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LL5β Directs the Translocation of Filamin A and SHIP2 to Sites of Phosphatidylinositol 3,4,5-Triphosphate (PtdIns(3,4,5)P_3) Accumulation, and PtdIns(3,4,5)P_3 Localization Is Mutually Modified by Co-recruited SHIP2*[S]

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Phosphatidylinositol 3,4,5-triphosphate (PtdIns(3,4,5)P_3) accumulates at the leading edge of migrating cells and works, at least partially, as both a compass to indicate directionality and a hub for subsequent intracellular events. However, how PtdIns(3,4,5)P_3 regulates the migratory machinery has not been fully elucidated. Here, we demonstrate a novel mechanism for efficient lamellipodium formation that depends on PtdIns(3,4,5)P_3 and the reciprocal regulation of PtdIns(3,4,5)P_3 itself. LL5β, whose subcellular localization is directed by membrane PtdIns(3,4,5)P_3, recruits the actin-cross-linking protein Filamin A to the plasma membrane, where PtdIns(3,4,5)P_3 accumulates, with the Filamin A-binding Src homology 2 domain-containing inositol polyphosphate 5-phosphatase 2 (SHIP2). A large and dynamic lamellipodium was formed in the presence of Filamin A and LL5β by the application of epidermal growth factor. Conversely, depletion of either Filamin A or LL5β or the overexpression of either an F-actin-cross-linking mutant of Filamin A or a mutant of LL5β without its PtdIns(3,4,5)P_3-interacting region inhibited such events in COS-7 cells. Because F-actin initially polymerizes near the plasma membrane, it is likely that membrane-recruited Filamin A efficiently cross-links newly polymerized F-actin, leading to enhanced lamellipodium formation at the site of PtdIns(3,4,5)P_3 accumulation. Moreover, we demonstrate that co-recruited SHIP2 dephosphorylates PtdIns(3,4,5)P_3 at the same location.

Directed migration is crucial in many biological events, including morphogenesis during development, accumulation of immune cells to the site of infection, and metastasis of cancer cells. A lamellipodium (a sheet-like process composed of actin filaments) must be formed correctly at the leading edge of a cell for smooth and efficient migration. Following stimulation with a chemoattractant, phosphatidylinositol 3-kinase (PI3K)[6] is locally activated, resulting in the transient accumulation of PtdIns(3,4,5)P_3 on the leading edge of directed migrating amoebas and leukocytes (1). PtdIns(3,4,5)P_3 works, at least in part, as a cell compass that translates external signals into directed cell movement (2). However, how PtdIns(3,4,5)P_3 contributes to the appropriate formation of a lamellipodium, or conversely, how lamellipodium formation influences PtdIns(3,4,5)P_3 accumulation has not been fully elucidated. For proper lamellipodium formation, the intracellular network of actin filaments (F-actins) must be regulated both dynamically and precisely. Such networks are composed primarily of cross-linked and branched F-actins. Previous work has shown that Filamin A cross-links F-actins and is indispensable for lamellipodium formation, whereas the Wiskott-Aldrich syndrome protein family verprolin homologous (WAVE) proteins are involved in branch formation (3–5).

Proteins involved in signal transduction and cytoskeletal dynamics interact with membrane phosphoinositides through their pleckstrin homology (PH) domains; such interactions

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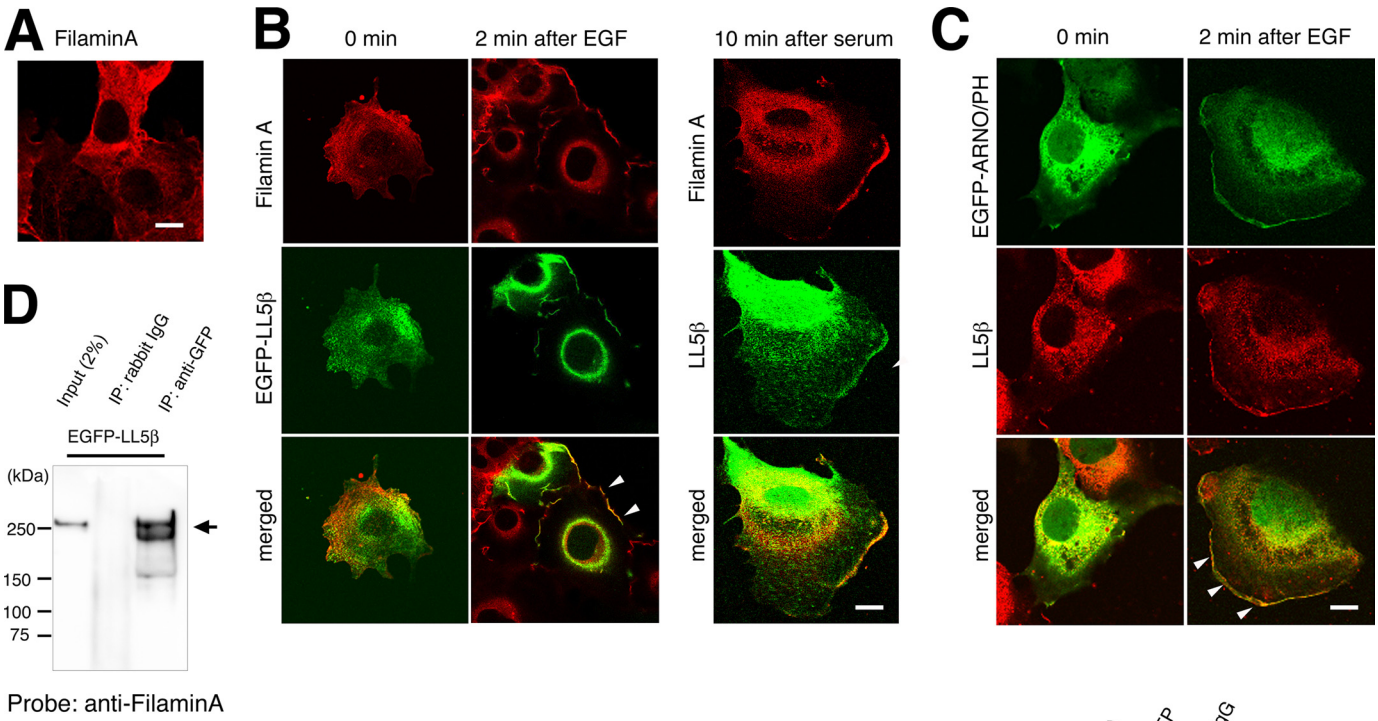
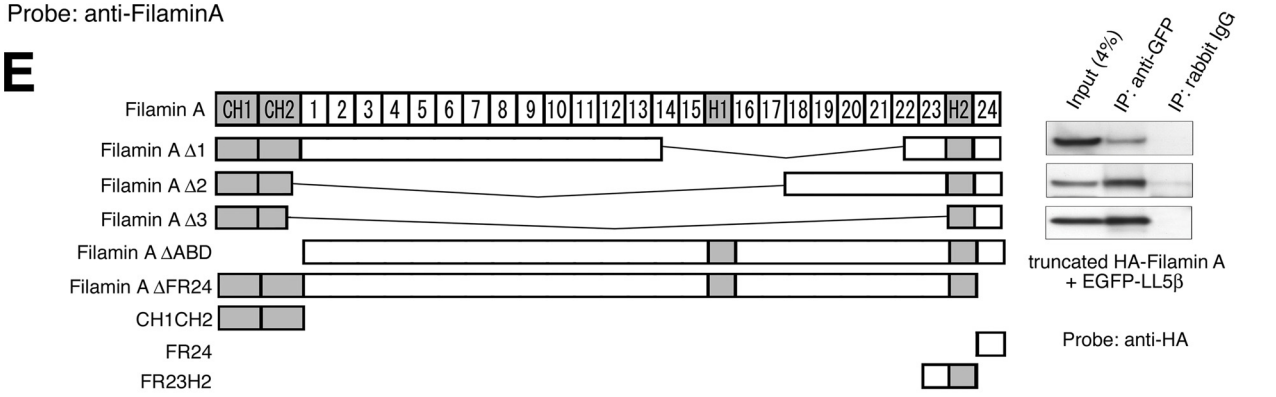
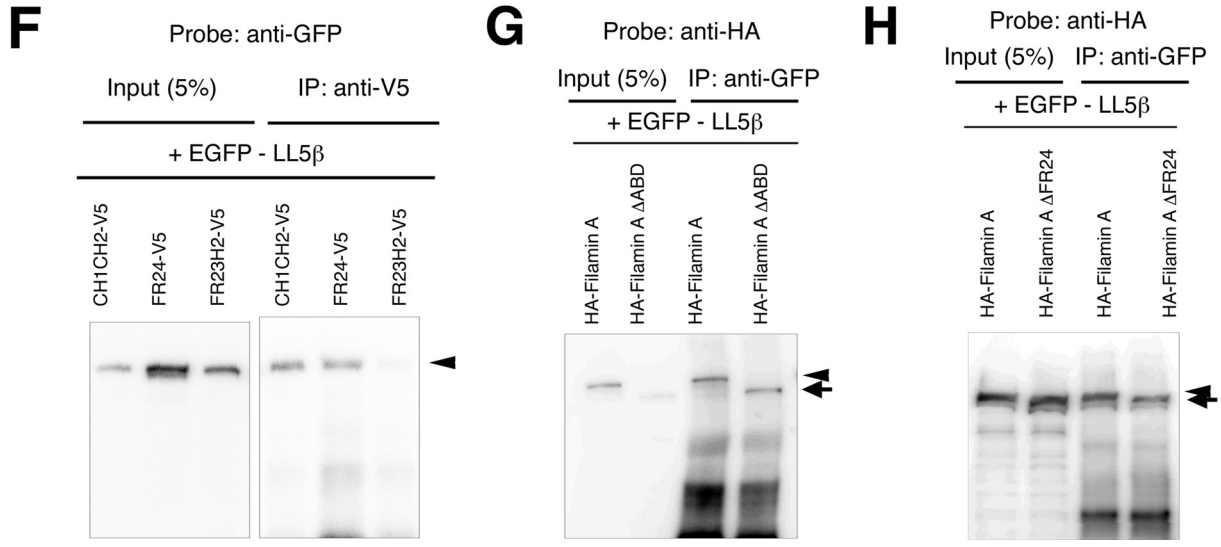
[6] The abbreviations used are: PI3K, phosphatidylinositol 3-kinase; PtdIns, phosphatidylinositol; PtdIns(3,4,5)P_3, phosphatidylinositol 3,4,5-triphosphate; ABD, actin-binding domain of Filamin A; ARNO, ARF nucleotide-binding site opener; PH, pleckstrin homology; FR, filamin repeats; COS-7, African green monkey SV40-transfected kidney fibroblast cell line; EGF, epidermal growth factor; GFP, green fluorescent protein; EGFP, enhanced green fluorescent protein; F-actin, actin filament; HA, hemagglutinin; SHIP2, Src homology 2 domain-containing inositol polyphosphate 5-phosphatase 2; MALDI-TOF, matrix-assisted laser desorption ionization/time of flight; RNAi, RNA interference; shRNA, short hairpin RNA; VSV, vesicular stomatitis virus.

