Regulation of Focal Adhesion Dynamics and Cell Motility by the EB2 and Hax1 Protein Complex

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Cell migration is a fundamental cellular process requiring integrated activities of the cytoskeleton, membrane, and cell/extracellular matrix adhesions. Many cytoskeletal activities rely on microtubule filaments. It has been speculated that microtubules can serve as tracks to deliver proteins essential for focal adhesion turnover. Three microtubule end-binding proteins (EB1, EB2, and EB3) in mammalian cells can track the plus ends of growing microtubules. EB1 and EB3 together can regulate microtubule dynamics by promoting microtubule growth and suppressing catastrophe, whereas, in contrast, EB2 does not play a direct role in microtubule dynamic instability, and little is known about the cellular function of EB2. By quantitative proteomics, we identified mammalian HCLS1-associated protein X-1 (HAX1) as an EB2-specific interacting protein. Knockdown of HAX1 and EB2 in skin epidermal cells stabilizes focal adhesions and impairs epidermal migration in vitro and in vivo. Our results further demonstrate that cell motility and focal adhesion turnover require interaction between Hax1 and EB2. Together, our findings provide new insights for this critical cellular process, suggesting that EB2 association with Hax1 plays a significant role in focal adhesion turnover and epidermal migration.

Cell migration is an essential process for developmental morphogenesis, wound healing, and tumor metastasis. The intricate, multistep process of cell migration requires integrated activities of the cytoskeleton, membrane, and cell/extracellular matrix adhesions (1, 2). During the process, focal adhesion plays a critical role in establishing a connection between the extracellular matrix and the actin cytoskeleton and serves as a point of traction for the cell (1, 3–6).

Microtubules are polar filaments with two structurally and functionally distinct ends, the plus end and the minus end. Interestingly, it has been well documented that microtubules can target peripheral focal adhesions (7–10), a process mediated by the mammalian spectraplakin protein ACF7 (11, 12). Furthermore, accumulating evidence has shown that the growth of microtubules can promote focal adhesion turnover by serving as tracks to deliver proteins essential for focal adhesion disassembly (7–10). For example, recently, a mitogen-activated protein kinase kinase kinase kinase 4 (MAP4K4)2 has been identified as a focal adhesion regulator that associates with microtubules. Knockout of MAP4K4 stabilizes focal adhesions and impairs cell migration (13).

Microtubule plus end tracking proteins are a diverse group of evolutionarily conserved proteins that enrich at the growing ends (plus ends) of microtubules (14, 15). Plus end proteins have been shown to participate in different aspects of cell architecture through their function in regulating microtubule dynamics and the interaction of microtubules with other cellular structures. It has been established that the three microtubule end-binding proteins (EB1, EB2, and EB3) in mammalian cells can track the plus ends of growing microtubules. They also share substantial sequence homology. EB1 and EB3 together can regulate microtubule dynamics by promoting microtubule growth and suppressing catastrophe, whereas, in contrast, EB2 does not play a direct role in microtubule dynamic instability, and little is known about the cellular function of EB2 (16, 17). Interestingly, our recent work has demonstrated that EB2 plays an essential role in the regulation of focal adhesion dynamics and cell migration via its interaction with MAP4K4 (13).

To dissect the roles of different EB proteins during cell motility, we determined the interactomes of EB1, EB2, and EB3 by a quantitative proteomics approach (18, 19). Our MS analysis revealed an intriguing interaction partner, HAX1, which is specifically associated with EB2 but not EB1 or EB3. Hax1 was initially identified as a binding partner of HS1, the hematopoietic homologue of cort actin (20). It has been suggested that deficiency in Hax1 leads to neutropenia by regulating neutrophil apoptosis (21). However, Hax1 is actually a ubiquitous protein that regulates the actin cytoskeleton and cell migration. Hax1 has been shown to associate with various cell adhesion molecules, including β6 integrin, cortactin, and HS1 (22, 23). Most interestingly, it has been shown that loss of Hax1 in neutrophils enhances integrin-mediated cell adhesion, strongly suggesting that Hax1 is critically involved in cell adhesion dynamics (24).

