PHD3-mediated prolyl hydroxylation of nonmuscle actin impairs polymerization and cell motility

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ABSTRACT Actin filaments play an essential role in cell movement, and many posttranslational modifications regulate actin filament assembly. Here we report that prolyl hydroxylase 3 (PHD3) interacts with nonmuscle actin in human cells and catalyzes hydroxylation of actin at proline residues 307 and 322. Blocking PHD3 expression or catalytic activity by short hairpin RNA knockdown or pharmacological inhibition, respectively, decreased actin prolyl hydroxylation. PHD3 knockdown increased filamentous F-actin assembly, which was reversed by PHD3 overexpression. PHD3 knockdown increased cell velocity and migration distance. Inhibition of PHD3 prolyl hydroxylation activity by dimethyloxalylglycine also increased actin polymerization and cell migration. These data reveal a novel role for PHD3 as a negative regulator of cell motility through posttranslational modification of nonmuscle actins.

INTRODUCTION Cell movement is a fundamental biological process that is critical for the development and maintenance of multicellular organisms. Dysregulation of cell movement is associated with disease processes, most notably cancer (Ridley et al., 2003). Actin is a highly conserved cytoskeletal protein that plays an essential role in eukaryotic cell movement (Pollard and Cooper, 2009). It consists of six isoforms in vertebrates: three α isoforms and one γ isoform are selectively expressed in skeletal, cardiac, and smooth muscle cells, whereas the nonmuscle β- and γ-actin isoforms are present in most cell types (Herman, 1993). Actin isoforms differ by only a few amino acids, and their protein structures are similar, but their biological functions are distinct in different tissues and cellular compartments (Herman, 1993).

Actin exists in monomeric (G-actin) and filamentous (F-actin) forms in cells (Dominguez and Homes, 2011). Polymerization of G-actin into double-stranded F-actin provides intracellular mechanical support that facilitates cell migration (Pollard and Cooper, 2009). Actin polymerization is highly dynamic, and many actin-binding proteins regulate this process (Insall and Machesky, 2009). A large body of data indicates that posttranslational modifications also regulate the balance between G-actin and F-actin (Terman and Kashina, 2013). Phosphorylation of Tyr-53, ADP-ribosylation of Arg-177 and Arg-206, oxidation of Cys-374, Cys-285, Cys-272, and Met-44, covalent cross-linking between Glu-270 and Lys-50, and glutathionylation, carbonylation, or nitrosylation of Cys-374 inhibit actin polymerization (Terashima et al., 1995; Dalle-Donne et al., 2000, 2003; Liu et al., 2006; Aldini et al., 2007; Lassing et al., 2007; Kudryashov et al., 2008; Visschedyk et al., 2010; Farah et al., 2011; Hung et al., 2011). Transglutamination of Lys-50, cross-linking between Gln-41 and Lys-50, methylation of His-73, ADP-ribosylation of Thr-148, and arginylation stabilize F-actin (Hegyi et al., 1992; Nyman et al., 2002; Lang et al., 2010; Saha et al., 2010).

Prolyl hydroxylases (PHDs) are α-ketoglutarate–dependent dioxygenases, which were originally identified as negative regulators of hypoxia-inducible factors (HIFs) and are present in cells of all...
metazoan species (Epstein et al., 2001; Hampton-Smith and Peet, 2009; Leonarz et al., 2011). PHDs transfer one oxygen atom from O₂ to the carbon-4 position of a prolyl residue (Pro) to form 2S, 4R-4-hydroxyproline, and the other oxygen atom reacts with α-ketoglutarate to form succinate and CO₂; in addition to utilizing α-ketoglutarate and O₂ as substrates, the enzymatic reaction requires iron (II) and ascorbate as cofactors (Gorres and Raines, 2010; Rose et al., 2011). Prolyl hydroxylated HIF-1α is bound by the von Hippel–Lindau tumor suppressor protein (VHL), which is a key component of the ubiquitin E3 ligase complex that targets HIF-1α for degradation in the 26S proteasome (Kaelin and Ratcliffe, 2008). Three PHD family members (PHD1–3) have been identified. PHD2 is the primary prolyl hydroxylase that regulates HIF-1α protein stability in nonhematopoietic cells, whereas knockdown of PHD1 or PHD3 does not affect HIF-1α protein levels in many cancer cell lines (Berra et al., 2003).