are critical for the subcellular localization of these proteins and the regulation of their activities (6, 7). Although the core tertiary structure is conserved among PH domains, high sequence variability among their primary structures (~120 amino acids) gives rise to different specificities of PH domains for different phosphoinositides (8, 9). LL5β (PHLDB2; pleckstrin homology-like domain, family B, member 2) is an ~160-kDa protein that contains two predicted coiled-coil domains







and a PH domain that is highly specific for PtdIns(3,4,5)P_3 (10, 11). It has been reported that LL5β binds to exogenous Filamin A, as well as Filamin C (12), *in vitro*. However, the LL5β and Filamin A regions required for binding and the biological significance of LL5β-Filamin A binding have not been experimentally explored.

It has been shown that SHIP2 is a critical component of a negative feedback loop that regulates PtdIns(3,4,5)P_3 levels in nerve growth factor-stimulated PC12 cells, together with the constitutively active phosphatidyl inositol-3 (PtdIns-3) phosphatase, phosphatase and tensin homologue deleted on chromosome 10 (PTEN) (13). This suggests that SHIP2 contributes to establishing a narrow accumulation of PtdIns(3,4,5)P_3 in the membrane. Because PtdIns(3,4,5)P_3 works as an internal compass of polarity in directed migrating cells, SHIP2 is likely to help a cell respond accurately and dynamically to external signals that impart directionality by increasing PtdIns(3,4,5)P_3 levels in a leading edge of the cell. Previous studies have demonstrated that SHIP2 binds to Filamin A and that Filamin A is essential for the translocation of SHIP2 to the membrane ruffle. Moreover, recombinant SHIP2 regulates PtdIns(3,4,5)P_3 levels and the distribution of submembranous actin at membrane ruffles following growth factor stimulation (14). However, it remains elusive as to how and what molecules control SHIP2 and Filamin A translocation to the membrane, where in the cell periphery SHIP2 translocates, how membranous PtdIns(3,4,5)P_3 levels are regulated by translocation of SHIP2, and how submembranous actins are controlled.

EXPERIMENTAL PROCEDURES

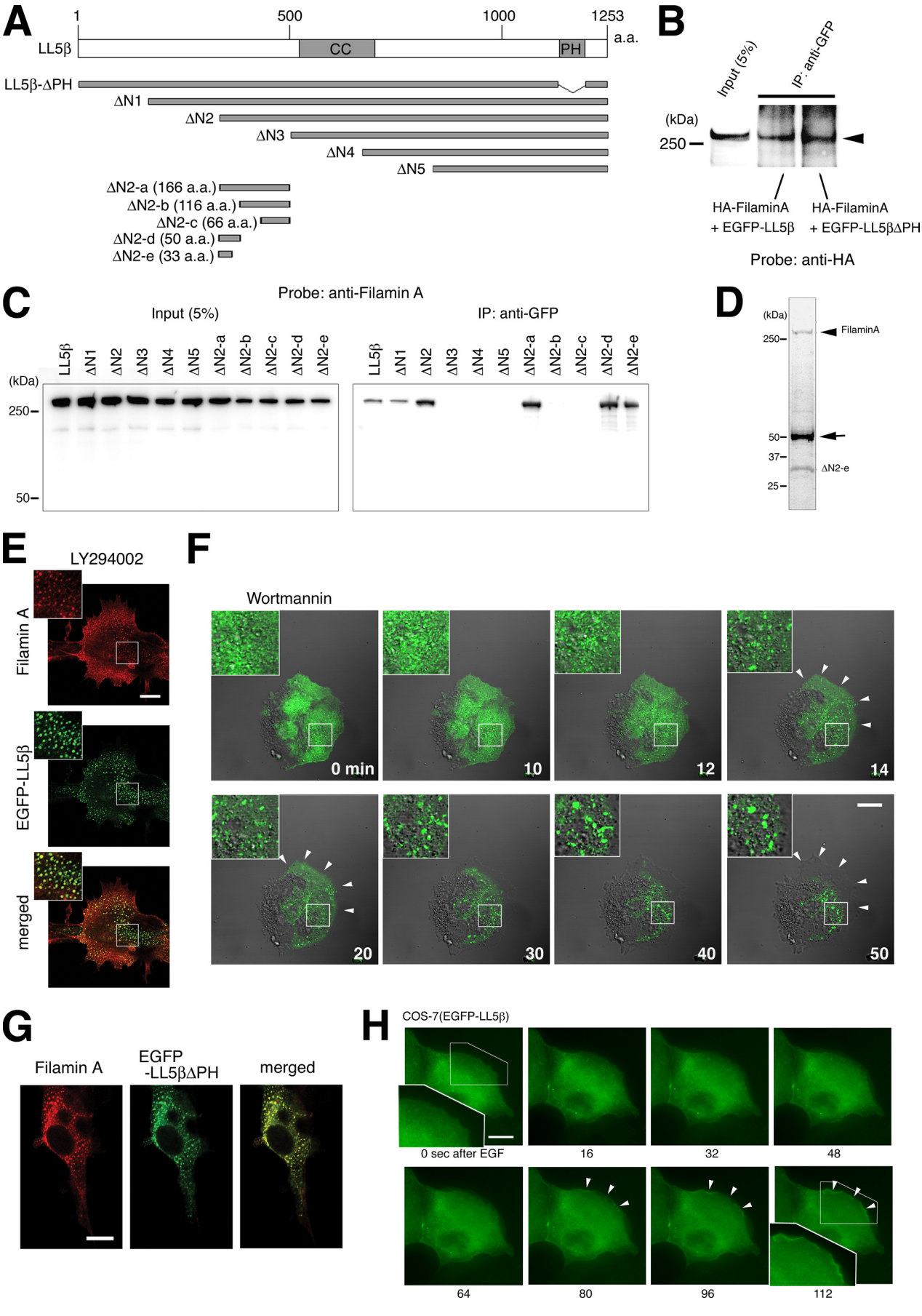
Details of experimental procedures are provided in the supplemental Experimental Procedures.