Mammalian skin provides a versatile and accessible platform to investigate cytoskeletal dynamics and cell migration in vivo (12, 25, 26). Impaired movement of epidermal cells can delay skin wound healing and have dire consequences for animal sur-

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† This article contains supplemental Tables 1–3 and Movies 1–4.

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2 The abbreviations used are: MAP4K4, mitogen-activated protein kinase kinase kinase kinase 4; EB, end-binding; TIRF, total internal reflection fluorescence microscopy.
vival. In this report, we found that knockdown of HAX1 or EB2 in skin keratinocytes leads to aberrant focal adhesion dynamics and impaired cell migration. With a skin grafting model, we further show that both HAX1 and EB2 play an essential role in skin wound healing and epidermal migration in vivo. Taken together, our study unravels an important mechanism whereby microtubule plus ends regulate focal adhesion turnover and directional cell movement through interaction between EB2 and Hax1.

**Experimental Procedures**

**Stable Isotope Labeling by Amino Acids in Cell Culture and LC-MS/MS Analysis**—HEK293T cells were labeled with either “heavy” or “light” isotopic lysine using a stable isotope labeling by amino acids in cell culture protein quantification kit (Thermo Scientific, Rockford, IL) according to the instructions of the manufacturer. Briefly, cells were grown in DMEM:F12 medium supplemented with 10% dialyzed fetal bovine serum and either the heavy form of L-lysine-2HCl (4,4,5,5-D4) or light L-lysine for more than six generations to achieve more than 98% labeling efficiency (19). Cells were transiently transfected with expression vectors encoding N-terminal triple HA-tagged EB1, EB2, EB3, or GST as a “spike-in” control.

Protein lysates were subjected to purification with an α-HA affinity column, and nonspecific binding proteins were washed off with washing buffer containing increasing amount of salts. Protein bands were excised by sterile razor blade and chopped into ~1-mm³ pieces. MS analysis was carried out by the proteomics core of the University of Chicago. Each sample was washed in water and destained using 100 mM ammonium bicarbonate (pH 7.5) at 20 °C for 30 min. Gel samples were washed in water, then in acetonitrile, and washed with 5% formic acid and vacuum-dried.

The peptide samples were loaded to a 0.25-μm OptiPak trapping cartridge custom-packaged with Michrom Magic C8 (Optimize Technologies), washed, and then switched in-line with a 20 cm × 75 μm C18 packed spray tip nanocolumn packed with Michrom Magic C18AQ for a two-step gradient. Mobile phase A was water/acetonitrile/formic acid (98/2/0.2), and mobile phase B was acetonitrile/isopropanol/water/formic acid (80/10/10/0.2). Using a flow rate of 350 nl/min, a 90-min, two-step LC gradient was run from 5% B to 50% B in 60 min, followed by 50–95% B over the next 10 min, and then for 10 min at 95% B, back to starting conditions, and re-equilibrated. The samples were analyzed via LC-MS/MS on a Q-Exactive (Thermo Scientific) mass spectrometer using a 60,000 reversed phase survey scan, m/z 375–1950, with lockmasses, followed by 15 higher-energy collisional dissociation collision-induced dissociation scans on only doubly and triply charged precursors between 375 Da and 1950 Da. Inclusion lists of expected acetylated or phosphor-ylated tryptic in silico peptide ion masses were also used. Ions selected for MS/MS were placed on an exclusion list for 60 s.

Tandem mass spectra were extracted by MSConvert (ProteoWizard 3.0.3768) All MS/MS samples were analyzed using MaxQuant (Max Planck Institute of Biochemistry, Martinsried, Germany; version 1.2.2.5). MaxQuant was set up to search the 140204_SPROT_HUMAN database (unknown version, 47496 entries) also assuming strict trypsin. MaxQuant and X! Tandem entries also assuming strict trypsin. MaxQuant and X! Tandem were searched with a fragment ion mass tolerance of 20 parts per million and a parent ion tolerance of 20 PPM. Carbamidomethyl of cysteine was specified in MaxQuant as a fixed modification. Label:2H(4) of lysine, oxidation of methionine, acetyl of the N terminus, and phospho of serine, threonine, and tyrosine were specified in MaxQuant as variable modifications.