PHD3-dependent hydroxylation promotes the degradation of several other proteins, including ATF-4, β2-adrenergic receptor, Paired box 2, and Sprouty 2 (Köditz et al., 2007; Xie et al., 2009; Anderson et al., 2011; Yan et al., 2011). In contrast, PHD3 blocks VHL-mediated ubiquitination of myogenin, thereby stabilizing myogenin in differentiated C2C12 cells (Fu and Taubman, 2010).

In addition to its role in regulating protein stability, PHD3 also exerts other cellular functions. PHD3 induces prolyl hydroxylation of pyruvate kinase M2 (PKM2) to stimulate interaction with HIF-1α, leading to increased HIF-1 transactivation (Luo et al., 2011). PHD3 mediates HCL2K prolyl hydroxylation to activate the ATR/CHK1 signaling pathway in response to DNA damage (Xie et al., 2012). PHD3 has also been reported to promote apoptosis (Tennant et al., 2010; Xie et al., 2012). Recent studies demonstrated that overexpression of PHD3 inhibits invasion of pancreatic cancer cells (Su et al., 2010). PHD3 knockdown or knockout increases migration of pancreatic cancer cells and macrophages (Kiss et al., 2012; Place et al., 2013). However, the molecular mechanism underlying PHD3-mediated inhibition of cell migration has not been delineated.

In the present study, we find that nonmuscle β- and γ-actin are subject to hydroxylation at Pro-307 and Pro-322, which is catalyzed by competing with dimethyloxalylglycine (DMOG), which inhibits PHDs (SILAC) proteomic screen (Figure 1A). HeLa human cervical cancer cells and macrophages (Kiss et al., 2012; Place et al., 2013). However, the molecular mechanism underlying PHD3-mediated inhibition of cell migration has not been delineated.

β-Actin was chosen as the representative nonmuscle actin for further evaluation. To identify the prolyl hydroxylase that modifies β-actin, we performed coimmunoprecipitation (co-IP) assays with HeLa cells. Endogenous β-actin was pulled down from whole-cell lysates (WCLs) by an anti-PHD3 antibody but not by control immunoglobulin G (IgG; Figure 2A). In contrast, IP of either overexpressed FLAG-PHD1 or endogenous PHD2 failed to provide evidence of interaction with β-actin under either nonhypoxic (20% O₂) or hypoxic (1% O₂) conditions (Supplemental Figure S2). Endogenous HIF-1α was coimmunoprecipitated with FLAG-PHD1 or PHD2 from lysates of hypoxic cells, validating the IP assays (Supplemental Figure S2). Thus β-actin selectively interacts with PHD3 in HeLa cells.

We next performed in vitro hydroxylation assays to determine whether PHD3 directly hydroxylates β-actin. Wild-type (WT) glutathione S-transferase (GST)–β-actin fusion protein was expressed in Escherichia coli, purified, and incubated for 30 min with or without recombinant PHD3 in the presence of O₂, α-ketoglutarate, FeSO₄, and ascorbate. The anti-hydroxyproline antibody detected WT GST–β-actin selectively interacts with PHD3 in HeLa cells. We next performed in vitro hydroxylation assays to determine whether PHD3 directly hydroxylates β-actin. Wild-type (WT) glutathione S-transferase (GST)–β-actin fusion protein was expressed in Escherichia coli, purified, and incubated for 30 min with or without recombinant PHD3 in the presence of O₂, α-ketoglutarate, FeSO₄, and ascorbate. The anti-hydroxyproline antibody detected WT GST–β-actin when PHD3 was coincubated, indicating that WT GST–β-actin is prolyl hydroxylated by PHD3 in vitro (Figure 2B). To further determine whether PHD3 hydroxylates Pro-130, Pro-307, or Pro-322 of β-actin, we generated mutant GST–β-actin constructs in which Pro-130, Pro-307, or Pro-322 was mutated to alanine. Mutant GST–β-actin fusion proteins were purified from bacteria and incubated with or without recombinant PHD3 in an in vitro hydroxylation assay. PHD3 induced prolyl hydroxylation of GST–β-actin (P322A), GST–β-actin (P307A), or GST–β-actin (P130A), comparable to WT GST–β-actin (Figure 2B). In contrast, double-mutant GST–β-actin (P307/322A) had significantly reduced levels of prolyl hydroxylation (Figure 2B). Triple mutation of Pro-130, Pro-307, and Pro-322 to alanine reduced prolyl hydroxylation of GST–β-actin by 30%, comparable to double-mutant GST–β-actin (P307/322A; Figure 2B). Next, WT and mutant β-actin were expressed in HeLa cells as V5 epitope–tagged proteins. IP of double-mutant β-actin (P307/322A) by anti-hydroxyproline antibody was decreased compared with WT β-actin despite comparable expression levels (Figure 2C). Taken together, these in vitro and cell-based assays indicate that PHD3 hydroxylates Pro-307 and Pro-322 of human β-actin.

