Nonmuscle Myosin II is Required for Internalization of the Epidermal Growth Factor Receptor and Modulation of Downstream Signaling*

Jong Hyun Kim¹,², Aibing Wang¹, Mary Anne Conti¹ and Robert S. Adelstein¹

¹ Laboratory of Molecular Cardiology, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, Maryland
² Present address: Medicinal Bioconvergence Research Center, Seoul National University, Seoul 151-742, Korea

*Running title: Internalization of EGFR by NM II

Address correspondence to: Robert S. Adelstein M.D. National Institutes of Health (NIH), Bldg 10, Rm 6C-103B, 10 Center Dr., Bethesda, MD 20892-1583. Tel: 301-496-1865; Fax: 301-402-1542; E-mail, adelster@mail.nih.gov or to nr.kimjohn@gmail.com.

Key words: EGFR internalization, nonmuscle myosin II, pERK, pAKT signaling

Background: Cellular internalization of the epidermal growth factor receptor (EGFR) is essential to its downstream signaling.

Results: Nonmuscle myosin II (NM II) is required for the internalization of the EGFR and modulates the EGFR-dependent activation of ERK and AKT.

Conclusion: NM II binds to the EGFR, expedites internalization and helps to initiate its role in signaling.

Significance: These findings elucidate an important step in the function of the EGFR.

SUMMARY

Ligand-induced internalization of the epidermal growth factor receptor (EGFR) is an important process for regulating signal transduction, cellular dynamics, and cell-cell communication. Here, we demonstrate that nonmuscle myosin II (NM II) is required for the internalization of the EGFR and to trigger the EGFR-dependent activation of ERK and AKT. The EGFR was identified as a protein that interacts with NM II by co-immunoprecipitation and mass spectrometry analysis. This interaction requires both the regulatory light chain 20 (RLC20) of NM II and the kinase domain of the EGFR. Two paralogs of NM II, NM II-A and NM II-B can act to internalize the EGFR, depending on the cell type and paralog content of the cell line. Loss (siRNA) or inhibition (25 μM blebbistatin) of NM II attenuates the internalization of the EGFR and impairs EGFR-dependent activation of ERK and AKT. Both internalization of the EGFR and downstream signaling to ERK and AKT can be partially restored in siRNA-treated cells by introduction of wild type (WT) GFP-NM II, but cannot be restored by motor mutant NM II. Taken together, these results suggest that NM II plays a role in the internalization of the EGFR and EGFR-mediated signaling pathways.

Binding of EGF to the epidermal growth factor receptor (EGFR) at the cell surface initiates a signal transduction process that involves a dynamic network of molecular interactions and modifications ultimately leading to cell survival, proliferation, and differentiation (1-3). The EGFR is a member of the tyrosine kinase receptor family and is comprised of an extracellular ligand-binding domain, a transmembrane domain and a cytoplasmic domain containing a tyrosine kinase module (4, 5). The dimerization and internalization of the EGFR are induced following binding of EGF to the extracellular domain in response to changes in the environment (6, 7). In many cases, downstream signaling events are initiated by internalization of the EGFR and terminated by its degradation. Although acceleration of internalization and lysosomal targeting lead to down-regulation of the EGFR,
and decrease the number of activated EGFRs at the cell surface, the activated EGFR can continue to signal from endosomes (8, 9). The internalization of the EGFR regulates the normal duration and intensity of EGFR signaling to extracellular signal-regulated protein kinase (ERK), c-Jun N-terminal kinase (JNK), and p38 MAP kinase (10, 11). Indeed, sustained ERK activation requires the presence of internalized EGFR in the endosomes (12, 13). Therefore, the internalization of the EGFR is an important process for signal propagation and amplification. Myosins are molecular motor proteins that crosslink and translocate actin filaments, using energy from ATP hydrolysis. Some classes of myosin can transport vesicles, organelles, and protein complexes, to various sites in the cell (14, 15). So far, over thirty different classes of myosin have been identified, some of which are important for multiple cellular processes, including endocytosis, exocytosis, and cell movement (16-19). One class, nonmuscle myosin II (NM II) is a hexamer composed of a pair of distinct heavy chains (II-A, II-B, or II-C) and two pairs of light chains (20kDa and 17kDa) (20-22). Although NM II plays important roles in a wide variety of cellular processes, the specific physiological roles of the three paralogs are still unclear. Recent publications have reported that NM II interacts with several kinds of receptors including CXCR4 (chemokine receptor), N-methyl-D-aspartate (NMDA receptor), DDR1 (as a collagen receptor), and the Ins (1,4,5)P3 receptor (23-26). However, to date, the potential involvement of NM II in EGFR-mediated intracellular signaling has not been studied in depth. Here, we identify the EGFR as a NM II-interacting molecule and show that NM II is required for internalization of the EGFR and modulates the EGFR-dependent activation of downstream signals including ERK and AKT.

**EXPERIMENTAL PROCEDURES**

**Materials** – siRNAs specific for human NMHC II-A (accession number NM_002473, GGCCAAAGAGAACGAGAAG), NMHC II-B (accession number NM_005964, AAGGAUCGCUACUAUUCAGGA), and NMHC II-C (accession number NM_028021, UCCGUCAGCAGCGUGUUAU) were chemically synthesized by Dharmacon Research, Inc. (Lafayette, CO, USA). SiRNA for RLC20 was obtained from Santa Cruz (SC-106242) (Santa Cruz, CA, USA). Purified EGFR intracellular domain (PKA-335) was obtained from ProSpec, East Brunswick, NJ, USA. Polyclonal antibodies for NMHC II-A, B, and C were generated as described previously (20, 27, 28). The EGFR antibody (06-847) was from Millipore, Billerica, MA.

**Identification of a 180kDa protein using mass spectrometry** – After immunoprecipitation with NM II-B antibodies from COS-7 cell lysates, immunocomplexes were separated by SDS-PAGE. The gel was stained with Coomassie Brilliant Blue and the bands excised and digested in gels with trypsin. The masses of the tryptic peptides obtained were measured with an Applied Biosystems 4700 MALDI-TOF-MASS. A compiled protein database was searched for protein matches.

**Cell culture and transfection** – COS-7, HEK-293, and A431 cells were maintained in DMEM supplemented with 10% (v/v) fetal bovine serum (FBS) at 37°C in a humidified, CO2-controlled (5%) incubator. COS-7 cells and HEK-293 cells were transfected using Lipofectamine 2000 for the transient expression of the indicated siRNAs or plasmids. A431 cells were transfected using Nucleofector kits according to the manufacturer’s instructions (Amaxa, Walkersville, MD, USA) for the transient expression of the indicated siRNAs and plasmids.