**Antibodies, Reagents, and Plasmid DNA Constructions**—The mouse monoclonal antibody against HAX1 was obtained from BD Biosciences. The rat monoclonal antibody against EB2 was obtained from Thermo (Waltham, MA). Human plasma fibronectin, HA-conjugated agarose, mouse monoclonal Vinculin, and β-tubulin antibodies were obtained from Sigma. Mouse monoclonal antibodies against Myc and rabbit polyclonal antibodies against HA were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Other chemicals or reagents were obtained from Sigma unless indicated otherwise.

Plasmids encoding DsRed-Zyxin, GFP-paxillin, and EB2 have been described previously (11, 27). Full-length HAX1 cDNA was gBlocked from IDT (Coralville, IA) and cloned into the mammalian expression vectors pHK3S and pHANS (with an N-terminal HA or Myc tag). The plasmid encoding full-length EB2 cDNA was a gift from Dr. Yulia Komarova (University of Illinois at Chicago). The EB2 coding sequence was cloned to other mammalian expression vectors, including pHK3s. Mutations in HAX1 were created by the following primers: GCG GGA TCC ATG AGC CTC TTT GTA GTT CTC TTC CG, CGG AAT TCC TAC TAC TGT GCA GGA CCT GGA AG; GCG GGA TCC ATG ACA CCT GGT GAG AGA CTA CGG, CGG AAT TCC TAC CGG GAG CGG AACCAA C; GCG GGA TCC GAT GAC ACC TGG TGA GAG ACT AGC G; CGG AAT TCC TAG GCT GGA GGT CTT GTT GAT TC; CGG AAT TCC TAT CTG GTC GAT TCT CTA GGA TGG GGC; CGC GGA TCC ATG GAG GAC ATC TCG AGA TTT AGT GCT TTC GCT TTT TG, cga aCAA AAA AGC AGA AGC TAC GAG ACT CAA TCG CTA GAT TGA GTG TCG TAG TGT CTT G; aCC GGC CAG CCC AAA TCG TAT TTC AAC ATC TCG TGT G; cga aCAA AAA AGC CCA AAT CGT AT TCA ACT CTA CGA GGT TTA TGT GTC GTT GGG CTG Gc; aCC GGT CAG CTT TGG TGA GTT TGT TTG, cga aCAA AAA AGC ATC TCG AGA AAG GGA GTC TCA AAA AAT GGT TTT TG T, cga aCAA AAA ATC GGT CTT TGG ATG TCT CTT TTC TCG AGA AAG GGT CAT CCA AAG CTG C. The mutation at the EB2 shRNA recognition site was created by overlapping PCR with the following primers: AGC ATC TGT TAA ACG GAT GAA CGT CCA AGT CAT CCC AGT GGA GAA G, CTT CTC CAC TGG GAT GAC TTT GTC.
GAC GTT CAT CCG TTT AAA GGA TGC T. The mutations in EB2 were created by overlapping PCRs with the following primers: AAG GAA AAA AGC GGC CGC TCA TGG CGG TCA ATG TGT ATT CTA C, GGA ATT CTT AAT ACT CTT CTT GTT CCT GTT GTG GCC CCC CCT CAT CAG GTA TCA CAA AGC CCT CAT CGG AAG GTT AGA GCA C, TCC TAA GGC TGG CCC CCC AAT GTT GCG AAA GAA TCC TGG TGT GGG CAA TGG AGA TGA TGA AGC TGA ACT ACA CGA GCA GGT ACA TCC, CCC CAG AGA CCC ATT GCA ACA CAG AGC ACT GCA GCT CCT AAG GCT GCC CCC GGA ATG, GAA GAT CTT CAA CCT GCC CAA GAA GCC TCT CGG TCT CAC TAC TGC AGC CCC ACA GAG ACC CAT TGC AAC. For the EB2/EB1 swapping mutant, the internal region close to the coiled-coil domain from EB2 was changed to the corresponding sequence from EB1 (PLGSSTAAPQRPIAT-QRTTAAPKAGPGMVRRKPVNGDDEAEE), or the acidic C terminus of EB2 (QEGQTEPEEAEQQHDPQqqqQqQY) was changed to the corresponding sequence from EB1 (GFV-IPDEGPGQEEY).