Isolation of LL5β cDNA—The full-length human LL5β cDNA (GenBank™ accession number AJ496194) was cloned from a human placenta cDNA library (BD Biosciences).

Plasmid Vectors—Full-length LL5β cDNA was subcloned into pEGFP-C3 (plasmid vector for expressing enhanced green fluorescent protein-C3, Clontech) to generate pEGFP-LL5β. In addition, expression vectors for human LL5β lacking the first 156 amino acid residues (pEGFP-LL5βΔN1, we designated this vector as ΔN1), 323 amino acid residues (pEGFP-LL5βΔN2 as ΔN2), and 488 amino acid residues (pEGFP-LL5βΔN3 as ΔN3) were generated using pEGFP-C3. pEGFP-C3 was also used for enhanced green fluorescent protein (EGFP) expression in COS-7 cells. Procedures for generating ΔN4, ΔN5, Δ2-a to Δ2-e, LL5βΔPH, and LL5β-VSV are described in the supplemental Experimental Procedures. Other protein expression vectors were constructed using pCAGGS so that the cDNAs would be driven under a modified chicken β-actin (CAG) promoter. We used pCAGGS-hemagglutinin (HA)-tagged Filamin A (pCAGGS-HA-Filamin A) (15). The following vectors were also generated: pCAGGS-HA-Filamin A truncated mutant 1 (Filamin AΔ1), pCAGGS-HA-Filamin A truncated mutant 2 (Filamin AΔ2), pCAGGS-HA-Filamin A truncated mutant 3 (Filamin AΔ3), and pCAGGS-HA-Filamin without repeat 24 (Filamin A ΔFR24). In addition, various vectors that expressed Val-Val-Val-Val-Val (V5)-His-tagged Filamin A fragments were constructed: CH1CH2, which contains the actin-binding domain 1 (CH1) and domain 2 (CH2) of Filamin A; FR24, which contains the filamin repeat (FR, also called immunoglobulin-like-β-sheet motif) 24 of Filamin A; and FR23H2, which contains the filamin repeat 23 and hinge 2 (H2) of Filamin A. The generation of Filamin AΔABD (ABD, actin-binding domain) has been described previously (15). For Filamin C expression, we used pBudCE4-Filamin C (made using pZP7-Filamin C, a generous gift from Dr. D. W. Chung, and pBudCE4 from Invitrogen). Full-length rat SHIP2 (GenBank accession number NM_022944) cDNA was subcloned into pCAGGS-GFP or pmCherry-N1 (Clontech) to generate SHIP2-expressing vector, where mCherry is a mutant fluorescent protein derived from the tetrameric *Discosoma* sp. red fluorescent protein (DsRed). The PH domain of rat ADP-ribosylation factor (ARF) nucleotide-binding size opener (16, 17) (ARNO, GenBank accession number NM_053911, gift from Dr. T. Balla) was subcloned into pCAGGS-GFP vector to generate EGFP-ARNO/PH-expressing vector. The PH domain of mouse general receptor for phosphoinositides 1 (18) (GRP1, GenBank accession number BC035296.1) was subcloned into pEGFP-C1 to generate EGFP-GRP1/PH-expressing vector.

Antibodies—The following antibodies were used: anti-GFP antibody that can recognize EGFP (MBL Medical and Biological Laboratories, Nagoya, Japan), anti-Filamin A antibody (MAB1678, Chemicon), anti-HA antibody (sc-805, Santa Cruz Biotechnology, Santa Cruz), anti-α-tubulin (DM1A, Sigma), anti-VSV antibody (P5D4, Sigma), anti-pan-cadherin antibody (CH-19, Sigma), anti-glyceraldehyde-3-phosphate dehydrogenase antibody (14C10, Cell Signaling Technology, Danvers, MA), anti-rabbit IgG (Sc-2027, Santa Cruz Biotechnology), anti-V5 antibody (Invitrogen), anti-β actin antibody (AC-40, Sigma), anti-FLAG antibody (F3165, Sigma), anti-PtdIns(3,4,5)P_3 antibody (F3165, NN111.1.1, MBL Medical and Biological Laboratories), Alexa

FIGURE 1. **LL5β binds to Filamin A through its CH1CH2 or repeat 24.** *A*, distribution of Filamin A in COS-7 cells without exogenous LL5β. *Scale bar* = 10 μm. *B*, *left* and *middle columns*, following EGF application, exogenous EGFP-LL5β (*green*) and endogenous Filamin A (*red*) colocalized at the plasma membrane (*arrowheads*). *Right column*, endogenous Filamin A (*red*) and endogenous LL5β (*green*) were visualized in COS-7 cells that had been cultured without serum for 18 h. They accumulated near the plasma membrane at 10 min after the addition of 10% serum. *Scale bar* = 10 μm. *C*, following EGF application, exogenous EGFP-tagged ARNO/PH (EGFP-ARNO/PH) (*green*) and endogenous LL5β (*red*) colocalized at the plasma membrane (*arrowheads*). *Scale bar* = 10 μm. *D*, lysates from COS-7 cells expressing EGFP-LL5β were subjected to immunoprecipitation (*IP*) with an anti-GFP antibody or rabbit IgG (control). Endogenous Filamin A (~280 kDa, *arrow*) was co-immunoprecipitated with EGFP-LL5β. *E*, schematic drawings show the various truncated Filamin A mutants. They were tagged with HA at their amino termini. EGFP-LL5β was co-expressed in COS-7 cells with one of the truncated Filamin A mutants, as shown on the *left*, followed by immunoprecipitation with anti-GFP antibody or rabbit IgG (control) and detection with anti-HA antibody. Filamin A mutants (Filamin AΔ1-Δ3) were co-immunoprecipitated. *CH1* and *CH2*, actin-binding site 1 and 2, respectively. *H1* and *H2*, hinge 1 and 2. *Numbers* (1–24) indicate the FR number. *F*, CH1CH2, FR24, or FR23H2 (Filamin repeat 23 and hinge 2) tagged with V5 were co-expressed with EGFP-LL5β. EGFP-LL5β was co-immunoprecipitated with CH1CH2 or FR24 but not FR23H2 (*arrowhead*). *G*, full-length Filamin A (*arrowhead*) or Filamin AΔABD (*arrow*) tagged with HA was co-immunoprecipitated with EGFP-LL5 β. *H*, full-length Filamin A (*arrowhead*) or Filamin A ΔFR24 (*arrow*) tagged with HA was co-immunoprecipitated with EGFP-LL5β.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EGFP-LL5β
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G
Filamin A
EGFP -LL5βΔPH
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COS-7(EGFP-LL5β)
0 sec after EGF
16
32
48
64
80
96
112

Fluor 568 phalloidin (Invitrogen). Anti-LL5β polyclonal antibody was generated in cooperation with SIGMA Genosys Japan (Ishikari, Japan).

Cell Culture and DNA Transfection—COS-7 cells were used. Vectors were transfected using PolyFect transfection reagent (Qiagen, Hilden, Germany) or FuGENE 6 transfection reagent (Roche Diagnostics). To increase PtdIns (3,4,5)P_3 in the plasma membrane, cells were cultured without serum for 16–18 h and then cultured with epidermal growth factor (EGF) for 2 min or serum for 10 min. Human melanoma cell lines M2 and A7 were maintained as reported previously (4). FuGENE HD (Roche Diagnostics) was used to transfect these cells.

F-actin Staining, Immunocytochemistry, Analyses of Cell Motility, and Reverse Transcription-PCR—Conventional protocols were used.