**Construction of flag and GST-tagged proteins** – Flag-tagged myosin light chain 20 (RLC20) cDNA (chicken) was used as template for PCR amplification to generate deletion mutants (SMART program) (29). In brief, flag-tagged RLC20 was amplified by PCR using specific primers, digested with restriction enzymes BamHI and Xhol, and ligated into pcDNA3.1(+) vector (Invitrogen, Carlsbad, CA, USA). The cytoplasmic domain of the EGFR was subcloned using PCR amplification from clone ID 30528231 (Open Biosystem, Huntsville, AL, USA), subsequently amplified by PCR using specific primers, digested with restriction enzymes BamHI and Xhol, and ligated into pcDNA3.1(+) vector (Invitrogen, Carlsbad, CA, USA). The cytoplasmic domain of the EGFR was subcloned using PCR amplification from clone ID 30528231 (Open Biosystem, Huntsville, AL, USA), subsequently amplified by PCR using specific primers, digested with restriction enzymes BamHI and Xhol, and ligated into pcDNA3.1(+) vector (Invitrogen, Carlsbad, CA, USA). The cytoplasmic domain of the EGFR was subcloned using PCR amplification from clone ID 30528231 (Open Biosystem, Huntsville, AL, USA), subsequently amplified by PCR using specific primers, digested with restriction enzymes BamHI and Xhol, and ligated into pcDNA3.1(+) vector (Invitrogen, Carlsbad, CA, USA). The sequence of the indicated siRNAs and plasmids.

**Purification of GST-fusion proteins** – GST-EGFR fusion proteins were purified as previously...
described (30). The protein concentration was determined by Bradford assay using BSA as a standard.

Receptor internalization assay – Receptor internalization assays were conducted as previously described, with minor modification (31). COS-7 cells and A431 cells were incubated at 37°C for the indicated time in the presence or absence of EGF (10ng/ml). Receptor internalization was then stopped by transferring cells to 4°C. After labeling with NHS-SS-biotin, treating with reducing solution (15.5 mg/ml glutathione, 75 mM NaCl, 75 mM NaOH, and 10% FBS) and subsequently with 5 mg/ml iodoacetamide (Sigma, St. Louis, MO, USA) in PBS containing 0.8 mM MgCl₂, 1.0 mM CaCl₂ plus 1% bovine serum albumin (BSA), the cells were lysed in TNE (10 mM Tris, pH 7.5, 150 mM NaCl, 0.5% Nonidet P-40, and 1 mM EDTA). Equal quantities of the cell lysate were used to precipitate the biotinylated proteins with streptavidin beads (Pierce, Rockford, IL, USA). Biotinylated proteins were eluted from the beads by incubation at 95°C for 5 minutes in Laemmli sample buffer.

Internalization of 125I-EGF – The internalization of 125I-EGF was measured as previously described (32). COS-7 cells and A431 cells were serum starved for at least 4 h in binding buffer (DMEM, 0.1% BSA, 20 mM Hepes pH 7.4), and then incubated at 37°C in the presence of 18.5ng/ml cold EGF plus 1.5ng/ml 125I-EGF (2x10⁵ cpm/ng) in 300μl binding buffer. After incubation for the indicated times, cells were put on ice, washed twice in cold PBS, and then incubated for 5 min at 4°C in 300 μl of acid wash solution (0.2 M acetic acid, 0.5 M NaCl pH 2.5). Radioactive 125I in the acid solution was measured. This sample represented the amount of 125I-EGF bound to the receptor on the cell surface. The remaining cells were dried at room temperature for 5 min, lysed with 300 μl of 1N NaOH and the radioactivity of collected lysates was measured. This sample represented the amount of internalized 125I-EGF. After being corrected for non-specific binding, the rate of internalization (kₑ) was expressed as the ratio between internalized and surface-bound radioactivity. The EGFR internalization rate (kₑ) is the slope of the line (calculated based on linear regression) at early time points, 1-5 min.

Normalization of Alexa-488-EGF – Cells were serum starved for 24h, and incubated in the presence of Alexa 488-EGF (10ng/ml) at 4°C and then at 37°C for 10 min. After fixation, images of the cells were captured by confocal microscopy. EEA-1 was used as an early endosome marker.

Immunoprecipitation and Immunoblot analyses – For immunoprecipitation, supernates from cell lysates were incubated with anti-NMHC II-A, or II-B antibodies for 9h or anti-FLAG antibody for 4h in binding buffer (50mM Tris-HCl, pH 8.0, 0.3 M NaCl, 1% Triton X-100, 0.25% Na-deoxycholate, 10 mM MgCl₂, 5 mM EDTA, 1 mM EGTA, 1 mM DTT, 10 mM ATP, 3 mM NaN₃, 1 mM PMSF, protease inhibitor cocktail, and phosphatase inhibitor cocktail and these immunocomplexes were recovered by incubation with protein A Sepharose beads for 2h. Immunoprecipitates were denatured by incubation at 95°C for 5 min in Laemmli sample buffer, and then separated by SDS 6% or 4-20% PAGE, and finally transferred to nitrocellulose membranes. Detection of peptides was performed using an enhanced chemiluminescence kit according to the manufacturer’s instructions (Pierce, Rockford, IL, USA). The images of immunoblots such as pERK, ERK, pAKT, AKT were quantified with ImageJ software (NIH). The values of pERK/ERK or pAKT/AKT are shown in graphic formats. Actin was used to normalize differences in loading. The protein concentration was determined by Bradford assay.

Fluorescence microscopy – COS-7 cells and A431 cells grown on 2 well chamber slides were transfected with the indicated siRNAs directed against NMHC II-A, II-B or II-C. The images were captured using a Zeiss LSM 510 META confocal microscope (Carl Zeiss, Thornwood, NY, USA).

In vitro kinase assay – The EGFR in vitro kinase reaction was performed as described previously (34) with minor modifications. Purified NM II-A (0.5μg/μl) or NM II-B (0.5μg/μl) were obtained following baculovirus expression (the details will be published elsewhere). They were incubated with active EGFR kinase domain (0.05μg/μl) in kinase assay buffer (20 mM HEPES, pH 7.5, 0 mM MgCl₂, and 2 mM MnCl₂). Kinase activity was tested by adding 5μCi [γ-32P]ATP (specific activity, 3000 Ci/mmol) and 10μM cold ATP.
After 20 minutes incubation at 37°C, the reaction was stopped by heating and adding Laemmli buffer, proteins were resolved by SDS-PAGE, and transferred to nitrocellulose for autoradiography. The same nitrocellulose membrane was rinsed and then subjected to immunoblotting.