**Skin Grafting and Wound Healing**—All mice used in this study were bred and maintained at the Animal Resource Center of the University of Chicago in accordance with institutional guidelines.

Skin grafting was carried out essentially as described previously (28). Briefly, dermis was prepared from newborn skin, and primary keratinocytes were seeded onto the dermis and cultured. Skins were then grafted on the back of adult nude mice. For skin wound healing assays, nude mice were anesthetized, and two full-thickness excisional wounds were made on both sides of the dorsal midline (28) where skins were grafted. Mice were housed separately, and no self-induced trauma was observed in mice. Tissue was collected 2–6 days after wounding, and wound re-epithelialization was evaluated by histological analyses. Hyperproliferative epidermis was identified by hematoxylin and eosin staining, and the length of the hyperproliferative epidermis that extended into the wounds was measured and quantified.

**Histology and Immunofluorescence**—Skin or wound samples were embedded in optimal cutting temperature, frozen, sectioned, and fixed in 4% formaldehyde. For paraffin sections, samples were incubated in 4% formaldehyde at 4 °C overnight, dehydrated with a series of increasing concentrations of ethanol, and then embedded in paraffin. Paraffin sections were rehydrated in decreasing concentrations of ethanol and subjected to antigen unmasking in 10 mM citrate (pH 6.0). Sections were subjected to hematoxylin and eosin staining or immunofluorescence staining as described previously (28). Antibodies were diluted according to the instructions of the manufacturer unless indicated otherwise.

For immunofluorescence staining of cultured keratinocytes, cells were fixed in 4% formaldehyde and then permeabilized in 0.5% Triton X-100. Processed cells were subjected to immunostaining as described previously (28). For staining of EB2 and microtubules, an optimized staining procedure was used to preserve microtubule structure. Cells were fixed in microtubule fixation buffer containing 80 mM PIPES (pH 6.9), 50 mM NaCl, 2 mM MgCl2, 0.4 mM CaCl2, 1% glutaraldehyde, 3% paraformaldehyde, 0.2% Triton X-100. Autofluorescence of glutaraldehyde was quenched by treatment of NaBH4 after staining. Stained cells were subjected to imaging with confocal or TIRF microscopy.

**Cell Culture and Transfections**—Primary mouse keratinocytes were isolated from the epidermis of newborn mice using trypsin after prior separation of the epidermis from the dermis by an overnight dispase treatment. Keratinocytes were plated on mitomycin C-treated 3T3 fibroblast feeder cells until passage 3. Cells were cultured in E-medium supplemented with 15% serum and a final concentration of 0.05 mM Ca2+. All experiments were performed using primary cells with less than 10 passages.

For transient transfections, primary keratinocytes were transfected with FuGENE6 according to the protocol of the manufacturer. Cells were usually examined 24–48 h post-transfection.

**Cell Migration Assays and Time-lapse Video Microscopy**—The scratch wound healing assay was performed essentially as described previously (12). Briefly, keratinocytes were plated on 35-mm tissue culture dish coated with fibronectin. After cells reached confluency, wounds were created by manual scraping of the cell monolayer with a pipette tip. The dishes were then washed with PBS, replenished with media, and photographed using a phase-contrast microscope. Afterward, dishes were placed in the tissue culture incubator, and the matched wound regions were photographed 12 and 24 h after wounding. To trace the movement of individual keratinocytes, cells were plated on fibronectin-coated dishes and imaged with an Olympus phase-contrast microscope (×20) for 3 h at 2 frames/min and tracked manually in ImageJ.

**Focal Adhesion Assembly/Disassembly Measurements**—Kinetics of focal adhesion assembly and disassembly were performed essentially as described previously (12). Keratinocytes were plated on fibronectin-coated dishes and transfected with a plasmid encoding GFP-paxillin. Time series of images were acquired on a spinning disc confocal microscope equipped with a ×100 α-plane (1.45 oil) lens and an EM charge-coupled device camera. The rate constants for focal adhesion assembly and disassembly were obtained by calculating the slope of relative fluorescence intensity increases or decreases of individual focal adhesion on a semilogarithmic scale against time.