Immunoprecipitation, Western Blotting (Immunoblotting) Analyses, and MALDI-TOF Mass Spectrometry—Immunoprecipitation was performed with Dynabeads (Dynal, Hamburg, Germany) coated with antibodies specific to rabbit or mouse IgG. Matrix-assisted laser desorption ionization/time of flight (MALDI-TOF) mass spectrometry (Bruker Daltonics, Billerica, MA) was used for protein identification.

RNA Interference—Three different constructs of LL5β short hairpin RNA (shRNA) were prepared in the mouse U6 snRNA promoter (mU6pro) vector (mU6pro-LL5β-RNAi), which has a mouse U6 promoter (15). Their sequence targets were nucleotides 177–197, 2246–2266, and 3647–3667 of the human LL5β cDNA. These shRNAs were termed LL5β-shRNA1, LL5β-shRNA2, and LL5β-shRNA3 and such vectors were called LL5β-RNAi1, LL5β-RNAi2, and LL5β-RNAi3, respectively. For co-transfection, a molar ratio of 1 (EGFP expression plasmid) to 2 (LL5β-RNAi or empty mU6pro vector) was employed. Empty shRNA vector was used as a control.

Wound-healing Assay—Overconfluent COS-7 cells were co-transfected with pEGFP-C3 and mU6pro-LL5β-RNAi vector or pEGFP-C3 and control vector. Empty shRNA vector was used as the control vector. After 36–48 h, the confluent cell layer was disrupted followed by a 3-h incubation. After fixing and staining with rhodamine-phalloidin, cells at the defect edge expressing EGFP were observed, and the numbers of cells with or without lamellipodia were counted.

PI3K Inhibition and Time-lapse Observation—For PI3K inhibition experiments, cells were cultured for 18–24 h followed by the addition of wortmannin (Sigma, final concentration 100 nM) or LY294002 (Sigma, final concentration 10 μM). Time-lapse observation was started (time 0) in a CO_2 incubation chamber (5% CO_2 at 37 °C) fitted onto a confocal microscope. Images were obtained every 1–2 min for 15 min to 1 h. Confocal images or live cell images were captured. Images were obtained using an AxioCam (Carl Zeiss, Oberkochen, Germany).

Subcellular Fractionation—Subcellular fractionation was performed using the ProteoExtract subcellular proteome extraction kit (Calbiochem) in accordance with the manufacturer's instructions. Utilizing the different extraction buffers in the kit, four different fractions were separated. The Triton-insoluble membrane/organelle fractions are shown in Fig. 5*E*. The amounts of the pan-cadherin (as reference for membrane proteins) and glyceraldehyde-3-phosphate dehydrogenase were used as internal controls.

Statistical Analyses—Significant differences, as indicated by $p < 0.01$, were determined by the non-parametric Mann-Whitney *U* test or *t* test.

RESULTS

First of all, we experimentally examined the relationship between LL5β and Filamin A (Fig. 1*A*) and their regions responsible for interaction. We expressed LL5β, tagged with EGFP at its amino terminus (EGFP-LL5β), in COS-7 cells to determine whether LL5β colocalizes with endogenous Filamin A (Fig. 1*B*). EGFP-LL5β and endogenous Filamin A colocalized primarily at the cell periphery after EGF application (final concentration, 100 ng/ml; Fig. 1*B*, *left* and *middle columns*), as reported previously (11). Endogenous LL5β (supplemental Fig. S1) colocalized with endogenous Filamin A at the cell periphery by serum stimulation (Fig. 1*B*, *right column*). Previous work has demonstrated that PI3K is activated by EGF (17) or by serum stimulation (19). EGFP-tagged ARNO-PH, which specifically binds to PtdIns(3,4,5)P_3, has been used as a PtdIns(3,4,5)P_3 detector (16, 17). Two minutes after EGF application, ARNO-PH accumulated at the cell periphery together with endogenous LL5β (Fig. 1*C*), suggesting that LL5β translocates where PtdIns(3,4,5)P_3 localizes. EGF-induced accumulation of PtdIns(3,4,5)P_3 at the cell periphery was further confirmed by another PtdIns(3,4,5)P_3 detector GRP1/PH (18) or immunocytochemical techniques (supplemental Fig. S2).

The interaction between LL5β and Filamin A was confirmed by immunoprecipitation. Cell lysates from COS-7 cells expressing EGFP-LL5β, an anti-GFP antibody that is capable of recognizing EGFP, and an anti-Filamin A antibody were used. This anti-Filamin A antibody did not cross-react with Filamin C in lysates from Filamin C-overexpressing COS-7 cells (data not shown). Filamin A, which is ~280 kDa (3, 20), was co-immu-

FIGURE 2. **The LL5β-Filamin A complex changes its subcellular localization depending on the level of membrane phosphatidylinositol 3,4,5-triphosphate.** *A*, schematic drawings show full-length and various truncated mutants of LL5β. *a.a.*, amino acids, *CC*, putative coiled-coil region. *B*, HA-Filamin A was co-immunoprecipitated with EGFP-LL5βΔPH as well as EGFP-LL5β (*arrowhead*). *C*, endogenous Filamin A was co-immunoprecipitated (*IP*) in with LL5β or LL5β mutants ΔN1, ΔN2, ΔN2-a, ΔN2-d, or ΔN2-e. *D*, ΔN2-e tagged with EGFP was expressed in COS-7 cells and then immunoprecipitated with anti-GFP antibody. Immunoprecipitates were subjected to SDS-PAGE. Filamin A detected by MALDI-TOF mass spectrometry is noted (*arrowhead*). The *arrow* indicates the IgG heavy chain band. *E*, the PI3K inhibitor LY294002 was applied to COS-7 cells expressing EGFP-LL5β. Exogenous EGFP-LL5β (*green*) as well as endogenous Filamin A (*red*) exhibited a punctate distribution with little expression at the cell periphery. The two proteins colocalized well. Each *inset* shows higher magnification views of the area indicated by the *square* in the same panel. *Scale bar* = 10 μm. *F*, temporal profiles of subcellular localization of EGFP-LL5β (*green*). The PI3K inhibitor wortmannin (100 nM) was applied to COS-7 cells at time 0. Each *inset* shows higher magnification views of the area indicated by the *square* in the same panel. Translocation of EGFP-LL5β to the cytosol as well as puncta formation began in about 14 min. The cell edge is indicated by *arrowheads*. *Scale bar* = 10 μm. *G*, EGFP-LL5βΔPH (*green*) showed a punctate distribution in COS-7 cells, colocalizing well with endogenous Filamin A (*red*), which also exhibited a punctate distribution. *Scale bar* = 10 μm. *H*, EGFP-LL5β (*green*) accumulated in the membrane in 80 s following the application of EGF (final concentration of 100 ng/ml) in COS-7 cells that had been serum-starved for 18 h. Each *inset* shows higher magnification views of the area indicated by the *pentagon* in the same panel. *Arrowheads* indicate the recruitment of EGFP-LL5β near the plasma membrane. *Scale bar* = 10 μm.