In vitro binding analysis – Purified NM II-A or II-B (see Fig 3B,C for concentrations) was incubated with 1 μM purified EGFR (amino acids 672-1210) for 4h at 4°C in binding buffer (see above). NM II-A or II-B antibodies were added, incubated for 4h at 4°C and subsequently Protein A Sepharose was added to the immunocomplexes for 2h and then complexes bound to beads were sedimented and washed 3 times in PBS. Proteins were detected by immunoblot as described above.

For detection of EGFR and NMII-A in immunoprecipitate and supernate, baculovirus expressed NMII-A (0.15 mg/ml) was incubated with EGFR (0.1 mg/ml) in 50mM Tris-HCl, pH 8.0, 150mM NaCl, 5mM MgCl2, 1mM EGTA, 1mM DTT, 1mM ATP, 1mM PMSF, and protease inhibitor cocktail (Sigma) on a tumbling mixer at 4° for 2 hours. NMHCII-A antibody was incubated with Protein G conjugated Dynabeads (Invitrogen) in PBS on a tumbling mixer at 4° for one hour. The tubes were combined and incubation continued at 4° for another 2 hours. The mixture was separated into pellet and supernate and proteins separated by SDS-PAGE. Immunoblots with NMII-A antibody and EGFR antibody were carried out as described above and signal was detected with fluorescent-conjugated secondary antibodies (LiCor, Lincoln, NE) using an Odyssey imaging system (LiCor).

Statistical analysis – The results are expressed as the mean ±SE of data obtained from the indicated number of experiments performed. Statistical significance was determined using Student’s t test. Values of p< 0.01 are considered as a statistically significant difference. In many cases statistics are included in the figure legends.

RESULTS

Identification of the EGFR as a NM II-B binding partner in COS-7 cell.

To detect proteins that associate with NM II-B, we used COS-7 cells which express NM II-B as the major isoform (92% NM II-B, 8% NM II-C determined by mass spectrometry) (35, 36). Using antibodies specific for NMHC II-B, a 180 kDa protein was co-immunoprecipitated with NMHC II-B and separated by SDS-PAGE. A Coomassie stained gel of the peptides co-immunoprecipitating in the pellet is shown in Supplemental Fig. 1. One of the proteins interacting with NM II-B was identified as the EGFR by mass spectrometry. Peptide sequences of the p180 kDa protein, all of which correspond to EGFR sequences, are shown in Table 2 of the Supplemental data.

To confirm this association, immunoprecipitates from COS-7 cell lysates were analyzed using antibodies to NMHC II-B, the EGFR or the transferrin receptor (TfR). As shown in Fig. 1A, antibodies to NMHC II-B co-immunoprecipitated the EGFR along with NMHC II-B. Fig. 1B shows that NMHC II-B was co-immunoprecipitated using antibodies to the EGFR. In contrast, only trace amounts of the NMHC II-B were co-immunoprecipitated under the same conditions with the TfR, which is also present in COS-7 cells. To rule out NM II-B binding to the EGFR through actin, we made use of an antibody to actin. As shown in Fig. 1C, when actin was pre-cleared from the lysates before immunoprecipitation with anti-NMHC II-B, no actin was found in the complex of EGFR and NMHC II-B. This confirms that the interaction between NMHC II-B and the EGFR in COS-7 cell extracts was not mediated through actin.

NMHC II-B silencing disrupts the internalization of the EGFR in COS-7 cells.

Since normal endocytotic trafficking of activated EGFR is required to achieve full activation of EGF-dependent intracellular signals, the internalization of the EGFR was investigated after stimulation with EGF-Alexa which allows visualization of the EGFR. As shown in Fig. 2A, COS-7 cells transfected with siRNAs directed against luciferase (control), NMHC II-B, or NMHC II-C were treated with EGF-Alexa, fixed with paraformaldehyde, and prepared for immunofluorescence microscopy. At 10 min after stimulation with EGF-Alexa, the EGFR colocalized with EEA-1, a marker of the early endosome (37) in cells transfected with control siRNA but did not colocalize with EEA-1 in cells transfected with NMHC II-B siRNA. When the cells were transfected with siRNAs directed against NMHC II-C, the EGFR still colocalized with EEA-1 after treatment with EGF-Alexa (Fig. 2A). These results indicate that NM II-B but not...
NM II-C is required for the internalization of the EGFR in COS-7 cells. To corroborate and quantify the results of experiments in which fluorescent EGF was used to follow internalization, we performed a cell surface biotinylation assay using sulfo-NHS-SS-biotin after treatment with EGF. As shown in Fig. 2B, which is an immunoblot of the cell surface proteins (quantified in the graph below the blot) the loss of the EGFR from the cell surface was more complete at 20 min in cells transfected with control siRNA (red line) compared with NMHC II-B siRNA (green line). The silencing of NMHC II-C (blue line) showed a similar pattern to that of control siRNA. The internalization of the TfR, (used as a control receptor), was not altered by the treatment with EGF under the same conditions (Fig. 2B, immunoblot). To measure the internalization rate of the EGFR, cells were exposed to 125I-EGF at 37°C for the indicated time (Fig. 2C). The redistribution of 125I-EGF from the acid-extractable (surface-bound) to the acid-resistant (internalized) pool was determined as described in Experimental Procedures. As shown in Fig. 2C, 125I-EGF was rapidly internalized in control siRNA-transfected cells (k_e= 0.587±0.011) whereas the internalization value for NMHC II-B siRNA-transfected cells was significantly slower (k_e= 0.193±0.009). The internalization value in NMHC II-C siRNA-transfected cells was 0.502±0.021, similar to the controls. These results suggest that NM II-B is important for internalization of the EGFR in COS-7 cells.

The EGFR interacts with NM II-A as well as NM II-B

HEK-293 cells which express NM II-A and NM II-B (74% NM II-A and 25% NM II-B, by mass spectrometry) were used to determine the specificity of the interaction of NM II paralogs with the EGFR. As shown in Fig. 3A, HEK-293 cell lysates were incubated with the indicated antibodies including anti-NMHC II-A and NMHC II-B. NM II-A and NM II-B were each co-immunoprecipitated with the EGFR in a concentration-dependent manner. No significant differences were observed in the interaction of the EGFR between NM II-A and NM II-B.