**Western Blots**—Western blotting was performed as described previously (12). Briefly, equal amounts of the cell lysates were separated on a 12% SDS-PAGE and electrobotted onto a nitrocellulose membrane. The immunoblot was incubated with Odyssey blocking buffer (Li-Cor) at room temperature for 1 h, followed by overnight incubation with primary antibody. Blots were washed three times with Tween 20/Tris-buffered saline (TBST) and incubated with a 1:10,000 dilution of secondary antibody for 1 h at room temperature. Blots were again washed three times with TBST. Visualization and quantification was carried out with the Li-Cor Odyssey scanner and software (Li-Cor Biosciences).

**Statistical Analysis**—Statistical analysis was performed using Excel or OriginLab software. Box plots are used to describe the entire population without assumptions on the statistical distri-
Results

Identification of HAX1 as an EB2-specific Binding Partner—Mammalian cells express three EB proteins (EB1, EB2, and EB3), among which the function of EB2 remains most unclear. To dissect the cellular function of different EB molecules, we engineered expression vectors encoding triple HA-tagged EB1, EB2, and EB3. The presence of N-terminal HA tags does not affect EB protein association with microtubules (Fig. 1A). We used affinity purification to isolate different EB complexes from transfected cells, and then employed stable isotope labeling by amino acids in cell culture coupled with LC-MS/MS to determine the interactome of each EB protein (Fig. 1, A and B). With GST as a common spike-in control, our quantitative proteomics revealed multiple binding partners for each EB molecule (Fig. 1C and supplemental Tables 1–3). Interestingly, in addition to EB proteins themselves (heterodimerization) and microtubule motor molecules, EB2 interacts with far more binding partners than EB1 or EB3. With a cutoff log2 ratio (heavy/light) of more than 2, we identified ~90 binding proteins for EB2, whereas EB1 and EB3 had less than 10 specific binding partners in our analysis (supplemental Tables 1–3). These results are consistent with our previous hypothesis that EB2 may act as a microtubule adaptor protein to recruit functional cargo molecules to the plus ends of growing microtubules (13).

Of all identified EB2 binding partners, Hax1 is particularly interesting to us. Our proteomics results suggest that it is an EB2-specific binding protein because the same protein was not retrieved from EB1 or EB3 pulldowns. Hax1 has a log2 (heavy/light) ratio of 19.32, suggesting high-affinity association with...
EB2. More importantly, previous studies have demonstrated a potential role of Hax1 in cell adhesion (22–24), which is consistent with the function of EB2 in the regulation of focal adhesion dynamics and cell motility. To verify the interaction between HAX1 and EB2, we co-expressed Hax1 with EB1, EB2, or EB3. Immunoprecipitation results suggest that Hax1 only associates with EB2 but not EB1 or EB3, as expected (Fig. 2A). We also carried out endogenous co-immunoprecipitation assays and detected a significant amount of EB2 in HAX1 immunoprecipitates but not in the control samples (Fig. 2B).

EB family members share significant sequence similarity. Alignment of three EB family members suggests three unique regions in EB2, the N terminus, the acidic C terminus (CT), and an internal region close to the coiled-coil (cc) domain (Fig. 2C). To examine their contribution to binding with Hax1, we introduced deletion or swapping mutations to EB2 by either removing the N terminus or replacing the different regions in EB2 with corresponding sequences from EB1 (Fig. 2C). We then carried out co-immunoprecipitation assays to determine their respective binding affinities to Hax1. Our results show that changes in any of the three regions lead to a significant reduction in Hax1 binding, whereas N terminus deletion almost abolished Hax1 interaction (Fig. 2C). These results suggest that Hax1 may associate with multiple regions in EB2, with the N terminus of EB2 harboring the most critical binding determinants.