To determine whether PHD3 hydroxylates endogenous β-actin, we analyzed the effect of PHD3 loss of function mediated by short hairpin RNA (shRNA). HeLa cells were transduced with a retrovirus encoding a scrambled control shRNA (shSC) or PHD3 shRNA (shPHD3). PHD3 protein levels in WCLs were markedly increased when HeLa-shSC cells were exposed to hypoxic conditions (1% O₂). Expression of shPHD3 reduced endogenous PHD3 protein levels in HeLa WCLs (Figure 2D). We previously demonstrated that exposure of HeLa cells to 1% O₂ for 24 h induces PHD3 expression, which compensates for reduced catalytic activity under hypoxic conditions, thereby maintaining constant levels of prolyl hydroxylated PKM2 (Luo et al., 2011). Analysis of anti-hydroxyproline immunoprecipitates revealed that levels of prolyl hydroxylated β-actin were reduced in HeLa-shPHD3 cells as compared with HeLa-shSC cells at
HeLa-shPHD3 cells as compared with HeLa-shSC cells (Figure 3A). Increased F-actin was also observed in HeLa-shPHD3-2244 cells, which were transduced with lentivirus encoding a second independent shRNA targeting PHD3 (Supplemental Figure S3), indicating a specific effect of PHD3 knockdown on F-actin formation. To determine whether PHD3 overexpression can reverse PHD3 knockdown-induced F-actin formation, we introduced an expression vector encoding shRNA-resistant PHD3 into HeLa-shPHD3 cells. Phalloidin staining demonstrated that expression of PHD3 reduced F-actin in HeLa-shPHD3 cells to baseline levels (Figure 3, B and C). We further analyzed G-actin and F-actin fractions from HeLa-shSC and HeLa-shPHD3 cells by sedimentation assays, which demonstrated that PHD3 knockdown significantly increased F-actin levels by 1.8-fold, whereas G-actin levels were not altered (Figure 3, D and E). Taken together, these data indicate that PHD3 inhibits actin polymerization in HeLa cells.

PHD3-mediated prolyl hydroxylation impairs actin polymerization

To determine whether prolyl hydroxylation regulates F-actin assembly, we stained HeLa-shSC and HeLa-shPHD3 cells with Alexa Fluor 555–conjugated phalloidin, which selectively binds filamentous F-actin but not monomeric G-actin (Lengsfeld et al., 1974). Fluorescence microscopy revealed increased F-actin in HeLa-shPHD3 cells as compared with HeLa-shSC cells (Figure 3A). Increased F-actin was also observed in HeLa-shPHD3-2244 cells, which were transduced with lentivirus encoding a second independent shRNA targeting PHD3 (Supplemental Figure S3), indicating a specific effect of PHD3 knockdown on F-actin formation. To determine whether PHD3 overexpression can reverse PHD3 knockdown-induced F-actin formation, we introduced an expression vector encoding shRNA-resistant PHD3 into HeLa-shPHD3 cells. Phalloidin staining demonstrated that expression of PHD3 reduced F-actin in HeLa-shPHD3 cells to baseline levels (Figure 3, B and C). We further analyzed G-actin and F-actin fractions from HeLa-shSC and HeLa-shPHD3 cells by sedimentation assays, which demonstrated that PHD3 knockdown significantly increased F-actin levels by 1.8-fold, whereas G-actin levels were not altered (Figure 3, D and E). Taken together, these data indicate that PHD3 inhibits actin polymerization in HeLa cells.

To determine whether the prolyl hydroxylase activity of PHD3 is required to inhibit β-actin polymerization, we treated HeLa cells with the hydroxylase inhibitor DMOG for 72 h. Compared to both 20 and 1% O2 (Figure 2D). Because prolyl hydroxylated β-actin levels were comparable at both 20 and 1% O2, we analyzed cells exposed to 20% O2 in subsequent experiments. Similar results were observed for PKM2, again validating our assay (Figure 2D).