To investigate whether NM II-A and NM II-B interact directly with the EGFR in vitro, pull down assays with NMHC II-A and NMHC II-B antibodies were performed. As shown in Figs 3B and C, baculovirus-expressed NM II-A as well as NM II-B, immunoprecipitated with purified EGFR in a concentration-dependent manner. In an effort to roughly estimate the relative affinity of the EGFR and NM II we isolated immunocomplexes and ran supernate and pellet in SDS-PAGE along with known amounts of NM II-A and the EGFR as standards for immunofluorescence. The molar ratio of the EGFR to NM II in the pellet was approximately 2:1 in two different experiments. A representative immunoblot is shown in Fig.3D. To examine the subcellular localization of NM II in HEK-293 cells during the internalization of the EGFR, immunofluorescence microscopy using Alexa 488-EGF and anti-NMHC II-A and NMHC II-B was carried out (Supplemental Fig. 2A,B). The internalized EGFR was visualized at 10 min after a temperature shift to 37°C following treatment with EGF-Alexa at 4°C. At 10 min, the internalized EGFR is observed as diffuse punctate regions mostly concentrated just inside the cell membrane where it is colocalized with NM II-A and II-B (merged images, Supplemental Fig. 2).

The RLC20 and the kinase domain of the EGFR are required for interaction with NM II-A or NM II-B.

To investigate which domain of NM II interacts with the EGFR several flag-fusion fragments of RLC20 were generated according to the SMART program (Supplemental Fig. 3A, left). The figure shows that antibodies to the flag peptide immunoprecipitate the EGFR together with NMHC II-A or NMHC II-B (Supplemental Fig. 3A, right). As shown in the figure, the C-terminus of the RLC20 is required for interaction with NMHC II-A or NMHC II-B since deletion of the C-terminal residues (RLC20-C1, RLC20-C2 and RLC20-Δ142) abolishes RLC20 binding to the NMHC. These deletion mutants of RLC20 also do not interact with the EGFR. On the other hand, the N-terminus of the RLC20 was not required for the binding of the RLC20 to NMHC II-A or NMHC II-B, since the N-terminal deletion mutant of RLC20 (RLC20-N1) still binds and immunoprecipitates with the NMHC. Despite interaction with the NMHC, the N-terminal deletion mutant of the RLC20 does not associate with the EGFR, although the RLC20-WT, which interacts with NMHC II-A and NMHC II-B also interacts with the EGFR. These results suggest that the intact-RLC20 is required for association of
NM II with the EGFR. It is also likely that the heavy chain is involved in this association. To examine whether phosphorylation of the RLC 20 on T18 and S19 can affect the interaction with the EGFR, we used phosphorylation mimic (Asp [D]), and deficient (Ala [A]) mutants of the RLC: T18D/S19D and T18A/S19A (Supplemental Fig. 3B). As shown in Supplemental Fig. 3C, our results indicate that neither the phosphorylation mimic nor Ala mutant of the RLC has a significant effect on the interaction with the EGFR compared to the RLC-WT although, as shown in Supplemental Fig. 3D EGF activation of the EGFR does lead to phosphorylation of the S19 site of the RLC.

To identify the domains of the EGFR involved in the interaction with NM II-A or NM II-B, several GST-fusion constructs of the EGFR were generated according to the SMART program (Supplemental Fig. 4A). As shown in Supplemental Fig. 4B, the GST-EGFR-cytoplasmic domain (a.a. 623-1091) as well as GST-EGFR-M (a.a. 667-923) interact with NM II-A or NM II-B. Neither GST-EGFR-N (a.a. 623-667) or GST-EGFR-C (a.a. 923-1091), which do not contain the kinase domain interact with NM II-A or NM II-B under the same conditions. To confirm that the tyrosine kinase domain of the EGFR is required for these interactions, a competition assay using GST-fusion proteins was performed. After transfection of HEK-293 cells with Flag-RLC20-WT, RLC20 was immunoprecipitated with Flag antibodies. The immunocomplexes containing Flag-RLC20-WT were incubated with purified GST-EGFR-M, or GST-EGFR-C to compete out endogenous EGFR bound with RLC20. Although GST-EGFR-M competed out endogenous EGFR in a concentration-dependent manner, GST-EGFR-C which isn’t able to interact with NM II-A or NM II-B did not (Supplemental Fig. 4C). These results indicate that the tyrosine kinase domain of the EGFR is involved in an interaction with NM II-A or NM II-B. The presence of the EGFR kinase domain bound to the RLC20 raised the question as to whether the light chain (and/or NMHC) could act as a substrate for the EGFR kinase. Supplemental Fig. 5 shows that although the kinase undergoes autophosphorylation, neither the RLC20 or NMHC II-A or NMHC II-B is a substrate.

NM II-A and NM II-B can both contribute to internalization of the EGFR in A431 cells. Because NM II-B (and not NM II-C) is required for the internalization of the EGFR in COS-7 cells (Fig. 2), it was of interest to investigate the internalization of the EGFR in human A431 cells, a squamous cell carcinoma line which expresses a large number of EGFRs and contains all three paralogs of NM II. The amount of NM II-A, NM II-B and NM II-C in these cells, using mass spectrometry is 84±1.9% II-A, 8.5±1.7% II-B and 6.5±0.6% II-C. A431 cells transfected with siRNAs for luciferase (control), NMHC II-A, or NMHC II-B were treated and fixed to visualize the EGFR. At 10 min after stimulation with EGF-Alexa, silencing of NMHC II-B interrupts the internalization of the EGFR, even though the expression of NM II-B is relatively low compared to NM II-A (Fig. 4A). Of note, siRNA directed against NMHC II-A also disrupts the internalization of the EGFR compared with control siRNA. To corroborate the results, a cell surface biotinylation assay was performed after treatment with EGF for the indicated time (Fig 4B). The EGFR was rapidly internalized in cells transfected with control siRNA, but it was only slowly internalized in cells transfected with NMHC II-A or NMHC II-B siRNAs and after 20 min only about one-half was internalized. 125I-EGF was used to measure the internalization rate of the EGFR, for the indicated times (Fig. 4C). The internalization rate is much lower in cells transfected with NMHC II-A or NMHC II-B siRNA (k_e=0.186±0.019 and 0.182±0.015, respectively) compared with control siRNA (k_e = 0.572±0.029). The internalization value in cells transfected with both NMHC II-A and NMHC II-B siRNA is significantly lower (k_e = 0.132±0.016) (Fig. 4D). The internalization rate in cells transfected with NMHC II-C siRNA (k_e = 0.517±0.012) is similar to that of control (k_e = 0.558±0.025) (Fig. 4D). These results suggest that both NM II-A and NM II-B play a role in the internalization of the EGFR in A431 cells. The ligand-induced internalization of the EGFR is controlled by multiple pathways. According to our results, NM II-A or NM II-B silencing does not completely disrupt the internalization of EGFR. Thus, a portion of the internalization of the EGFR is unaffected by NM II silencing and appears to be controlled by other pathways. The Grb2-Cbl
pathway in particular is known to be a major contributor to internalization of the EGFR (38).

