SUPPLEMENTAL DATA

Figure S1. Distribution of transfected myosins in siRNA-depleted cells. (A-F) Images depicting the distribution of GFP-myosins compared to that of the endogenous isoforms. siRNA-depleted WI-38 cells were transfected with the indicated GFP-tagged full-length or chimeric myosin II. Images show cells migrating on the edge of a wound and in all cases the cells are migrating to the left. In the merged images, the myosin IIs are pseudo-colored as indicated at the top. Those not labeled with "GFP-" represent the endogenous forms. (G) Immunoblot demonstrating depletion of endogenous myosins (arrowheads) and expression of transfected myosins (arrows) in WI-38 cells. The numbers below each lane indicate the percent depletion compared to control siRNA (GL2) transfected cells. Labels on the left indicate the antibody used for immunoblotting (IB), with β-Actin used as a loading control. Note that the myosins antibodies were produced against a peptide sequence derived from the C-terminal region. siIIA, IIA-depleted; siIIB, IIB-depleted.

Figure S2. Subcellular distribution of GFP-IIAΔacd and GFP-IIBΔacd mutants. Wound migrating WI-38 cells expressing the indicated myosin II deletion mutants were fixed 90 min after wounding and immunostained with antibodies specific for the isoform opposite to the one being expressed. In all images the cells are migrating downward. Theses images demonstrate that both isoforms, when lacking the C-terminal region, assume similarly diffuse subcellular distributions.

Figure S3. IIA and IIB expression in various cell lines. (A) Coomassie Blue-stained gel showing the purity of the bacterially purified protein standards for IIA and IIB, GST-IIAtail and GST-IIBtail respectively. Also loaded on the gel are known amounts of BSA as standards for the determination of the mass concentration of the myosin II protein standards (see the description of the method included below). (B) Known amounts of GST-IIAtail and GST-IIBtail, based upon quantification from (A), were mixed with various cell lysates, separated by SDS-PAGE and immunoblotted for IIA (top) or IIB (bottom). The lysates used were from 293T(T), A549(A), COS-7(C), MDA-MB-231(M) and WI-38(W). Full-length endogenous myosin is indicated by the arrows, while the standards are marked by the arrowheads. The numbers below each lane indicate the mass (ng) of GST-tagged protein standard loaded in that lane. Note that it was necessary to load different amounts of the different lysates in order to ensure that the amount of endogenous myosin II was within the range of the standards. (C) Table showing quantification of the amount of IIA and IIB in each cell lysate, and the ratio of IIA to IIB (see the description of the method included below for details). The values are for the immunoblots shown in (B). All the data shown here are from a single experiment. The experiment was repeated with freshly purified standards and separate cell lysates with similar results.

Video 1. Wound migrating A549 cells expressing mChe-IIA (red) and GFP-IIB (green). This video demonstrates the dynamic nature of the differential distribution of IIA and IIB in migrating cells. Note that prior to the formation of protrusions the signals overlap (yellow), but that as the

cell begins to move forward mChe-IIA quickly assumes a more anterior distribution than GFP-IIB. Later in the video (in particular time point 01:49), a region of the protrusion temporarily stalls and the signals for IIA and IIB overlap until protrusion resumes. Time after wounding is indicated as hh:mm.

Video 2. An A549 cell expressing GFP-IIA was videoed before and after treatment with 10 uM Y27632. Prior to treatment, GFP-IIA was found throughout the entire cell with some of this myosin II isoform forming puncta that organized into filamentous arrays, similar to fixed cells. However, after treatment the cell underwent a change in morphology characterized by increased spreading, and the GFP-IIA signal rapidly lost any organized distribution and spread diffusely throughout the entire cell. Time is indicated as mm:ss, relative to addition of Y27632. Bar, 10 um.

Video 3. An A549 cell expressing GFP-IIB was videoed before and after treatment with vehicle alone (DMSO) and 10 uM Y27632. GFP-IIB was initially observed to organize largely into filamentous arrays, which were unaffected by treatment with vehicle alone. However, after Y27632 treatment the cell underwent a change in morphology characterized by increased spreading, and the GFP-IIB signal rapidly became diffusely distributed throughout the entire cell. Time is indicated as mm:ss, relative to addition of Y27632. Bar, 10 um.