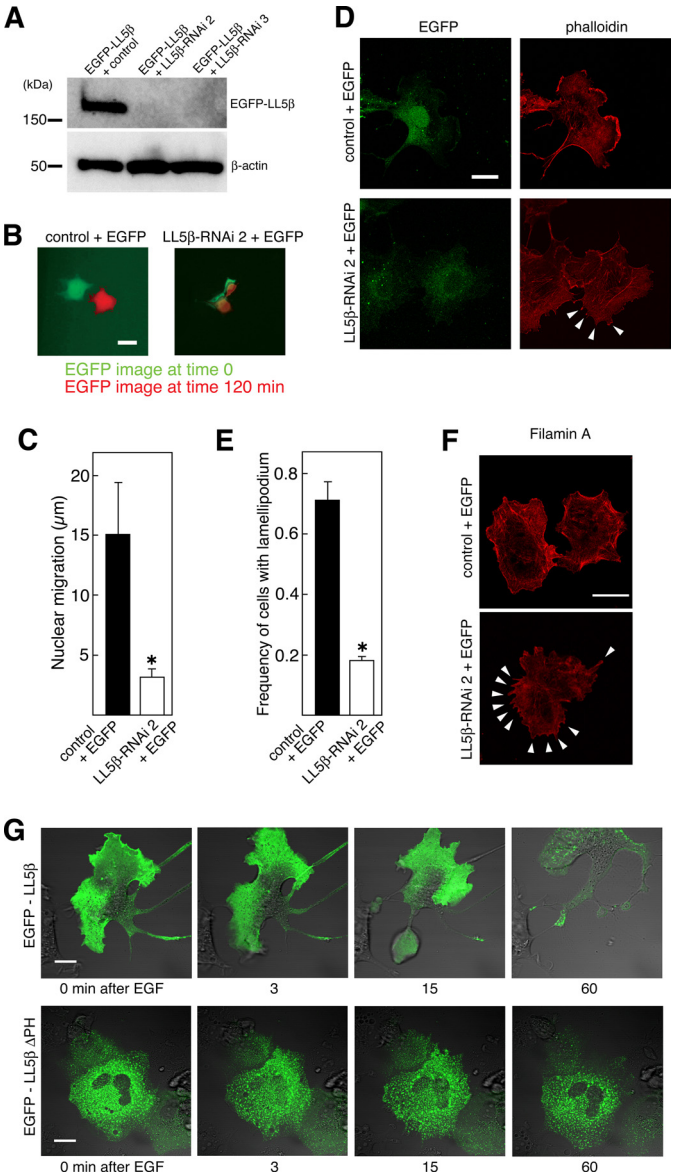

FIGURE 3. **Knockdown of LL5β decreases cell motility and lamellipodium formation.** *A*, LL5β-RNAi2 or LL5β-RNAi3 were co-transfected with the EGFP-LL5β expression vector in COS-7 cells. The knockdown efficiency of each RNAi was evaluated by estimating the EGFP signal intensity. *B*, EGFP expression vector and empty mU6pro vector (control) (*left*) or EGFP expression vector and LL5β-RNAi vector (LL5β-RNAi 2) (*right*) were transfected into COS-7 cells that had been cultured under low cell density conditions. EGFP images (*green*) were acquired at an interval of 120 min and merged together after the color of the later images had been converted to red. Knockdown of LL5β resulted in a decrease in cell motility. *Scale bar* = 50 μm. *C*, to quantify cell motility, the migrated distance (mean ± S.E.) of each nucleus during the 120-min interval was measured for each group ($n = 50$ each). * indicates statistical significance ($p < 0.01$). *D*, the EGFP expression vector was transfected into COS-7 cells together with an empty mU6pro vector (control) (*upper panels*) or LL5β-RNAi vector (*lower panels*). EGFP-positive (transfected) cells were studied. F-actin localization was visualized simultaneously with rhodamine-phalloidin or Alexa Fluor 568 phalloidin. Knockdown of LL5β resulted in the frequent formation of small protrusions (*arrowheads*) and in a decrease in lamellipodium formation. *Scale bar* = 10 μm. *E*, the number of cells with lamellipodia (with an edge line equal to or longer than 10 μm) in *panel D* was counted among randomly selected EGFP-positive cells (mean ± S.E.). $n = 90$ for both cases (three independent experiments, $n = 30$ each). *, $p < 0.01$. *F*, the peripheral distribution of endogenous Filamin A was impaired by knocking down LL5β. Only fiber-like Filamin A was observed in small protrusions (*arrowheads*). The EGFP expression vector was co-transfected, and EGFP-positive cells were studied (EGFP images were not shown). *Scale bar* = 10 μm. *G*, culture medium containing 100 ng/ml EGF was added (0 min) to COS-7 cells expressing EGFP-LL5β or EGFP-LL5βΔPH after being cultured without serum for 18 h. EGFP-LL5β-expressing cells responded strongly and dramatically changed their morphology, whereas those with EGFP-LL5βΔPH did not. *Scale bar* = 20 μm.

noprecipitated with EGFP-LL5β (Fig. 1*D*). We then searched for the region of Filamin A responsible for this interaction. Filamin A, which usually exists as a homodimer, contains an amino-terminal actin-binding domain that precedes 24 immunoglobulin-like β-sheet motifs (FR, filamin repeats) (Fig. 1*E*). The last FR (FR 24) is responsible for homodimerization (20). Using three deletion mutants of amino-terminally HA-tagged Filamin A that lack different regions of the immunoglobulin-like-β-sheet motifs (Filamin AΔ1-Filamin AΔ3), we identified the domain of Filamin A responsible for its interaction with LL5β (Fig. 1*E*). Even Filamin AΔ3, which includes hinge 1 but lacks FR1–23, was co-immunoprecipitated with EGFP-LL5β (Fig. 1*E*). We next assessed the ability of the two actin-binding motifs (CH1, CH2), FR24 and hinge 2 (H2) of Filamin A, to bind to EGFP-LL5β. Two Filamin A fragments, CH1CH2 and FR24, but not FR23H2, were co-immunoprecipitated with EGFP-LL5β (Fig. 1, *E* and *F*). Furthermore, HA-tagged Filamin A without actin-binding domain (HA-Filamin A ΔABD) or without FR24 (HA-Filamin A ΔFR24) was co-immunoprecipitated with EGFP-LL5β (Fig. 1, *E*, *G*, and *H*). These results suggest that both the actin-binding domain and the FR24 of Filamin A are responsible for an interaction with LL5β.

We next determined which domains of LL5β are required for this interaction (Fig. 2*A*). First, we generated a mutant of LL5β that lacks the PH domain (LL5βΔPH). HA-tagged Filamin A was co-expressed with EGFP-tagged LL5β (EGFP-LL5β) or EGFP-tagged LL5βΔPH (EGFP-LL5βΔPH) in COS-7 cells followed by lysis and immunoprecipitation with an anti-GFP antibody. Co-immunoprecipitated HA-tagged Filamin A was observed with the similar intensity in lysates from cells co-expressing either EGFP-LL5β or EGFP-LL5βΔPH (Fig. 2*B*), indicating that the PH domain is not required for binding to Filamin A. We then used various truncated forms of LL5β with N-terminal EGFP tags to search for regions of LL5β responsible for Filamin A binding. A Filamin A interaction occurred with ΔN1 and ΔN2, but not with the ΔN3, ΔN4, or ΔN5 mutants of LL5β, indicating that a domain present in ΔN2 but not ΔN3 is responsible for this interaction. We then further truncated this domain and found that ΔN2-a, but not ΔN2-b or ΔN2-c, could interact. Finally, we discovered that a 33-amino acid region (323–356, ΔN2-e) was sufficient for binding (Fig. 2*C*). This binding region of LL5β was different from what has been previously documented (12). To confirm our observation, we expressed ΔN2-e in COS-7 cells and identified co-immunoprecipitated proteins using MALDI-TOF mass spectrometry. Then, Filamin A was identified (Fig. 2*D*). We reconfirmed our results.

Because the PH domain of LL5β is specifically sensitive to PtdIns(3,4,5)P_3 (10), we next examined whether the recruitment of LL5β to the lamellipodia is driven by PtdIns(3,4,5)P_3. Because the phosphorylation state of PtdIns(3,4,5)P_3 is regulated by the phosphorylation of phosphatidylinositol 4,5-biphosphate by PI3K, COS-7 cells with exogenous EGFP-LL5β were treated with the PI3K inhibitor, LY294002. After this