Isoform specificity of NM II rescue of NM II-depleted cells.

The finding that depletion NM II-A and NM II-B had similar effects on the internalization of the EGFR despite the larger amount of NM II-A compared to NM II-B in A431 cells prompted us to see if one paralog could replace the other. To examine whether NM II-A could replace NM II-B (and vice versa) during receptor internalization, biotinylation assays and immunoblots were performed. As shown in Fig. 5A, when NMHC II-B was introduced into NMHC II-A silenced cells, the EGFR was only slightly internalized compared to the internalization found when NMHC II-A was introduced into NMHC II-A silenced cells (Fig. 5A, lanes 6 and 8). As shown in Fig. 5B, lanes 6 and 8, when NMHC II-A was expressed in NMHC II-B silenced cells, the EGFR was only partially internalized compared to the internalization seen when NMHC II-A was introduced into NMHC II-B silenced cells. These results indicate that the role of each NM II cannot be compensated for by a different NM II paralog and suggest that each plays a separate role in receptor internalization.

NM II-A or NM II-B modulates EGFR-dependent downstream signaling.

Since NM II is required for the internalization of the EGFR, the effect on potential downstream signaling pathways of EGFR after silencing of NMHC II-A or NMHC II-B was investigated. Previous studies have reported that stimulation of the EGFR triggers the Raf-1 kinase-ERK pathway or PI-3 kinase-AKT pathway during the internalization of the EGFR (39, 40). To determine whether NM II-A or NM II-B can affect the stimulation of ERK or AKT in HEK-293 cells, ERK activation, based on phosphorylation of ERK (pERK) and AKT activation, based on pAKT were investigated. Although ERK and AKT activation were significantly increased by treatment with EGF in cells transfected with control siRNA, they were only slightly increased in cells transfected with NMHC II-A or NMHC II-B siRNAs under the same conditions (Fig. 6A). To elucidate the effects of NMHC II-B depletion in cells in which it is the principal paralog, NMHC II-B siRNA was transfected into COS-7 cells which were treated with EGF for the indicated time and activation of ERK and AKT was then measured (Fig. 6B). The activation of ERK and AKT is attenuated in COS-7 cells transfected with NMHC II-B siRNA compared with control siRNA. However, the time courses of activation of ERK and AKT were significantly different indicating that ERK activation occurs relatively earlier than AKT activation.

To investigate whether ERK and AKT activation can be restored to COS-7 cells treated with siRNA, GFP-NMHC II-B was transfected into these cells. As shown in Fig. 6C, although ERK and AKT activation are attenuated in cells transfected with NMHC II-B siRNA, phosphorylation levels are significantly increased in cells transfected with GFP-NMHC II-B. However, phosphorylation isn’t completely restored (4.9-fold increase in ERK phosphorylation compared to 11.8-fold for control and a 9.1-fold increase in AKT phosphorylation compared to 16.9-fold for control). These results indicate that NM II-A or NM II-B contributes to EGFR-dependent activation of ERK and AKT. Of note ERK showed a maximal activation earlier in siRNA treated cells (at 2 min) compared to control cells (at 5 min) (Fig. 6B). Rescuing the NM II-B depleted cells with GFP-NMHC II-B restored increased activation at 5 min (Fig. 6C). Fig. 6C also provides evidence that the exogenously introduced GFP-NMHC II-B used in the rescue experiment is not degraded by the siRNA. The last three lanes of the top immunoblot show the expression of GFP-NMHC II-B which migrates more slowly than endogenous NMHC II-B.

The effect of blebbistatin and NM II mutations on the EGFR.

To investigate the effects of the NM II inhibitor blebbistatin on the internalization of the EGFR, A431 cells were examined by immunofluorescence microscopy. In the presence of 25 μM blebbistatin at ten minutes after stimulation with EGF-Alexa most of the EGFR is not internalized and does not colocalize with EEA-1 (compare Fig. 7A bottom and 7A top). Moreover blebbistatin significantly inhibited the activation of both ERK and AKT in a dose-dependent manner (Fig. 7B). To further investigate the role of NM II activity in rescuing internalization of the EGFR we made use of two NM II paralogs with point mutations, NM II-A N93K and NM II-B R709C. These mutant NM IIs are impaired in their enzymatic activity and ability to translocate actin filaments in an in vitro motility assay, although both mutants can bind to actin and are released by ATP (41, 42). Furthermore, these
mutations have been shown to cause defects in humans (43) and in mice (44). A cell surface biotinylation assay in the absence and presence of EGF was performed. As shown in Fig. 8A, the internalization of the EGFR was decreased in A431 cells transfected with NMHC II-A siRNA compared with control siRNA (Fig. 8A, lanes 2, and 4). When GFP-NMHC IIA-WT was introduced into A431 cells, the internalization of the EGFR was mostly restored (Fig. 8A, lanes 2 and 6). However, introduction of the motor-defective mutant GFP-NMHC IIA-N93K failed to restore the internalization of the EGFR (Fig. 8A, lanes 2, and 8). To investigate the effects of compromised NM II motor activity on the EGFR-dependent activation of ERK and AKT, GFP-NMHC IIA-N93K was introduced into A431 cells (Fig. 8B) following depletion of NMHC II-A using siRNA. Activation of ERK and AKT as monitored by the phosphorylation state is not restored following EGF treatment (Fig. 8B, lanes 2 and 8). When GFP-NMHC IIA-WT is introduced, EGFR-dependent activation of ERK is only marginally restored (lanes 2 and 6, not permitting quantification). This incomplete rescue of ERK most likely reflects the fact that NM II is only one of a number of proteins playing a role in the downstream signaling but further experimentation is required to establish the cause (see also below). On the other hand, activation of AKT by NMHC IIA-WT though partial, is more evident. (Fig. 8B, lanes 2 and 6; 4.3 for WT vs 9.1 for control). The role of NM II-B was also investigated in A431 cells. As shown in Fig. 8C, internalization of the EGFR was decreased in cells transfected with NMHC II-B siRNA compared with control siRNA as indicated by the increased amount of EGFR present at the cell surface of the transfected cells (Fig. 8C, lanes 2, and 4). When GFP-NMHC IIB-WT is introduced, the internalization of EGFR is restored (Fig. 8C, lane 2 and 6). However, when motor-impaired GFP-NMHC IIB-R709C is introduced, the internalization of the EGFR is not restored (Fig. 8C, lanes 2 and 8). Fig. 8D shows the results of introduction of WT and mutant NMHC II-B on the EGFR-dependent activation of ERK and AKT, following depletion of NMHC II-B in A431 cells. When GFP-NMHC IIB-WT is introduced, the activation of ERK and AKT is partially restored (Fig. 8D, lanes 2 and 6, only quantified for pAKT). Introduction of mutant GFP-NMHC IIB-R709C does not restore the activation of ERK and AKT at all (Fig. 8D, lane 2 and 8).