FIGURE 1: Nonmuscle β- and γ-actin are novel prolyl hydroxylated proteins. (A) Schematic representation of the SILAC proteome screening strategy. (B) Two prolyl hydroxylated peptides of the nonmuscle actin were identified by mass spectrometry. Hydroxyproline residues are underlined. (C, D) MS/MS spectrum of the prolyl hydroxylated nonmuscle actin peptide. Hydroxyproline residues are shown in lowercase. (E) Mapping hydroxylated proline residues Pro-307 and Pro-322 (red) in the protein structure of actin. Hydroxylated Pro-307 and Pro-322 were mapped by SWISS-MODEL (Arnold et al., 2006) using the protein structure of human β-actin (PDB ID 3BYH) as template (Galkin et al., 2008) and visualized using PyMOL (Schrödinger LLC, 2008).
PHD3 inhibits cell motility through its prolyl hydroxylase activity

To determine whether PHD3 regulates cell migration, we performed microfluidic assays with HeLa-shSC and HeLa-shPHD3 cells. Cells were seeded onto a multiple-channel microchip, and chemotaxis driven by a serum gradient was monitored for 10 h. The chemotactic migration of HeLa-shPHD3 cells was significantly increased 2.2-fold compared with that of HeLa-shSC cells (Figure 5, A and B, and Supplemental Videos S1 and S2). The mean velocity of HeLa-shPHD3 cells was 3.1-fold greater than that of HeLa-shSC cells (Figure 5C). Consistent with the microfluidic assays, scratch assays demonstrated that the cell-free area was much greater in cultures of HeLa-shSC cells compared with HeLa-shPHD3 cells after 48 h (Figure 5D). PHD3 knockdown did not alter the rate of cell proliferation (Supplemental Figure S4).

PHD3 knockdown HeLa cells assumed a spindle-shaped morphology that was distinct from that of HeLa-shSC cells (Supplemental Figure S5).

To determine whether the prolyl hydroxylase activity of PHD3 is required for inhibition of cell migration, HeLa cells were treated with DMOG for 72 h. Microfluidic assays demonstrated that DMOG significantly increased cell velocity 1.9-fold and chemotactic migration 2.0-fold compared with DMSO (Figure 6 and Supplemental Videos S3 and S4). These data indicate that PHD3 expression and prolyl hydroxylase activity are required for inhibition of cell motility.

DISCUSSION

A growing body of data indicates that PHD3 has a wider range of substrates than PHD1 or PHD2 (Jaakkola and Rantanen, 2013). Several proteins, including ATF-4, β2-adrenergic receptor, HCLK2, paired box 2, and PKM2, are prolyl hydroxylated specifically by PHD3 (Köditz et al., 2007; Xie et al., 2009, 2012; Luo et al., 2011; Yan et al., 2011). In the present study, we demonstrate that nonmuscle β- and γ-actin are also specific substrates of PHD3. Hydroxylation of nonmuscle actin by PHD3 negatively regulated actin polymerization and cell motility. Previous and current studies on β2-adrenergic receptor, HCLK2, PKM2, and actin revealed that there is no strong consensus sequence surrounding proline residues that are hydroxylated by PHD3 (Köditz et al., 2007; Xie et al., 2009, 2012; Luo et al., 2011; Yan et al., 2011). In the present study, we demonstrate that nonmuscle β- and γ-actin are also specific substrates of PHD3. Hydroxylation of nonmuscle actin by PHD3 negatively regulated actin polymerization and cell motility.
In vitro hydroxylation assays and mass spectrometry identified Pro-307 and Pro-322 of human β-actin as two target residues for hydroxylation by PHD3. Double mutation of Pro-307 and Pro-322 of β-actin significantly but incompletely reduced prolyl hydroxylation, and thus additional Pro residues of β-actin may be subject to hydroxylation. Structural analysis of actin demonstrated that Pro-322 is located within a structural hinge (residues 321–324 in subdomain 3) that rotates as a rigid unit, leading to a conformational change (Page et al., 1998). Electron cryomicroscopy also revealed that interaction of Pro-322 with Gly-245 of a neighboring actin molecule is important for actin polymerization (Fujii et al., 2010), suggesting that prolyl hydroxylation may interfere with this interaction to block polymerization. Further studies are required to determine how prolyl hydroxylation impairs actin polymerization and cell motility.
Actin plays a critical role in metastasis (Pollard and Cooper, 2009; Carnell and Insall, 2011). Inhibition of PHD3 markedly increased cell motility. PHD3-knockdown cells also assumed a more spindle-shaped morphology. Thus PHD3 may alter the physical properties of cells by disrupting F-actin formation, thereby impairing cell migration. Expression of PHD3 is down-regulated in pancreatic cancer, colon cancer, and metastatic melanoma (Qi et al., 2008; Xue et al., 2010; Place et al., 2011). PHD3 expression is also negatively