To further characterize Hax1 interaction with EB2, we generated different truncation mutants of Hax1. Co-immunoprecipitation analysis showed that a C-terminal part other than the N-terminal part of HAX1 is necessary for the interaction with EB2 (Fig. 2D). To narrow down the potential domain involved in the interaction between HAX1 and EB2, we further generated and examined different HAX1 C-terminal deletion mutants on the basis of previous literature (22–24), including the ∆183 mutant (deletion of the C-terminal 97 residues that include the potential transmembrane domain) and ∆TMD (deletion of 18 residues at the transmembrane domain) mutant. Co-immunoprecipitation results show that ∆183, but not ∆TMD,
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Abolished EB2 interaction (Fig. 2D), suggesting that the corresponding C-terminal region is essential for EB2 binding. Hax1 has been shown to regulate integrin-based cell adhesion in neutrophils (24). To test subcellular localization of Hax1, we carried out immunofluorescence staining of endogenous Hax1 together with focal adhesions in cultured primary keratinocytes. Confocal imaging of stained keratinocytes indicates a punctate localization of Hax1 at the ventral surface of the cell, with enriched staining at the cell periphery, where focal adhesions localize (Fig. 3A; the complete Z stacks of the confocal images are shown in supplemental Movies 1 and 2). Staining of Hax1-depleted cells with the same antibody shows strongly diminished signals, supporting the specificity of our staining procedures (Fig. 3A and supplemental Movies 3 and 4). To specifically image Hax1 localization at cell adhesions and suppress the signals from cytosolic Hax1, we employed total internal reflection fluorescence microscopy (TIRF), which can selectively illuminate and excite fluorophores in the restricted region immediately adjacent to the cell-contacting surface (29). With TIRF microscopy, our staining shows significant enrichment of Hax1 around focal adhesions in WT keratinocytes, and depletion of EB2 disrupts this colocalization (Fig. 3B; quantification in Fig. 3C).

Our recent study has demonstrated the potential role of EB2 as an adaptor molecule to deliver key proteins through microtubules for regulation of focal adhesion dynamics (13). To determine the localization of EB2 and Hax1 in keratinocytes, we imaged both endogenous and exogenously expressed Hax1 and EB2 with TIRF microscopy. Because microtubule network and EB2 staining are sensitive to fixation, we examined the localization of Hax1 and EB2 under different conditions. Our results demonstrate colocalization between Hax1 and EB2 in both formaldehyde-fixed (Fig. 4A) and glutaraldehyde-fixed (Fig. 4B) keratinocytes. In addition, transiently expressed Hax1 and EB2 display a similar colocalization under TIRF (Fig. 4B).

To test the potential association between Hax1 and microtubules, we performed microtubule pulldown assays. Our data indicate microtubule association of Hax1 in WT cells but not in EB2-depleted cells (Fig. 4C). Depletion of Hax1 in cultured keratinocytes, however, does not affect EB2 association with microtubules (Fig. 4C). Together, our results suggest that EB2 can recruit Hax1 to microtubule growing ends and focal adhesions through its interaction with Hax1.