DISCUSSION

The intensity, duration, and specificity of signal transduction through receptor tyrosine kinases (RTKs) are regulated by ligand-mediated endocytosis and post-endocytic trafficking of activated receptors (45). The impairment of activated RTK internalization results in dysregulation of RTK activity and malfunction of downstream signaling processes. Despite the important role of internalization of RTKs, the molecular mechanisms controlling this process remain poorly understood. In this study, we provide evidence that the motor proteins NM II-A, and NM II-B are required for the ligand-induced internalization of the EGFR, a member of the RTK family. The disruption of NM II-A or NM II-B expression decreases internalization of the EGFR and subsequently impairs downstream signaling. To show that the role of NM II is important in this process, we made use of a number of cell lines expressing different amounts of the NM II paralogs.

The roles of the cytoskeletal proteins in the internalization of receptors have not been fully addressed, although there have been several reports about an association between NM II and various receptors. These receptors including CXCR4, Ins(1,4,5)P3-R, and NMDA-R are not single trans-membrane receptors with the exception of the discoidin domain receptor 1 (DDR1) which is a tyrosine kinase receptor activated by type I collagen (23-26). The EGFR is identified as an NM II binding partner in this study. NM II forms complexes with the EGFR, and utilizes its ATPase activity for internalization of the EGFR as indicated by the inability of NM IIs with compromised ATPase activity to rescue internalization (Fig. 8A,C). Although the precise amino acid residues responsible for the interaction of NM II with the EGFR remain to be established, we show that the association between the NM II domain in the area of the RLC20 and the EGFR kinase domain is an important part of the internalization of the EGFR.

Myosins are a large family of diverse molecular motors. NM II is comprised of three genetically distinct heavy chains referred to as NMHC II-A, II-B, and II-C.
NMHC II-B, and NMHC II-C (20-22). Several reports suggest that NM II paralogs may have individually important roles for which other paralogs cannot compensate (37, 46, 47). Here, we estimated that the amount of NM II-B in A431 cells is less than 10% of the total NM II. Despite the relatively low amount, NM II-B plays a significant role in the internalization of the EGFR because NM II-B silencing (as well as NM II-A) disrupts the internalization of the EGFR (Fig. 2, 4). It is of note that the rate of internalization ($k_e$) is greater for the combined reduction of both NM II-A and NM II-B in A431 cells than for each one separately, suggesting that they play different roles during receptor internalization. This idea is further supported by our finding that neither paralog can replace depletion of the other although each can replace itself in rescue experiments following siRNA depletion. Our results also indicate that both NM II-A and NM II-B can participate in receptor internalization, but not NM II-C.

There are several proteins regulating the internalization of the EGFR by interacting with its tyrosine kinase domain. Eps 15 (EGFR pathway substrate 15) is a multifunctional adaptor protein regulating intracellular trafficking, a known substrate for the EGFR, and is also involved in regulation of receptor signaling by endocytosis (48). Eps 8 is also a substrate of the EGFR, and coordinates EGFR signaling through Rac and trafficking through Rab5 (49). Although we show that the EGFR interacts with RLC20 of NM II through its kinase domain, we failed to observe phosphorylation of RLC20 or NMHC II by the EGFR. These results imply that NM II is not a direct substrate of the EGFR although a functional NM II motor is required for the internalization of the EGFR.

Our finding that NM II-A and II-B but not NM II-C can participate in receptor internalization is consistent with the NMHC as well as the RLC20 participating in the binding of the EGFR, since the RLC20 is thought to be common to all three heavy chain paralogs. On the other hand our data also suggest that phosphorylation of RLC20 does not affect the binding to the EGFR although RLC20 phosphorylation does occur during receptor internalization.

Although the acceleration of internalization and lysosomal targeting leads to receptor down-regulation, which serves to decrease the number of activated receptors in the cell, the internalized EGFR continues to signal from endosomes. Specifically endocytosis has been proposed as necessary for the normal duration and intensity of EGFR signaling to ERK, c-Jun N-terminal kinase (JNK), and p38 MAP kinase (10, 11). There is growing evidence showing that kinase activation not only happens at the plasma membrane but also in endosomes (50, 51). Several of the EGFR downstream signaling factors such as Grb2, Shc and Sos are recruited to endosomes shortly after internalization of the EGFR (52). Sustained ERK activation also requires the presence of an active EGFR in endosomes (12, 13). Therefore, the internalization of the EGFR is an important process for regulating signal propagation and amplification. Although we have established an important role for NM II in receptor internalization, its role in signal propagation is not as clear since the ability of NM II-WT to restore activation of ERK and AKT is only partial. This raises the possibility that other factors are involved. The ligand-induced internalization of the EGFR is controlled by multiple pathways. The EGFR remains in the plasma membrane in the absence of ligand. Upon the binding of ligand to the receptor, EGFR is internalized to the endosomal compartments. As shown in Supplemental Fig. 2 NM II-A or NM II-B colocalized with the internalized EGFR. Fig. 3B-D also demonstrates that NM II directly interacted with the EGFR in vitro. These results imply that this interaction is important to transduce EGFR signaling. We speculate that disruption of this interaction results in impairment of downstream EGFR signaling in the endosomal compartment. Therefore, we suggest that NM II contributes to the propagation of endosomal-derived signaling during the internalization of EGFR.

