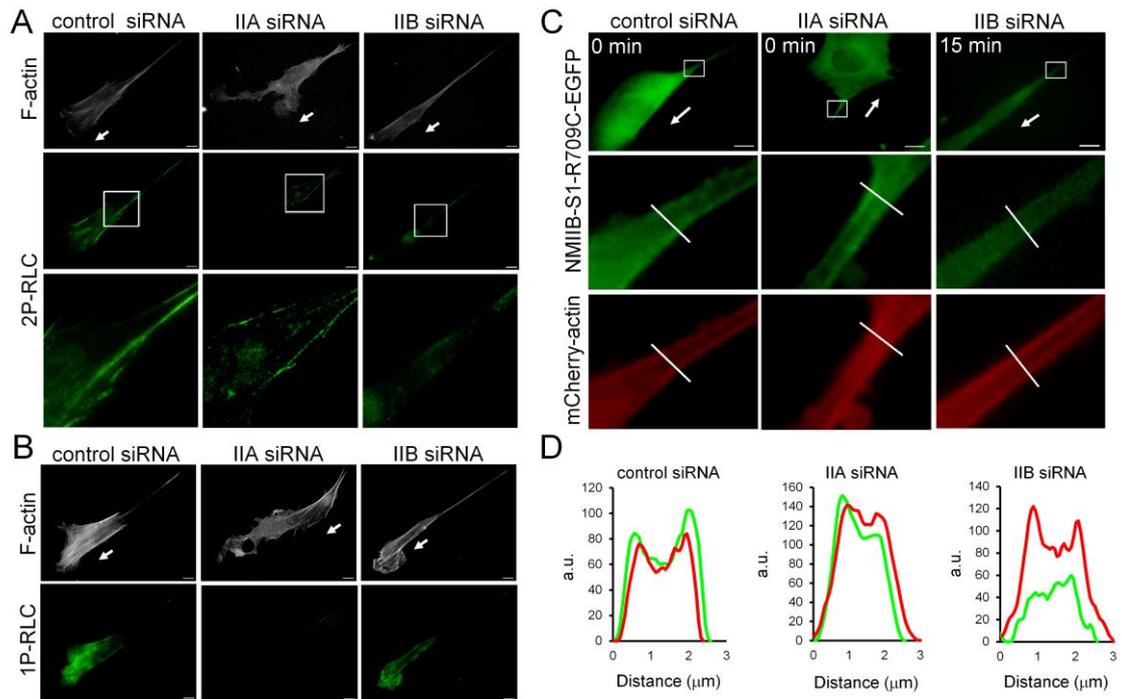
**Fig. 2.** Effects of KD of each NMII isoform on dynamics of the tail region.

(A) Time series of images showing morphological changes in the posterior regions of the indicated siRNA-treated cells from Supplementary Movies S6–8. The white arrowhead indicates protrusions from the posterior region of a NMIIB-KD cell. (B) Immunofluorescence images of the posterior region of NMIIB-KD cells. F-actin was stained with TRITC-phalloidin. Yellow arrows indicate protrusions from the posterior region. White arrowheads indicate the edge of the original tail. Bar, 10  $\mu$ m.



**Fig. 3.** Effects of KD of each NMII isoform on maintenance of cell shape during migration.

(A) Direct immunofluorescence of NMIIA and NMIIIB in the indicated siRNA-treated TIG-1 cells. White arrows indicate the expected direction of migration. F-actin was stained with Alexa Fluor 350-conjugated phalloidin. (B) A still image showing mCherry-NMHC-IIA and EGFP-NMHC-IIB fluorescence from Supplemental Movie S12 at 0 min. The white arrowhead indicates the accumulation of NMHC-IIB in the stretched tail region. (C and D) Cell area (C) and axis ratio (major axis/minor axis) (D) of the indicated siRNA-treated cells ( $n > 140$  cells/condition). \*\*\* $P < 0.0005$ . Bar, 10  $\mu\text{m}$ .



**Fig. 4.** NMIIB is responsible for diphosphorylation of RLC and tension generation in the posterior region.

(A and B) Immunofluorescence of F-actin and 2P-RLC (A) or 1P-RLC (B) in the indicated siRNA-treated TIG-1 cells. F-actin was stained with Alexa Fluor 350-conjugated phalloidin. White arrows indicate the expected direction of migration. In A, the bottom panels are enlarged images of the boxed regions in the middle panels. (C) Still images showing the indicated siRNA-treated cells expressing NMIIB-S1-R709C-EGFP and mCherry-actin from Supplementary Movies S13–15. White arrows indicate the direction of migration. The middle panels are enlarged images of the boxed regions in the top panels. (D) Fluorescence intensity profiles along the lines in the middle panels of C showing the posterior regions of the indicated siRNA-treated cells expressing NMIIB-S1-R709C-EGFP and mCherry-actin. Lines were drawn

at a position where the tail had a similar width in each condition. Green and red lines indicate the fluorescence intensities of NMIIB-S1-R709C-EGFP and mCherry-actin, respectively. Bar, 10  $\mu\text{m}$ .