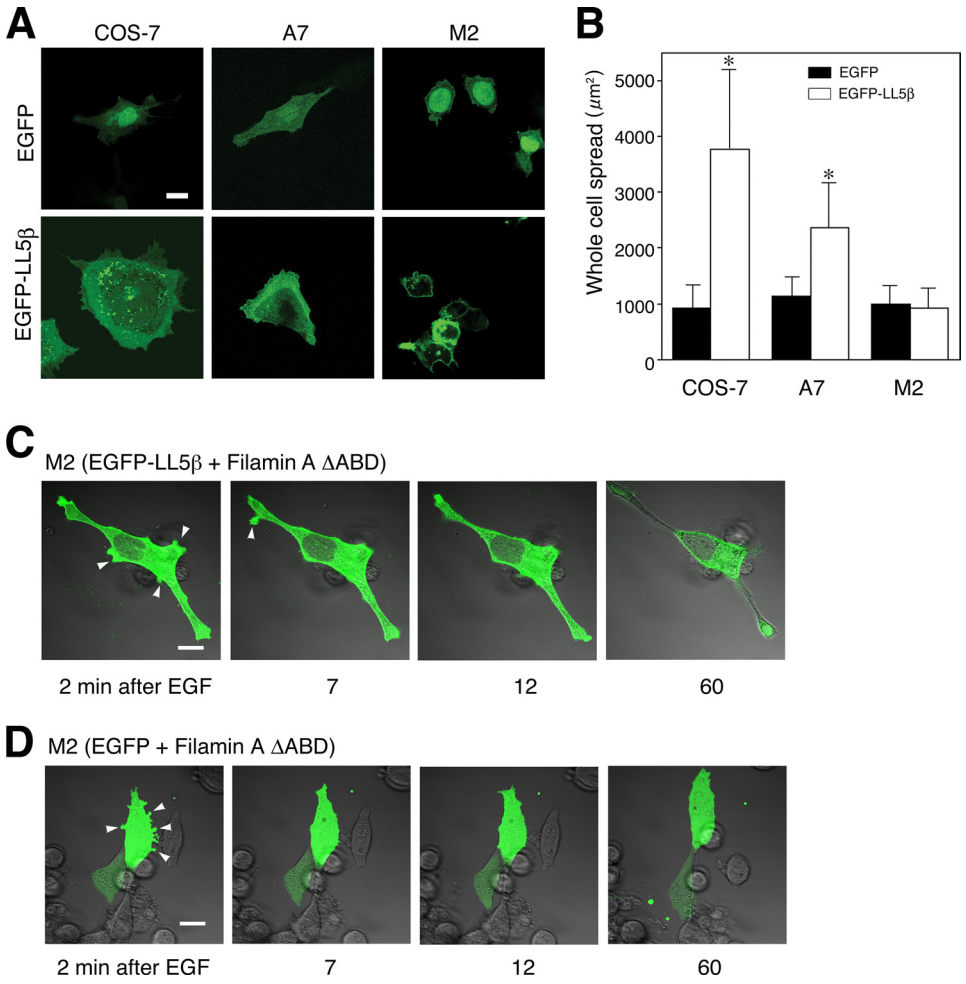

FIGURE 4. **Filamin A is necessary for induction of lamellipodium formation by LL5β.** *A*, EGFP expression vector or EGFP-LL5β expression vector was transfected into COS-7 cells, A7 cells (which contain Filamin A), and M2 cells (which lack Filamin A). Very large lamellipodia were developed in the presence of EGFP-LL5β in COS-7 cells and A7 cells but not in the M2 cells. *Scale bar* = 10 μm. *B*, a whole-cell area was measured from 50 cells; cells were randomly selected in each case (mean ± S.E.). * $p < 0.01$. *C* and *D*, M2 cells expressing EGFP-LL5β and Filamin AΔABD or EGFP and Filamin AΔABD were cultured without serum for 18 h. EGF (final concentration 100 ng/ml) was then added into the culture medium (time 0). Approximately 2 min after EGF addition, small pseudopods (*arrowheads*) were transiently generated. The images were merged with their differential interference contrast images. *Scale bars* = 10 μm.

treatment, EGFP fluorescence was distributed in a punctate manner and colocalized with endogenous Filamin A (Fig. 2*E*; supplemental Fig. S3; supplemental Video 1). No accumulation of Filamin A was observed at the cell periphery. Our time-lapse study gave us more detailed information on how this redistribution took place. Puncta were formed in COS-7 cells about 14 min after treatment with the PI3K inhibitor, wortmannin (Fig. 2*F*). The EGFP-LL5β signal was not observed in lamellipodia after this treatment (Fig. 2*F*). This observation was further investigated by overexpressing EGFP-LL5βΔPH. EGFP-LL5βΔPH did not localize to the plasma membrane but colocalized well with endogenous Filamin A in a punctate distribution in the cytoplasm (Fig. 2*G*). This result is consistent with the demonstration of LL5βΔPH binding to HA-tagged Filamin A (Fig. 2*B*). We further examined the spatiotemporal regulation of LL5β in response to PtdIns(3,4,5)P_3. We added EGF to the culture medium after serum depletion for 16–18 h and continuously observed changes in EGFP-LL5β localization. Recruitment of EGFP-LL5β to the plasma membrane was first observed ~80 s after EGF application, and by 112 s, EGFP-LL5β accumulated along the plasma membrane (Fig. 2*H*; supplemental Video 2). These data suggest that LL5β changes its subcellular localization quickly enough to chase changeable PtdIns(3,4,5)P_3 distribution (supplemental Fig. S4 and supplemental Video 3).

The ability of LL5β to bind to Filamin A, a protein crucial for cell motility (20), suggests that LL5β is also involved in motility control. To investigate the function of LL5β, we carried out LL5β knockdown experiments using RNA interference (RNAi). Of the three different shRNA constructs against LL5β, we found that LL5β-RNAi2 and LL5β-RNAi3 suppressed the expression of EGFP-LL5β with similar efficiency (Fig. 3*A*; supplemental Fig. S5). In the following experiments, we primarily used LL5β-RNAi2 to suppress LL5β expression in COS-7 cells, but LL5β-RNAi3 gave us similar results (supplemental Fig. S6). LL5β-RNAi reduced cell motility when compared with control cells under low cell density conditions where cells could move freely (Fig. 3, *B* and *C*). We then employed a wound-healing assay to determine whether there was any alteration to the cell-crawling machinery (Fig. 3, *D* and *E*). In this assay, quiescent cells in an overconfluent state usually develop lamellipodia in response to the removal of neighboring cells. We found that "typical lamellipodia" (defined as edge lines equal to or more than 10 μm long) were not properly formed in cells with LL5β-RNAi. Instead, small protrusions that contained F-actin (with edge lines less than 10 μm long) were observed (Fig. 3*D*). The frequency of cells with lamellipodia was estimated with or without LL5β-RNAi. Knockdown of LL5β resulted in a decrease in lamellipodium formation (Fig. 3*E*), suggesting that LL5β is essential for lamellipodium formation in COS-7 cells. Moreover, when LL5β expression was down-regulated by the RNAi technique in COS-7 cells, the distribution of Filamin A near the plasma membrane was significantly altered (Fig. 3*F*). Reduced formation of lamellipodia and a fiber-like distribution of Filamin A were observed in the small protrusions seen in LL5β knockdown cells. In contrast, LL5β overexpression resulted in the formation of large lamellipodia (Figs. 3*G*, *upper row*, and 4*A*, *left* and *middle panels*). In response to EGF, COS-7 cells with exogenous LL5β became very active (supplemental Video 4), whereas those with exogenous LL5βΔPH did not (Fig. 3*G*, *lower row*), indicating that the