Here, we demonstrate for the first time that NM II interacts with the EGFR and that NM II motor activity is required for the internalization of the EGFR and contributes to propagation of the downstream signals, ERK and AKT. We also describe how NM II acts to internalize the EGFR in both COS-7 cells and A431 cells, which show distinct expression patterns of two different paralogs of NM II.
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ACKNOWLEDGEMENTS
We thank the members of the Laboratory of Molecular Cardiology (NHLBI, National Institutes of Health) for reagents and for helpful discussions. We thank Drs. Christian A. Combs and Daniela A. Malide (Light Microscopy Core Facility, NHLBI, ) and Dr. Guanghui  Wang (Proteomics Core Facility, NHLBI, NIH) for their professional help and skills. We also thank to Dr. Tom Egelhoff (Cleveland Clinic Foundation) for mutant T18A/S19A and T18D/S19D plasmids.

FOOTNOTES
*This work was supported by the Division of Intramural Research, NHLBI, NIH.
1,2Address correspondence to: Robert S. Adelstein M.D. National Institutes of Health (NIH), Bldg 10, Rm 6C-103B, 10 Center Dr., Bethesda, MD 20892-1583. Tel: 301-496-1865; Fax: 301-402-1542; E-mail adelster@mail.nih.gov or to nr.kimjohn@gmail.com.
3The abbreviations used are: AKT, protein kinase B; BSA, bovine serum albumin; DMEM, Dulbecco’s modified Eagle’s medium; DTT, dithiothreitol; EDTA, ethylene diaminetetraacetic acid; EGFR, epidermal growth factor receptor; ELC, essential light chain; ERK, extracellular signal-regulated protein kinase; FBS, fetal bovine serum; NM II, nonmuscle myosin II; NMHC II, nonmuscle myosin heavy chain II; PMSF, phenylmethylsulfonyl fluoride; RLC, regulatory light chain; RTK, Receptor tyrosine kinase; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; WT, wild type.
FIGURE LEGENDS

Fig. 1. Co-immunoprecipitaion of NM II-B and the EGFR from COS-7 cell lysates.

A. Cells were harvested, lysed, and immunoprecipitated with NMHC II-B antibody. Immunocomplexes were separated by SDS-PAGE, and immunoblotted with the indicated antibodies. Normal serum (N. S.) was used as a negative control for immunoprecipitation. B. Cells were harvested, lysed, and immunoprecipitated with the EGFR or transferrin (TfR) antibodies. The immunocomplexes were separated by SDS-PAGE, and immunoblotted with the indicated antibodies. C. COS-7 cells were harvested, lysed, and immunoprecipitated with NMHC II-B antibody after preclearance with actin antibody. Immunocomplexes were separated by SDS-PAGE, and immunoblotted with the indicated antibodies. Normal serum (N. S.) was used as a negative control for immunoprecipitation.

Fig. 2. NMHC II-B silencing disrupts EGFR internalization in COS-7 cells.

A. Cells were transfected with the indicated siRNAs for luciferase (Control), NMHC II-B, or NMHC II-C. Cells were serum starved for 24h, and incubated in the presence or absence of Alexa 488-EGF (10ng/ml) at 37°C for 10 min. After fixation, images of the cells were captured by confocal microscopy. EEA-1 (red) was used as an early endosome marker. DAPI staining (blue) marks nuclei. Bars, 10μm. B. After treatment with EGF (10ng/ml) for the indicated time, biotinylation of the cell surface proteins was performed as described in Experimental Procedures. Cells were harvested, lysed, and precipitated with streptavidin. Precipitated complexes were separated by SDS-PAGE, and immunoblotted with the indicated antibodies. The transferrin receptor (Tfr) was used as a negative control. The graph below the immunoblots shows the relative amount of EGFR at the cell surface at the indicated time. C. Cells were transfected with the indicated siRNAs for luciferase (Control), NMHC II-B, or NMHC II-C. Cells were serum starved for 24h, and incubated with 125I-EGF (1.5ng/ml) plus cold EGF (18.5ng/ml) at 37°C for the indicated time before measuring internalized and surface-bound 125I-EGF. The ke values are 0.587±0.011 for luciferase (Control) siRNA, 0.193±0.009 for NMHC II-B siRNA, and 0.502±0.021 for NMHC II-C siRNA. p< 0.01 (n=3) The data represent the mean of three independent experiments.

Fig. 3. The EGFR interacts directly with NM II-A as well as NM II-B.

A. HEK-293 cells were harvested, and then lysed. Cell lysates were immunoprecipitated with NMHC II-A or NMHC II-B antibodies at the indicated concentrations. The immunocomplexes were separated by SDS-PAGE, and immunoblotted with the indicated antibodies. B. Purified baculovirus expressed NM II-A, at the concentrations indicated, was incubated with purified baculovirus expressed EGFR (1μM) for 4h at 4°C, immunoprecipitated with NMHC II-A antibody, separated by SDS-PAGE, and immunoblotted with the indicated antibodies. C. Purified NM II-B was immunoprecipitated using NMHC II-B antibodies, similar to the experiment described in panel B, and immunoblotted with the indicated antibodies. D. Baculovirus expressed NM-A was incubated with baculovirus expressed EGFR as detailed in Experimental Procedures, immunoprecipitated with NMHC II-A antibodies, separated by SDS-PAGE, and immunoblotted with the EGFR and NMHC II-A antibodies. Both supernate and pellet and aliquots of the purified proteins are shown. N.S. indicates normal serum.

Fig. 4. The effects of NMHC II silencing on the internalization of the EGFR in A431 cells.

A. Cells were transfected with the indicated siRNAs for luciferase (Control), NMHC II-A, or NMHC II-B. Cells were serum starved for 24h, and incubated in the presence of Alexa 488-EGF (10ng/ml) at 37°C. EEA-1 (red) was used as an early endosome marker. DAPI staining (blue) marks nuclei. Bars, 10μm. B. After treatment with EGF for the indicated time, biotinylation of the cell surface proteins was performed as described in Experimental Procedures. The graph below the immunoblot shows the relative amount of the EGFR at the cell surface at the indicated times. C. Time course of 125I-EGF internalization in A431 cells. Cells were starved for 24h, and incubated with 125I-EGF (1.5ng/ml) plus cold EGF (18.5ng/ml) at 37°C for the indicated time before measuring internalized and surface-bound 125I-EGF. The ke values are...
0.572±0.029 for luciferase (Control) siRNA, 0.186±0.019 for NMHC II-A siRNA, and 0.182±0.015 for NMHC II-B siRNA. p< 0.01 (n=3) The data represent the mean of three independent experiments. D. Time course of $^{125}$I-EGF internalization in A431 cells. The $k_e$ values are 0.558±0.025 for luciferase (control) siRNA, 0.132±0.016 for NMHC II-A siRNA+ NMHC II-B siRNA, and 0.517±0.012 for NMHC II-C siRNA. p< 0.01 (n=3) The data represent the mean ±SE of three independent experiments.