**EB2 and Hax1 Interaction Is Essential for Epidermal Cell Migration**—Our previous work established an important role of EB2 in regulation of focal adhesion turnover and cell motility (13). To examine whether EB2 and Hax1 interaction contributes to cell movement, we first monitored cell migration with EB2 and Hax1 knockdown keratinocytes. When introducing an ~500-μm scratch wound into the cell monolayer, EB2 and Hax1 knockdown cells both displayed a significant delay in the recovery of these scratches (Fig. 5, A and B). BrdU incorporation analysis shows comparable cell proliferation in control cells or cells depleted of EB2 or Hax1 (Fig. 5B), strongly suggesting that the delayed wound healing is likely due to cell movement defects. Video microscopy permitted imaging and monitoring the velocities of individual keratinocytes (Fig. 5C). Depletion of endogenous Hax1 or EB2 by shRNA leads to significant inhibition of cell motility (Fig. 5, C and D). Re-expression of exogenous EB2 or Hax1 in the corresponding knockdown cells can restore cell motility. However, expression of an EB2 mutant deficient for Hax1 interaction (EB2-DN) or a Hax1 mutant defective in EB2 interaction (Hax1-ΔC183) failed to recover the motility defect, strongly suggesting that EB2 and Hax1 interaction plays an essential role in cell migration.
To determine the role of EB2/Hax1 interaction in focal adhesion dynamics, we first examined cell/extracellular matrix adhesions after EB2 or Hax1 depletion. Immunofluorescence microscopy showed significantly enhanced labeling of focal adhesion in EB2 or Hax1 knockdown keratinocytes relative to controls (Fig. 6A). Quantification of the presence of vinculin showed a significant increase in the size of focal adhesions in the two knockdown lines. To further examine the role of HAX1/EB2 association in focal adhesion dynamics, we employed confocal video microscopy to trace and examine the behavior of individual focal adhesion in knockdown cells. To monitor this process, we transfected cells with plasmids encoding GFP-paxillin. Representative examples of the perturbations in focal adhesions dynamics arising from HAX1 depletion are shown in montages in Fig. 6B. During the interval of observation, focal adhesions in control keratinocytes usually underwent continuous bouts of formation, maturation, and disassembly, whereas focal adhesion in EB2- or Hax1-
depleted cells were significantly more static. Quantifications of the kinetics of individual focal adhesions revealed dramatic decrease in the disassembly rates of focal adhesions in knockdown cells (Fig. 6C). Exogenous expression of WT EB2 or Hax1 in the corresponding knockdown cells can restore focal adhesion turnover, whereas the EB2 mutant deficient for Hax1 interaction (EB2-ΔN) or the Hax1 mutant defective in EB2 interaction (Hax1-ΔC183) cannot (Fig. 6C). Together, our results provide compelling evidence that EB2 interaction with Hax1 plays a critical role in focal adhesion dynamics.
Both EB2 and Hax1 Are Essential for Skin Wound Repair in Vivo—Our in vitro analysis clearly demonstrated the critical role of EB2/Hax1 interaction in cell adhesion dynamics and cell movement. However, the in vivo relevance remains unclear. Although in vitro approaches to explore signaling cascades controlling cell migration are readily available, the development of corresponding animal models to determine their significance in vivo can be costly and time-consuming. In this regard, skin epidermis provides a unique platform to investigate cell migration in vivo (12, 25, 26). Primary skin epidermal stem cells can be grafted to nude mice via chamber grafting (28), allowing us to examine the role of EB2/Hax1 interaction in vivo in a rapid and cost-effective manner. Transplantation of skin epidermal cells to nude host led to efficient skin engraftments that are stable and could readily express exogenous genes that were transduced to keratinocytes (Fig. 7, A and B).

**FIGURE 6.** EB2 interaction with Hax1 regulates focal adhesion dynamics in vitro. A, box and whisker plot indicating the size distribution of focal adhesions in WT or EB2 or Hax1 knockdown (KD) cells (~50 focal adhesions were analyzed for each genotype). Statistical analysis with Student’s t test showed that knockdown of EB2 or Hax1 leads to a significant increase in focal adhesion size compared with mock cells (p < 0.01). B, representative time-lapse images (montages) of GFP-paxillin-expressing keratinocytes. Note the formation and dissolution of focal adhesions in WT cells and very static focal adhesion in hax1 knockdown cells. Scale bars = 10 μm. C, box and whisker plots revealing slow disassembly rates of focal adhesions in EB2 or Hax1 knockdown cells relative to their WT counterparts. EB2 but not the EB2 mutant and Hax1 but not the Hax1 mutant can rescue the defect in focal adhesion dynamics when introduced to express exogenously in the corresponding knockdown cells. For each genotype, ~50 focal adhesions were analyzed. Statistical analysis with Student’s t test showed that knockdown of EB2 or Hax1 leads to a significant decrease of focal adhesion disassembly compared with mock cells (p < 0.01). Re-expression of WT EB2 or Hax1, but not their corresponding mutants, leads to a significant increase in migration speed compared with the knockdown cells (p < 0.01).
The grafted skin displayed a stratification and differentiation program indistinguishable from that of the host skin (Fig. 7C).

To determine the role of EB2 and Hax1 in skin wound repair, we engrafted mock keratinocytes, EB2 knockdown cells, or Hax1 knockdown cells to nude mice. These cells were incorporated into the host skin with comparable efficiency, and no significant difference was identified in cell proliferation or differentiation in vivo (Fig. 7, C and E). When grafted skins were challenged to respond to injury, both EB2 and Hax1 knockdown skins exhibited a significant delay in repairing full-thickness wounds compared with mock skin (Fig. 7, D and E). Histological analysis and quantification revealed that the area of
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