Video 4. Wound migrating A549 cells expressing mChe-IIA (red) and GFP-IIA-Bacd (green). This video demonstrates that the chimera GFP-IIA-Bacd behaves similarly to GFP-IIB, in that the chimera exhibits a more posterior distribution relative to mChe-IIA. This is observed as a band of red at the front of the migrating cells. Time after wounding is indicated as hh:mm.

Purification of Bacterially Produced Protein Standards and Isoform Quantification

In order to accurately quantify the relative expression levels of myosin IIA and IIB, immunoblotting protein standards were generated. As the isoform-specific antibodies for myosin IIA and IIB were produced using peptides derived from the extreme C-terminal tail of each isoform, cDNAs encoding each isoform's C-terminal tail (amino acids 1259-1960 of IIA and 1248-1976 of IIB) were subcloned into the bacterial expression vector pGEX-6P-1 (Amersham #27-4597-01), which attaches an N-terminal GST tag to the protein. The standards are thus referred to as GST-IIAtail and GST-IIBtail. The standards were purified from bacterial lysate inclusion bodies, based upon the method described by Sambrook et al. (Sambrook, MacCullam et al. 2001**).** In brief, 1 L of bacterial culture expressing the GST-tagged protein standards were pelleted by centrifugation at 100 x g for 30 min, and the pelleted bacteria were resuspended in 50 ml Buffer A (50 mM Tris-HCl pH 8, 50mM NaCl, 1 mM DTT in PBS), and frozen in 8 ml aliquots at -70°C.

To purify the protein, one 8 ml aliquot was thawed and incubated on ice for 10 min, and Triton X-100 was added to lysate to a final concentration of 0.6%, then mixed thoroughly. DNase I was then added to a final concentration of 5 ug/ml and the lysate was rocked for 10 min at 4°C. Next the inclusion body protein was purified from the lysate by centrifugation at ~16,000 x g for 15 min at 4°C, after which the supernatant was discarded and the pellet was washed with 5 ml of Buffer B (50 mM Tris-HCl pH 8, 100mM NaCl, 10 mM EDTA pH 8, 0.5% TX-100 in PBS). The washed pellet, which now contained the purified standards, was resuspended in 1 ml deionized water. For quantification, the purified standards were diluted in

3X sample buffer, boiled for 10 min then separated SDS-PAGE along side known amounts of BSA. The gel was then stained with Coomassie Blue and scanned, and the amount of purified GST-IIAtail and GST-IIBtail present was quantified from a comparison to the known amounts of BSA by densitometry using Odyssey software (version 1.2).

To quantify the relative amount of myosin IIA and IIB in the various cell lines, whole cell extracts from 293T(T), A549(A), COS-7(C), MDA-MB-231(M) and WI-38(W) were produced. Known amounts of GST-IIAtail and GST-IIBtail were mixed separately with the various cell extracts and then separated by SDS-PAGE for immunoblotting. For accurate quantification, a particular amount of each cell extract was loaded such that the amount of endogenous myosin in each sample was within the range of the protein standards. The proper amounts of each cell extract to load were determined empirically. In the immunoblots shown in Figure S2 the loading was as follows: for IIA 2.5 ug of WI-38, 5 ug of A549 and 10 ug each of the others, while for IIB 25 ug of 293T and 50 ug of each of the others. The immunoblot was performed using LiCor for quantitative detection of protein, and the amount of endogenous myosin II and protein standard present in each lane was determined using Odyssey software (version 1.2). The signal in each band on the immunoblot is directly proportional to the moles of antigen present in each band. However, as the standards consist of truncated forms of each myosin II attached to GST, the mass to mol ratio in the immunoblot for each standard is different from that of the full-length endogenous protein. Therefore, the actual mass of endogenous protein in each band was determined by multiplying the value determined from the standards by 2.09 or 2.05 for IIA and IIB, respectively. These conversion factors are the ratio of the mass of full-length myosin II isoform to the corresponding GST-tagged standard. Finally, the amount of IIA and IIB in each cell lysate was standardized by dividing the amount of myosin (ng) by the amount of lysate loaded on the gel (ug), resulting in the values shown in Figure S2C.