A previous study showed that PHD2 knockdown increased F-actin formation in cancer cells (Vogel et al., 2010). PHD2 knockdown led to increased cofilin-1 phosphorylation, which inhibits F-actin disassembly. Our data demonstrate that PHD2 does not bind to β-actin, which excludes the possibility that PHD2 hydroxylates β-actin in HeLa cells.

In human cancers, metastasis is responsible for ~90% of cancer deaths. Invasion and metastasis require cancer cell motility, and actin plays a critical role in metastasis (Pollard and Cooper, 2009; Carnell and Insall, 2011). Inhibition of PHD3 markedly increased cell motility. PHD3-knockdown cells also assumed a more spindle-shaped morphology. Thus PHD3 may alter the physical properties of cells by disrupting F-actin formation, thereby impairing cell migration. Expression of PHD3 is down-regulated in pancreatic cancer, colon cancer, and metastatic melanoma (Qi et al., 2008; Xue et al., 2010; Place et al., 2011). PHD3 expression is also negatively

FIGURE 5: PHD3 knockdown increases HeLa cell motility. (A–C) Microfluidic assays were performed using HeLa-shSC and HeLa-shPHD3 cells. (A) Representative images from three independent experiments. The yellow asterisks indicate the position of cells after 600 min of migration, and the red asterisks indicate the initial starting position of cells at 0 min. Scale bar, 20 μm. (B) Quantification of chemotactic migration. Mean ± SEM, n = 297 (HeLa-shSC) or 266 (HeLa-shPHD3) cells. ***p < 0.001 vs. shSC. (C) Quantification of cell velocity. Mean ± SEM, n = 305 (HeLa-shSC) or 327 (HeLa-shPHD3) cells. ***p < 0.001 vs. shSC. (D) Scratch assays were performed with HeLa-shSC and HeLa-shPHD3 cells. Representative images at indicated time points from two independent experiments. Scale bar, 100 μm.
correlated with breast cancer grade and stage (Peurala et al., 2012). These findings suggest that PHD3 may inhibit metastasis of multiple human cancers by mediating prolyl hydroxylation of nonmuscle actins to block F-actin assembly. Further studies are required to determine the effect of modulating PHD3 activity on invasion and metastasis in murine cancer models.

MATERIALS AND METHODS

Plasmid constructs

Human β-actin or PHD3 cDNA was amplified by reverse transcription PCR and cloned into pcDNA3.1-V5-His or pGEX-6P-1 vector (GE Healthcare, Piscataway, NJ). Pro–mutant β-actin cDNA was generated using QuickChange Site-directed Mutagenesis Kit (Stratagene, La Jolla, CA) and subcloned to pcDNA3.1-V5-His or pGEX-6P-1 vector. shRNA-resistant PHD3 cDNA was generated using QuickChange Site-directed Mutagenesis Kit (Stratagene) and subcloned to lentiviral vector EF.v-CMV.GFP. Other constructs have been described previously (Luo et al., 2011).

Lentivirus production

HEK293T cells were cotransfected with transducing vector encoding PHD3 or empty vector or packaging vectors pMD.G and pCMVR8.91. After 48 h, lentivirus particles in the medium were harvested, filtered, and transduced into HeLa-shPHD3 cells.

Cell culture

HeLa cells (Scherer et al., 1953) were cultured in DMEM with 10% heat-inactivated fetal bovine serum (FBS) at 37°C in a 5% CO\textsubscript{2}/95% air incubator. For hypoxia exposure, cells were placed in a modular incubator chamber (Billups-Rothenberg, Del Mar, CA) that was flushed with a gas mixture containing 1% O\textsubscript{2}, 5% CO\textsubscript{2}, and balance N\textsubscript{2} and incubated for 24 h at 37°C.