**Fig. 5. Rescue of NM II-A depleted cells by NM II-B and vice versa.**

A. A431 cells were transfected with the indicated siRNAs and plasmids. Cells were serum starved for 24h, and incubated in the absence or the presence of EGF (10ng/ml) for 20 min. Biotinylation of cell surface proteins was performed as described in Experimental Procedures. B. After transfection with the indicated siRNAs and plasmids, A431 cells were starved for 24h. After EGF treatment, cells were harvested, lysed, separated by SDS-PAGE, and immunoblotted with the indicated antibodies.

**Fig. 6. The effects of NMHC II expression on downstream signaling from the EGFR.**

A. HEK-293 cells were transfected with the indicated siRNAs for luciferase (Con), NMHC II-A, or NMHC II-B. Cells were serum starved for 24h, and treated with EGF (10ng/ml) for the indicated times. Protein amounts were normalized to actin. B. COS-7 cells were transfected with the indicated siRNAs for luciferase (Control), or NMHC II-B. Cells were serum starved for 24h, and treated with EGF (10ng/ml) for the indicated times. Protein amounts were normalized to actin. C. COS-7 cells were transfected with luciferase (Control) siRNA, NMHC II-B siRNA, or NMHC II-B siRNA followed by GFP-NMHC II-B. Cells were serum starved for 24h, and treated with EGF (10ng/ml) for the indicated time. Protein amounts were normalized to actin. The data represent the mean of three independent experiments. Quantification for each panel is shown by bar graphs.

**Fig. 7. The effects of blebbistatin on downstream signals of the EGFR.**

A. A431 cells were serum starved for 24h. Cells were pre-treated with vehicle (DMSO) or blebbistatin (25μM) for 30 min and then treated with Alexa 488-EGF (10ng/ml) at 37°C for 10 min. After fixation, the images of cells were captured by confocal microscopy. EEA-1 (red) was used as an early endosome marker. DAPI staining (blue) marks nuclei. Bars, 10μm. B. A431 cells were pre-treated with the indicated concentration of blebbistatin. Cells were treated with EGF (10ng/ml) and then harvested, lysed, separated by SDS-PAGE, and immunoblotted with the indicated antibodies. Protein amounts were normalized to actin. The mean of three independent experiments along with the SE are shown by bar graphs. Some SEs cannot be visualized.

**Fig. 8. The effects of NMHC II mutants on the internalization and downstream signaling of the EGFR.**

A, C. A431 cells were transfected with the indicated siRNAs and plasmids. Cells were serum starved for 24h, and incubated in the absence or the presence of EGF (10ng/ml) for 20min. Biotinylation of cells surface proteins was performed as described in Experimental Procedures. Cells were harvested, lysed, and precipitated with streptavidin. Precipitated complexes were separated by SDS-PAGE, and immunoblotted with the indicated antibodies. B, D. A431 cells were transfected with the indicated siRNAs, and plasmids. Cells were starved for 24h, and incubated in the absence or the presence of EGF (10ng/ml) for 20min. Cells were harvested, lysed, separated by SDS-PAGE, and immunoblotted with the indicated antibodies. Actin serves as a loading control. Con, control; Vec, empty vector. pERK signals in 8B and D were too weak to quantitate. The mean of three independent experiments along with the SE are shown by bar graphs.
Fig. 1
Fig. 2
Fig. 2
B.

| siRNA       | Con  | NMHC II-B | NMHC II-C |
|-------------|------|-----------|-----------|
| EGF (min)   | 0    | 3         | 20        |

Cell surface

Pull down: streptavidin

Total

C.

Fig. 2
Fig. 4
B. siRNA Con NMHC II-A NMHC II-B
EGF 0 3 20 0 3 20 0 3 20 (min)
Cell surface
Pull down: streptavidin
Total
NMHC II-A
NMHC II-B
EGFR
TfR

C. 125I-EGF internalization (internalized/surface bound)

D. 125I-EGF internalization (internalized/surface bound)

Fig. 4
**Fig. 5.**

### A. siRNA and Cell Surface EGFR

| siRNA  | Con   | NMHC II-A | NMHC II-B |
|--------|-------|-----------|-----------|
| EGF    | -     | +         | +         |

- **Cell surface:**
  - **EGFR**
  - **TfR**

- **Total:**
  - **NMHC II-A**
  - **NMHC II-B**
  - **EGFR**
  - **TfR**

### B. siRNA and Cell Surface NMHC II

| siRNA  | Con   | NMHC II-B |
|--------|-------|-----------|
| EGF    | -     | +         |

- **Cell surface:**
  - **EGFR**
  - **TfR**

- **Total:**
  - **NMHC II-A**
  - **NMHC II-B**
  - **EGFR**
  - **TfR**
A.

| siRNA | Con | NMHC II-A | NMHC II-B |
|-------|-----|-----------|-----------|
| EGF   | 0   | 2         | 5         |
|       | 2   | 5         | 0         |
|       | 2   | 5         | 0         |

B.

| siRNA | Control | NMHC II-B |
|-------|---------|-----------|
| EGF   | 0       | 2         |
|       | 2       | 5         |
|       | 10      | 20        |

Fig. 6
C. Control siRNA + + + - - - - - -
NMHC II-B siRNA - - + + + + + + +
GFP-NMHC II-B - - - - + + + + +

EGF 0 2 5 0 2 5 0 2 5 (min)

GFP-NMHC II-B
endogenous NMHC II-B

GFP-NMHC II-B
pERK

Relative intensity of pERK

ERK

pAKT

Relative intensity of pAKT

AKT

Actin

Fig. 6
Fig. 7
B.

| EGF  | - | + | + | + | + | + | + |
|------|---|---|---|---|---|---|---|
| Blebbistatin | 0 | 0 | 5 | 10 | 25 | 50 | 100 | 200 (μM) |

**Fig. 7**

- NMHC II-A
- NMHC II-B
- pERK
- ERK
- pAKT
- AKT
- Actin
Fig. 8
Nonmuscle myosin II is required for internalization of the epidermal growth factor receptor and modulation of downstream signaling
Jong Hyun Kim, Aibing Wang, Mary Anne Conti and Robert S. Adelstein

J. Biol. Chem. published online June 20, 2012

Access the most updated version of this article at doi: 10.1074/jbc.M111.304824

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