Expression of GFP-Myosins in siRNA-depleted WI-38 Cells

The siRNA oligos used were the same as Sandquist et al., and the transfection was similar [REFERENCE]. Briefly, WI-38 cells were plated and 24 h later were transfected with 200 pmol siRNA using the Oligofectamine reagent according to the manufacturer's protocol. Then, 24 h after the first transfection, fresh media was added and the siRNA transfection was repeated. At 48 h after the initial siRNA transfection the cells were trypsinized and nucleofected with the GFP-tagged myosin II constructs using Cell Line Nucleofector Kit R, as described in the Materials and Methods. At 72 h after the initial siRNA transfection, the cells were trypsinized and replated into two dishes, with half of the cells being plated onto fibronectin coated glass coverlips and the remaining cells being plated for later use as immunoblotting extracts to verify myosin II depletion. Lastly, 96 h after the initial transfection, the time of maximum depletion, the siRNA-depleted cells were wounded, fixed and immunostained as described in the Materials and Methods.

Construction of Chimeric Myosins

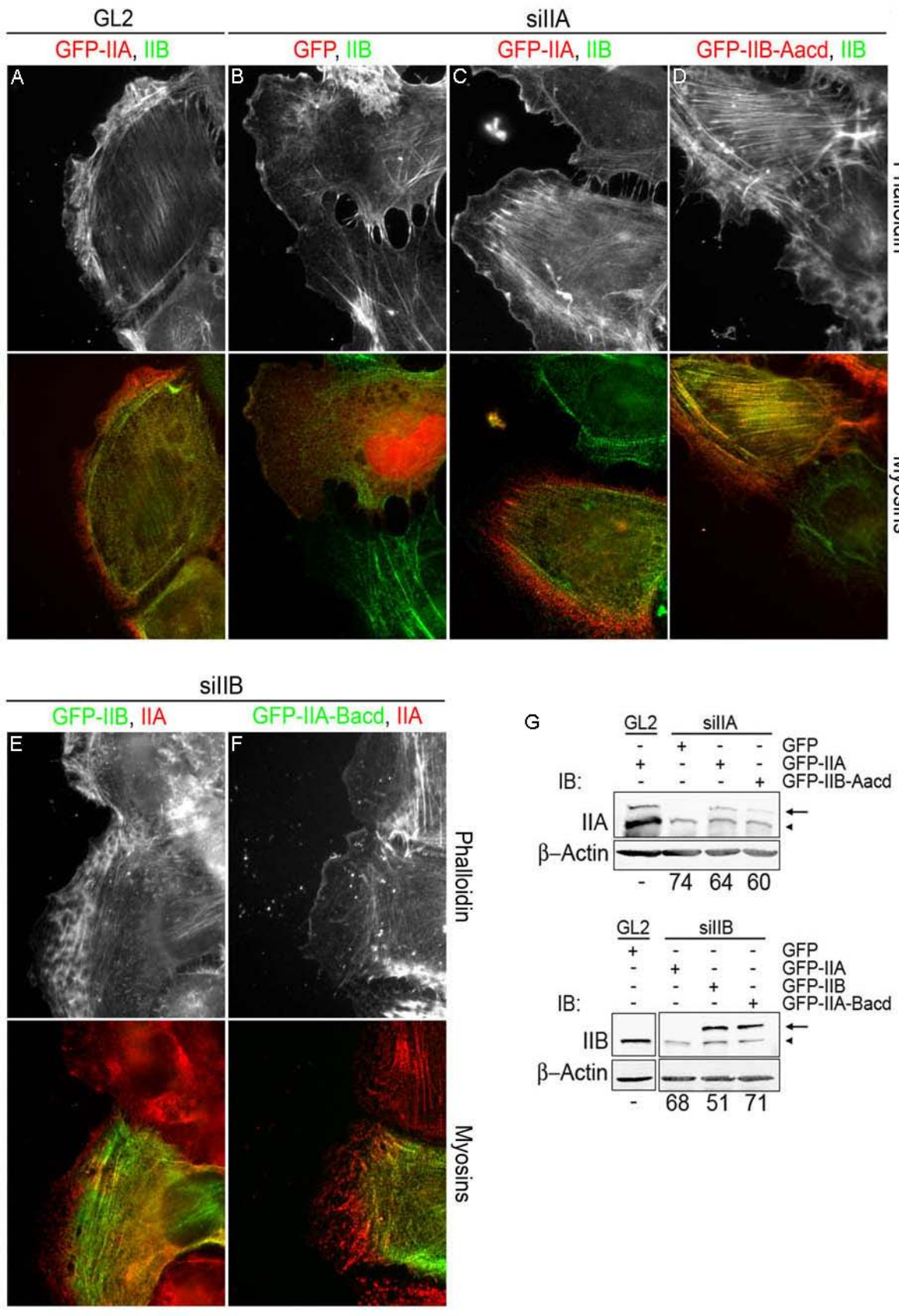
GFP-IIA-Btail – The N-terminal head domain encompassing amino acids 1-781 of IIA was PCR amplified from the GFP-IIA construct, introducing N-terminal ScaI and C-terminal EcoRV sites. These sites were then used to insert this fragment in frame into the existing ScaI/EcoRV sites in the GFP-IIB construct, replacing its head domain. The product was sequenced to verify correct frame and that no mistakes were introduced by PCR.