In vitro prolyl hydroxylation assays

WT or mutant GST–β-actin and GST-PHD3 fusion proteins were expressed in E. coli BL21-Gold (DE3) and purified by binding to glutathione-Sepharose beads (GE Healthcare). WT or mutant GST–β-actin was eluted from beads with 20 mM reduced glutathione. Recombinant PHD3 was obtained by removal of GST with PreScission protease at 4°C. WT or mutant GST–β-actin fusion protein was incubated at 30°C for 30 min with or without recombinant PHD3 protein supplemented with 50 mM Tris/HCl (pH 8.0), 100 mM NaCl, 100 μM DTT, 100 μM FeSO\textsubscript{4}, 5 mM ascorbate, and 1 μM α-ketoglutarate. The prolyl hydroxylation reaction was stopped by adding Laemmli sample buffer and analyzed by immunoblot assays using anti-hydroxyproline antibody (Abcam, Cambridge, MA).

Co-IP assays

Cells were lysed in modified radioimmunoprecipitation assay buffer, and WCLs were incubated overnight with anti-FLAG (Sigma-Aldrich, St. Louis, MO), anti-PHD2 (Novus Biologicals, Littleton, CO), anti-PHD3 (Novus Biologicals), or anti-hydroxyproline (Abcam) antibody in the presence of protein A–agarose beads (Novus Biologicals). After three washes, the bound proteins were fractionated by
SDS–PAGE and analyzed by immunoblot assays using antibodies against the following proteins or epitope tag: PHD2, PHD3, β-actin, PKM2 (Novus Biologicals), VS (Invitrogen, Carlsbad, CA), or FLAG (Sigma-Aldrich).

Microfluidic assays

Microfluidic experiments were performed as previously described (Lin et al., 2012). Briefly, cells were seeded onto microfluidic chips in DMEM containing 0.5% FBS and were allowed to attach for 8–12 h. A gradient was formed across the microchannels by flowing DMEM containing 10% FBS in one of the flanking flow arms and DMEM containing 0.5% FBS in the other arm. During experiments, cells were incubated under 37°C and 95% air/5% CO₂. Images were acquired under brightfield illumination every 10 min with a Zeiss Axiosvert 200M microscope coupled to a Cascade II:1024 electron-multiplying charge-coupled device camera (Photometrics, Tucson, AZ) using a 40×/1.3 numerical aperture (NA) oil immersion objective. Cells were tracked manually using custom scripts written in Matlab 2007b (MathWorks, Natick, MA). Velocity data were generated based on coordinates obtained from cell tracking. Chemotactic migration measurements were defined as the final displacement of cells from their initial positions. Positive values were assigned to distances toward the gradient, whereas negative values were assigned to distances away from the gradient. Cells migrating within microfluidic channels for <10 h were excluded from these measurements.

Scratch assays

Cells were seeded onto 10-cm dishes and cultured overnight to reach 80–90% confluence. The cell layer was scratched with a sterile 1-ml pipette tip, labeled, and photographed at the indicated time points with an inverted IX71 microscope (Olympus, Center Valley, PA) that was equipped with a digital camera (Olympus DP70) using a 10×/0.3 NA objective.

Fluorescence microscopy

After rinsing with phosphate-buffered saline (PBS), cells were fixed with 3.7% methylamine-formaldehyde for 10 min at room temperature, permeabilized with 0.1% Triton X-100 in PBS for 5 min, and incubated with 1% bovine serum albumin for 60 min. For phalloidin staining, cells were incubated for 120 min with Alexa Fluor 555–conjugated phalloidin in the dark. Cells migrating within microfluidic channels for <10 h were excluded from these measurements.

Actin sedimentation assays

Cells were homogenized in 0.5 ml of prewarmed lysis buffer containing 50 mM 1,4-piperazinediethanesulfonic acid (pH 6.9), 50 mM NaCl, 5 mM MgCl₂, 5 mM ethylene glycol tetraacetic acid, 5% glycerol, 0.1% Igepal CA-630, 0.1% Triton X-100, 0.1% Tween 20, 0.1% β-mercaptoethanol, 0.001% Antifoam C, 100 mM ATP, and protease inhibitor cocktail and centrifuged at 2000 rpm for 5 min to remove unbroken cells. The supernatant (0.4 ml) was centrifuged at 100,000 × g for 60 min at 37°C. The supernatants, which contained G-actin, were collected, and the pellets, which contained F-actin, were resuspended in 0.4 ml of lysis buffer and sonicated. Equal volumes of G-actin and F-actin fractions were analyzed by immunoblot assays using an anti–β-actin antibody (Novus Biologicals).

Statistical analysis

Data are expressed as mean ± SEM. Differences were analyzed by Student’s t test; p < 0.05 was considered significant.

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