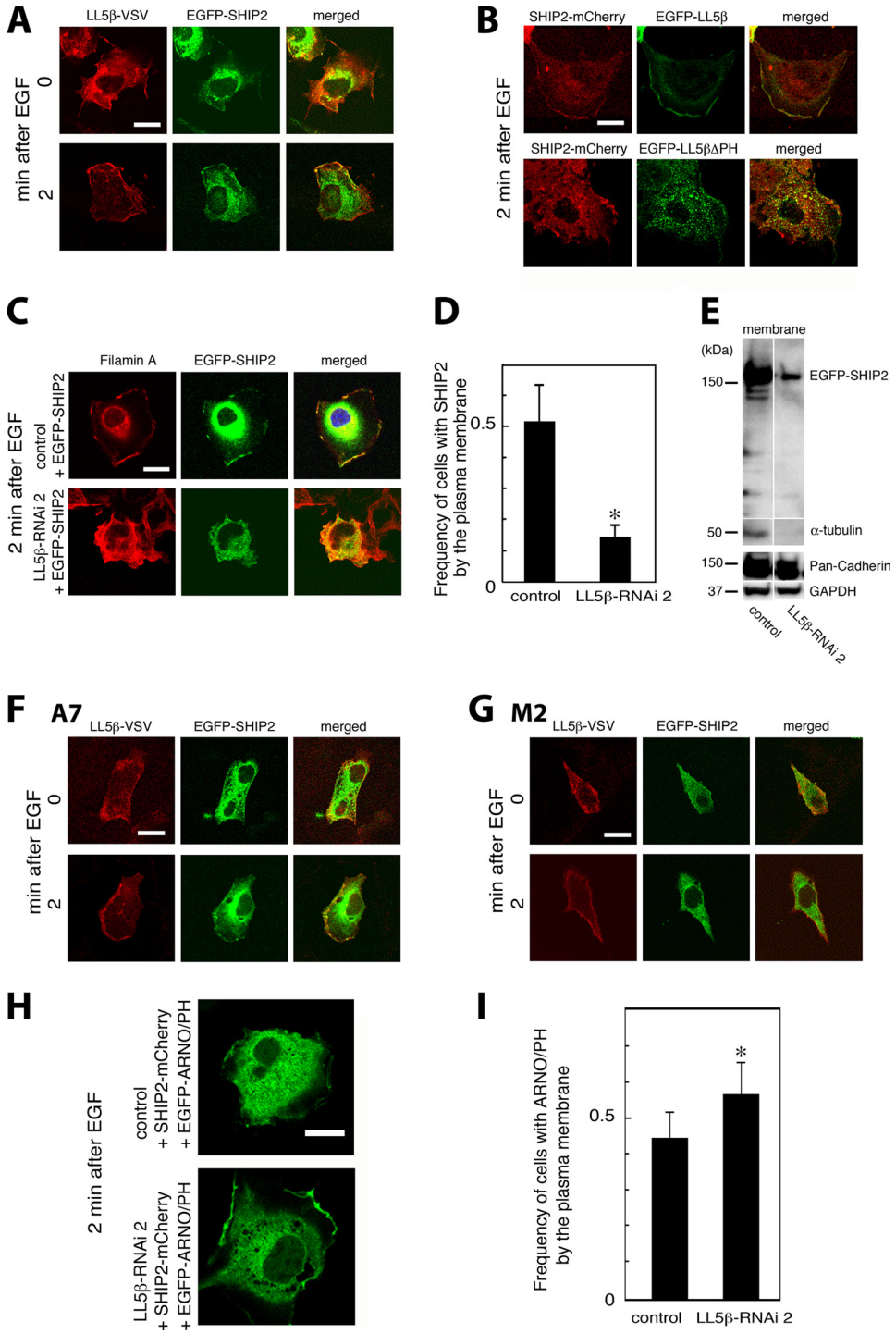

FIGURE 5. **LL5β directs membrane localization of SHIP2, and PtdIns(3,4,5)P_3 localization is mutually modified by co-recruited SHIP2.** In the following cases, cells were serum-starved for 16–18 h and then stimulated with the final concentration of 100 ng/ml EGF for 2 min. *A*, LL5β tagged with VSV (LL5β-VSV) was co-expressed with EGFP-SHIP2 in COS-7 cells. VSV signals (*red*) accumulated near the plasma membrane 2 min after EGF stimulation. *Scale bar* = 10 μm. *B*, SHIP2 tagged with mCherry (SHIP2-mCherry) and EGFP-LL5β or EGFP-LL5$\beta\Delta$PH were co-transfected into COS-7 cells. SHIP2-mCherry (*red*) and EGFP-LL5β (*green*) accumulated near the plasma membrane (*upper row*), whereas SHIP2-mCherry and EGFP-LL5$\beta\Delta$PH (*green*) did not (*lower row*). *Scale bar* = 10 μm. *C*, knockdown of LL5β resulted in a decrease in the membrane localization of SHIP2 (*lower row*). Empty mU6pro vectors were used for control. *Scale bar* = 10 μm. *D*, the frequency of cells with membrane-localized SHIP2 in *panel C* was determined among randomly selected EGFP-positive cells (mean ± S.E., three independent experiments, $n = 50$ cells each). *, $p < 0.01$. *E*, knockdown of LL5β resulted in reduced membrane translocation of EGFP-SHIP2. COS-7 cells in *panel C* were subjected to subcellular fractionation. The amount of SHIP2 in the membrane fractions was analyzed by immunoblotting with anti-GFP antibody. Unexpectedly, α-tubulin was decreased in LL5β knockdown cells. The amounts of cadherins (detected by pan-cadherin antibody) and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) were used as a reference. *F* and *G*, LL5β-VSV and EGFP-SHIP2 expression vector were transfected into A7 cells and M2 cells. LL5β-VSV (*red*) and EGFP-SHIP2 (*green*) accumulated near the plasma membrane in A7 cells (*F*), but not in M2 cells (*G*), by the addition of EGF. *Scale bar* = 10 μm. *H*, EGFP-ARNO/PH, SHIP2-mCherry, and control (empty mU6pro vector) or LL5β-RNAi2 were co-transfected into COS-7 cells. Knockdown of LL5β resulted in an increase in membrane-localized EGFP-ARNO/PH (*green*). *Scale bar* = 10 μm. *I*, the frequency of cells with membrane-localized EGFP-ARNO/PH in *panel H* was determined among randomly selected EGFP-positive cells (mean ± S.E., three independent experiments, $n = 50$ cells each). *, $p < 0.01$.

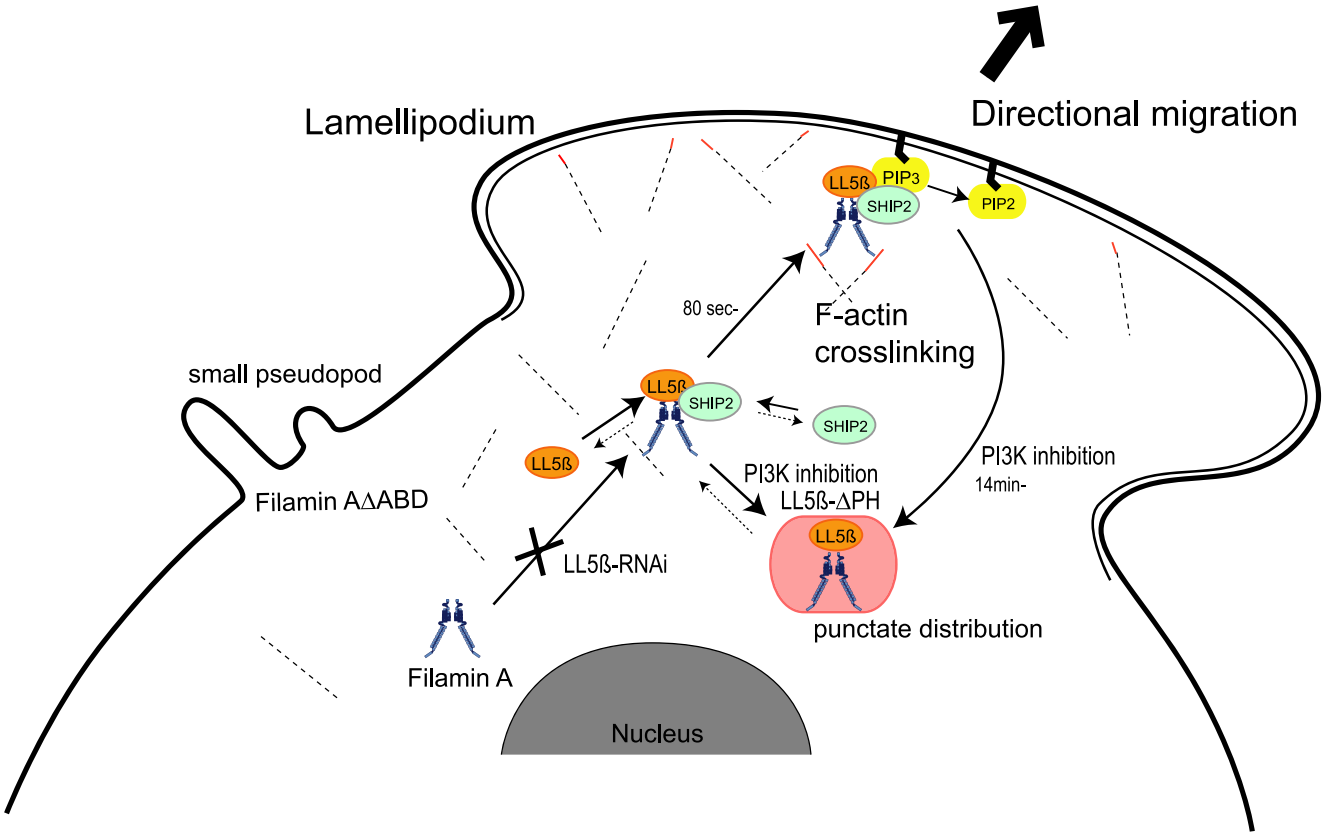

FIGURE 6. **Role of the LL5*β*-Filamin A-SHIP2 complex during PtdIns(3,4,5)P$_3$-mediated directed migration.** In directed migrating cells, PtdIns(3,4,5)P$_3$ accumulates in their leading edge. LL5*β* controls the spatial distribution and activity of Filamin A depending on the localization of membranous PtdIns(3,4,5)P$_3$ (shown as *PIP$_3$*). LL5*β* enhances lamellipodium formation by possibly increasing the cross-linking of newly polymerized F-actin at the plasma membrane (shown in *red*) by recruiting the F-actin-cross-linking molecule, Filamin A, to the plasma membrane. SHIP2 is co-recruited to sites of PtdIns(3,4,5)P$_3$ accumulation by LL5*β* via Filamin A, and then it reciprocally dephosphorylates PtdIns(3,4,5)P$_3$ to phosphatidylinositol 3,4-biphosphate (PtdIns(3,4)P$_2$, shown as *PIP$_2$*).