GFP-IIB-Atail - The N-terminal head domain encompassing amino acids 1-788 of IIB was PCR amplified from the GFP-IIB construct, introducing N-terminal AseI and C-terminal AatII sites. These sites were then used to insert this fragment in frame into the existing AseI/AatII sites in the GFP-IIA construct, replacing its head domain. The product was sequenced to verify correct frame and that no mistakes were introduced by PCR.

GFP-IIA-Bacd and GFP-IIB-Aacd – In order to make these constructs an AflII site was introduced into GFP-IIB by making a G5391T point mutation by site-directed mutagenesis. The mutagenesis product was sequenced to verify that no other mutations were introduced. Next, the "acd" fragments of both GFP-IIA and GFP-IIB were excised via restriction with AflII/HpaI, followed by ligation of these fragments into the opposite constructs. The products were sequenced to verify correct frame.

GFP-IIAΔacd – In order to remove the final 170 residues of IIA, GFP-IIA was digested with AflII/SalI, followed by blunting with Klenow and religation. This truncates IIA at Lue1790 and adds Asn-Ser-Lys-His at the end of the polypeptide.

GFP-IIBΔacd – In order to remove the final 178 residues of IIB, GFP-IIB (G5391T) was digested with AflII, followed by blunting with Klenow and religation. This creates a stop codon after Asn1798 of IIB.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siIIA
GFP-IIA, IIB
GFP, IIB
GFP-IIA, IIB
GFP-IIB-Aacd, IIB
A
B
C
D
Phalloidin
Myosins
siIIB
GFP-IIB, IIA
GFP-IIA-Bacd, IIA
E
F
Phalloidin
Myosins
G
GL2
siIIA
GFP
GFP-IIA
GFP-IIB-Aacd
IB:
IIA
β–Actin
- 74 64 60
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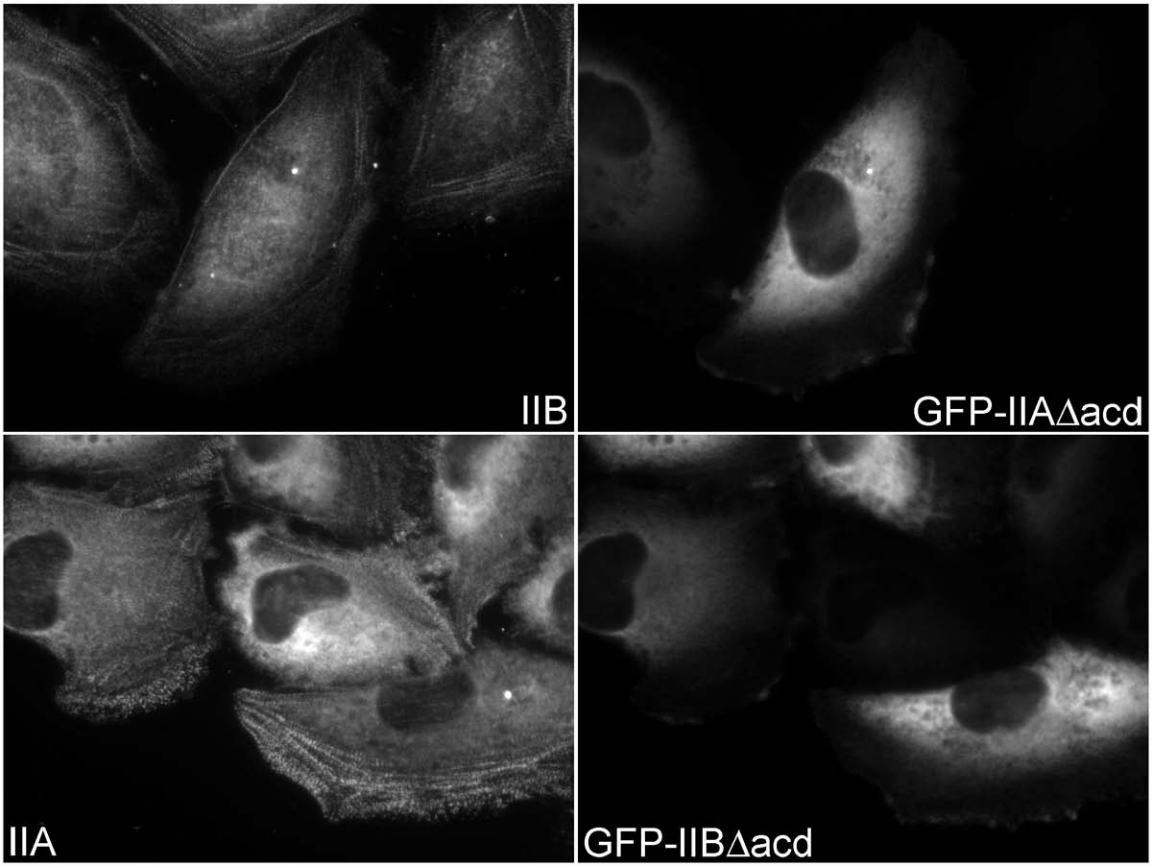
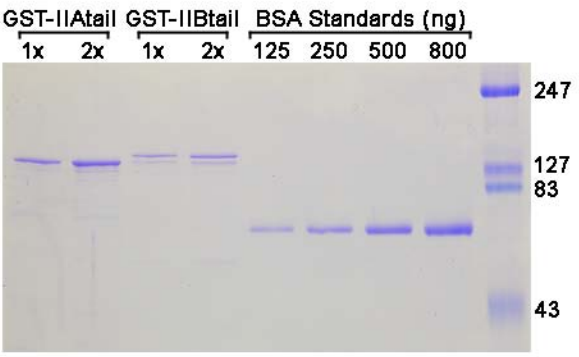
β–Actin
- 68 51 71


IIB
GFP-IIAΔacd
IIA
GFP-IIBΔacd

A



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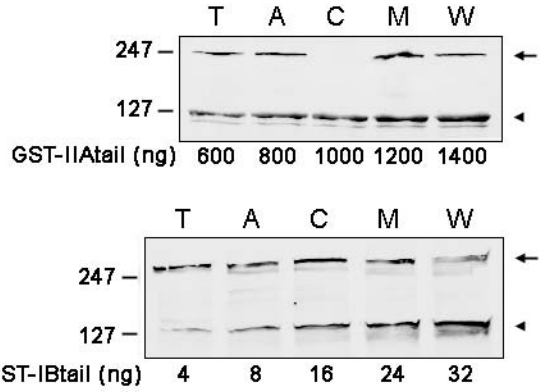

C

Myosin / Total Protein (ng/ug)

Cell Line	IIA	IIB	IIA/IIB
293	53	0.96	55
A549	139	0.22	623
COS-7	-	0.53	-
MDA-MB-231	107	0.33	328
WI-38	203	0.24	833