PH domain of LL5*β* is critical for this action. Because the PH domain of LL5*β* is primarily sensitive to PtdIns(3,4,5)P$_3$(10, 11) and because PtdIns(3,4,5)P$_3$ is generated at the plasma membrane upon EGF application (17), it is probable that the binding of LL5*β* to membrane PtdIns(3,4,5)P$_3$ induced this cellular response.

Because LL5*β* overexpression in COS-7 cells resulted in the enlargement of the peripheral area (including lamellipodia) of cells, we next examined whether Filamin A is involved in this response. M2 cells, in which LL5*β* mRNA was not detected (data not shown), are derived from a human melanoma and do not express Filamin A, whereas A7 cells are generated by stably transfecting Filamin A cDNA into M2 cells (4). These cells express a molar ratio of Filamin A to actin comparable with that found in cells normally expressing Filamin A (4). The LL5*β*-induced enlargement was observed in A7 cells as well as COS-7 cells, but not in M2 cells (Fig. 4, *A* and *B*), suggesting that enlargement induced by LL5*β* overexpression requires Filamin A. The versatility of Filamin A led us to ask whether the F-actin-cross-linking activity of Filamin A is essential for this enlargement. Filamin A dimerized through their carboxyl termini, and then dimerized Filamin A cross-linked two F-actin molecules through their ABD at their amino termini (15, 21). Thus, we transfected a Filamin AΔABD expression vector (15) into M2 cells and examined the Filamin A F-actin-cross-linking activity to that observed in A7 cells. Application of EGF resulted in the formation of small pseudopods, but not proper lamellipodia, in M2 cells transfected with EGFP-LL5*β* and Filamin AΔABD (Fig. 4*C*). Therefore, the actin binding activity, or probably the F-actin-cross-linking activity, of Filamin A is crucial for the LL5*β*-mediated formation of lamellipodia. Because EGF induced small pseudopods in M2 cells transfected with Filamin AΔABD even in the absence of LL5*β* (Fig. 4*D*), it is unlikely that LL5*β* is involved in the formation of these small pseudopods.

We next sought to determine whether LL5*β* directs translocation of SHIP2-Filamin A complex. As reported (14), SHIP2 interacts with Filamin A (data not shown). By contrast, direct interaction between SHIP2 and LL5*β* was not observed (supplemental Fig. S7), suggesting that LL5*β* directs subcellular localization of SHIP2 via Filamin A. Two minutes after EGF stimulation, EGFP-tagged SHIP2 (EGFP-SHIP2) or mCherry-tagged SHIP2 (SHIP2-mCherry) localized to the cell periphery with LL5*β* (Fig. 5, *A*, *lower row*, and *B*, *upper row*) but remained in the cytosol in the presence of LL5βΔPH (Fig. 5*B*, *lower row*). As expected, SHIP2 colocalized well with Filamin A (Fig. 5*C*, *upper row*), but translocation of SHIP2 to the cell membrane was observed less in LL5*β* knockdown COS-7 cells (Fig. 5, *C*, *lower row*, and *D*). Supporting this observation, the amount of SHIP2 in the membrane fraction was reduced in LL5*β* knockdown COS-7 cells (Fig. 5*E*). These data indicate that Filamin A is an intermediate between LL5*β* and SHIP2. Indeed, translo-

cation of EGFP-SHIP2 and LL5β to the cell periphery was observed in A7 cells (Fig. 5*F*). In M2 cells (Filamin A-deficient A7 cells), LL5β was translocated to the cell periphery, leaving SHIP2 in the cytosol, following EGF stimulation (Fig. 5*G*). It was of interest that the amount of α-tubulin was also decreased in LL5β knockdown cells (Fig. 5*E*). Because it has been reported that SHIP2 regulates PtdIns(3,4,5)P_3 at membrane ruffles (14) and because it has also been proposed that SHIP2 mediates negative feedback on the accumulation of PtdIns(3,4,5)P_3 (13), we examined whether SHIP2, which is directed by LL5β through Filamin A, regulates membrane PtdIns(3,4,5)P_3. We found that ARNO-PH, one of the widely used PtdIns(3,4,5)P_3 detectors (16, 17) (supplemental Figs. S2 and S4 and supplemental Video 3), showed a greater accumulation at the plasma membrane in LL5β knockdown cells (Fig. 5, *H* and *I*). This suggests that SHIP2, which is directed by LL5β, is very likely to regulate membrane PtdIns(3,4,5)P_3.

DISCUSSION

We demonstrate here that LL5β is essential for quick Filamin A-dependent SHIP2 translocation in response to membrane PtdIns(3,4,5)P_3, fast enough to chase PtdIns(3,4,5)P_3 changes, and the reciprocal control of membrane PtdIns(3,4,5)P_3 through SHIP2, as well as the enhanced lamellipodium formation induced by PtdIns(3,4,5)P_3 (Fig. 6). It has been reported that v-ral simian leukemia viral oncogene homolog A (RalA) targets Filamin A in filopodia (22). In addition, faint membrane localization of mutated SHIP2 that lacks Filamin A-interacting domain, is observed after 5 min of EGF stimulation (14). This is consistent with the fact that SHIP2 is capable of associating with $p130^{Cas}$ and localizes to membrane ruffles and focal adhesions (23).

Because F-actin primarily grows near the plasma membrane by polymerizing globular actins at its plus end (24) and because cells do not form lamellipodia (large protrusions) in response to EGF application in the presence of the non-membrane-associating LL5βΔPH mutant, it is likely that increasing the cross-linking of newly polymerized F-actins by membrane-recruited Filamin A helps a cell to form lamellipodia. It is of interest that Filamin A can regulate the formation of small membrane protrusions without its actin-cross-linking activity. Indeed, small pseudopods were formed in M2 cells transfected with Filamin AΔABD in response to EGF.

LL5β, as well as Filamin A, is apparently versatile because the amount of α-tubulin in the membrane fraction was decreased by LL5β knockdown. It has been reported that LL5β binds to cytoplasmic linker-associated proteins (CLASPs) (25), which attach to the plus ends of microtubules, thereby modulating microtubule stabilization (26). Therefore, it is possible that LL5β synchronizes actins and microtubules, thereby coordinating cytoskeletal components for efficient migration.

It has been shown that SHIP2 is a critical component of a negative feedback loop that regulates PtdIns(3,4,5)P_3 levels and is likely to help a cell respond accurately and dynamically to external signals that impart directionality (13). We show here that SHIP2 translocates directly to sites of PtdIns(3,4,5)P_3 accumulation with Filamin A, which is dependent on LL5β. Considering that LL5β accumulates to the membrane in response to EGF application within a few minutes, it is plausible that SHIP2 follows changes in PtdIns(3,4,5)P_3 without a substantial delay. Therefore, it is likely that SHIP2, Filamin A, and LL5β help a cell to move in one direction smoothly. Moreover, the ability of PtdIns(3,4,5)P_3 and SHIP2 to regulate various cellular responses, such as the insulin response (27), and the reciprocal regulation of PtdIns(3,4,5)P_3 with Filamin A-LL5β reported here suggest that these findings are significant for the general understanding of such events, and possibly, for the understanding of related diseases.

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