Cell, Volume *135*

Supplemental Data

ACF7 Regulates Cytoskeletal-Focal Adhesion Dynamics and Migration and Has ATPase Activity

Xiaoyang Wu, Atsuko Kodama, and Elaine Fuchs

Supplemental Experimental Procedures

Antibodies, Reagents, and Plasmid DNA Constructions

Generation and purification of the rabbit polyclonal ACF7, K5 and K6 antibodies (Abs) have been described (Karakesisoglou et al., 2000; Perez-Moreno et al., 2006). Rat monoclonal β4-integrin (CD104) and anti-mouse-β1-integrin (CD29, active form) Abs were obtained from BD Pharmingen (Franklin lakes, NJ). Human plasma fibronectin, rabbit polyclonal phospho-Histone3 and glu-tubulin Abs, rat monoclonal mouse-β1-integrin Abs and mouse monoclonal Paxillin, Src and Rac1 Abs were obtained from Millipore (Billerica, MA). Phalloidin, nocodazole, taxol, ATP, PEI-cellulose TLC plastic plates, HA-peptide, HA-conjugated Agarose, mouse monoclonal Vinculin, acetylated tubulin, and β-tubulin Abs were obtained from Sigma (St. Louis, MO). Texas Red-conjugated Phalloidin was obtained from Invitrogen (Carlsbad, CA). Rabbit monoclonal RhoA Abs were obtained from Cell Signaling (Danvers, MA). Mouse monoclonal Abs against EB1 and Clip170, and rabbit polyclonal Abs against Cdc42, HA and FAK were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Rabbit polyclonal Abs against FAK Y397 and Src Y418 were obtained from Biosource (Rue de l'Industrie 8, B-1400 Nivelles, Belgium). α-^{32}P-ATP was obtained from MP Biomedicals (Solon, OH). Purified muscle actin, tubulin, myosin II, and kinesin motor domain were obtained from Cytoskeleton (Denver, CO). The mouse monoclonal Ab pan Myosin, and the rabbit polyclonal Abs against K10 and Loricrin were obtained from Covance. Rabbit polyclonal Clasp2 Abs were generated by Covance. Rabbit polyclonal Clasp1 Abs were a generous gift from Dr. William Earnshaw at Wellcome Trust Centre for Cell Biology at the University of Edinburgh. Other chemicals or reagents were obtained from Sigma, unless otherwise indicated.

Plasmids encoding DsRed-Zyxin, GFP-Paxillin, and GFP-EB1 have been described (Kodama et al., 2003; Schober et al., 2007). Plasmids pEGFP-C1-Clasp1 and pEGFP-C1-Clasp2 were generous gifts from Dr. William Earnshaw at Wellcome Trust Centre for Cell Biology at the University of Edinburgh. Full-length ACF7 cDNA was assembled from 5 fragments and inserted to a modified pEGFP-C1 vector (pEGFP-C1A) between SacII and KpnI. The full-length fragment was then transferred into other mammalian expression vectors, including pKH3S (confers an N-terminal HA tag) and pRFP-C1S (confers an N-terminal RFP tag). ACF7-NC were generated by PCR, using primers: CGG GAT CCA TGT TTC CTG TTC TTT GGG CCG G, AAG GAA AAA AGC GGC CGC TTT GTT TGC AAT CTG TAG CAG C, AAG GAA AAA AGC GGC CGC TGA TCG GCT GGA GGA GTT GAA AG, CTA GCT AGC GGT ACC TTA TCG CTT GGG ACC TGG AGT CC. The DNA fragment was then inserted into pKH3S, pEGFP-C1S, and pRFP-C1S. To create CFP-tagged actin, coding sequence of actin was generated by PCR using primers: AAG GAA AAA AGC GGC CGC TCA TGG ATG ATG ATA TCG CCG CGC, CGG AAT TCC TAG AAG CAT TTG CGG TGG ACG. The fragment was then inserted to pKH3S-CFP between EcoRI/NotI. The walker B deletion mutatant was constructed by site-directed mutagenesis using primers: CCA TTA GCC TAC TCA ATT CAG AGC G, TGT GGA CTG GGA CAC GGC TCG CTG GCG CAC CTC CTC, GAG GAG GTG CGC CAG CGA GCC GTG TCC CAG TCC ACA, GGT TCA TGG CTT CCA AAC GGC TCC.

Histology and Immunofluorescence

Skin or wound samples were embedded in OCT, 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 xylene, 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 (Guasch et al., 2007). Abs were diluted according to manufacturer's instruction, unless indicated.

Cell Culture

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-media supplemented with 15% serum and a final concentration of 0.05 mM Ca^{2+}. All experiments were performed using primary cells with less than 10 passages. For suspension culture, 293F cells were cultured in sterile flasks at 37°C, 8% CO_2 and 125 rpm shaking, using serum-free 293 SFM II media (invitrogen).

Quantification of FA size and relative staining intensity

Keratinocytes were fixed and stained with mouse anti-Vin and FITC anti-mouse Ab to label focal adhesions. Pictures were acquired on a Carl Zeiss Apotome fluorescence microscope on a 16-bit scale. Focal adhesion characteristics were quantified using Image J. An integrated morphometric analysis was performed on thresholded images to select classified objects of a size greater than 10 $pixel^2$ as focal adhesions based on the anti-Vin staining. Non-specific nuclear staining was removed manually.

Small GTPase Activity Assay, Microtubule and Actin Pull Down Assay, Immunoprecipitation, gel-overlay assay and Western Blotting

Levels of GTP-bound Rho, Rac, and Cdc42 were determined using Rho, Rac, Cdc42 activation assay biochem kits from Cytoskeleton (Denver, CO) according to manufacturer's instruction. Actin and microtubule binding were examined using actin binding spin-down assay kit and microtubule binding protein spin-down assay kit from Cytoskeleton (Denver, CO), according to manufacturer's instructions. Immunoprecipitation and western blotting were performed as described previously (Wu et al., 2004). Gel-overlay (far-western) assay was performed essentially as described (Wu et al., 2007).

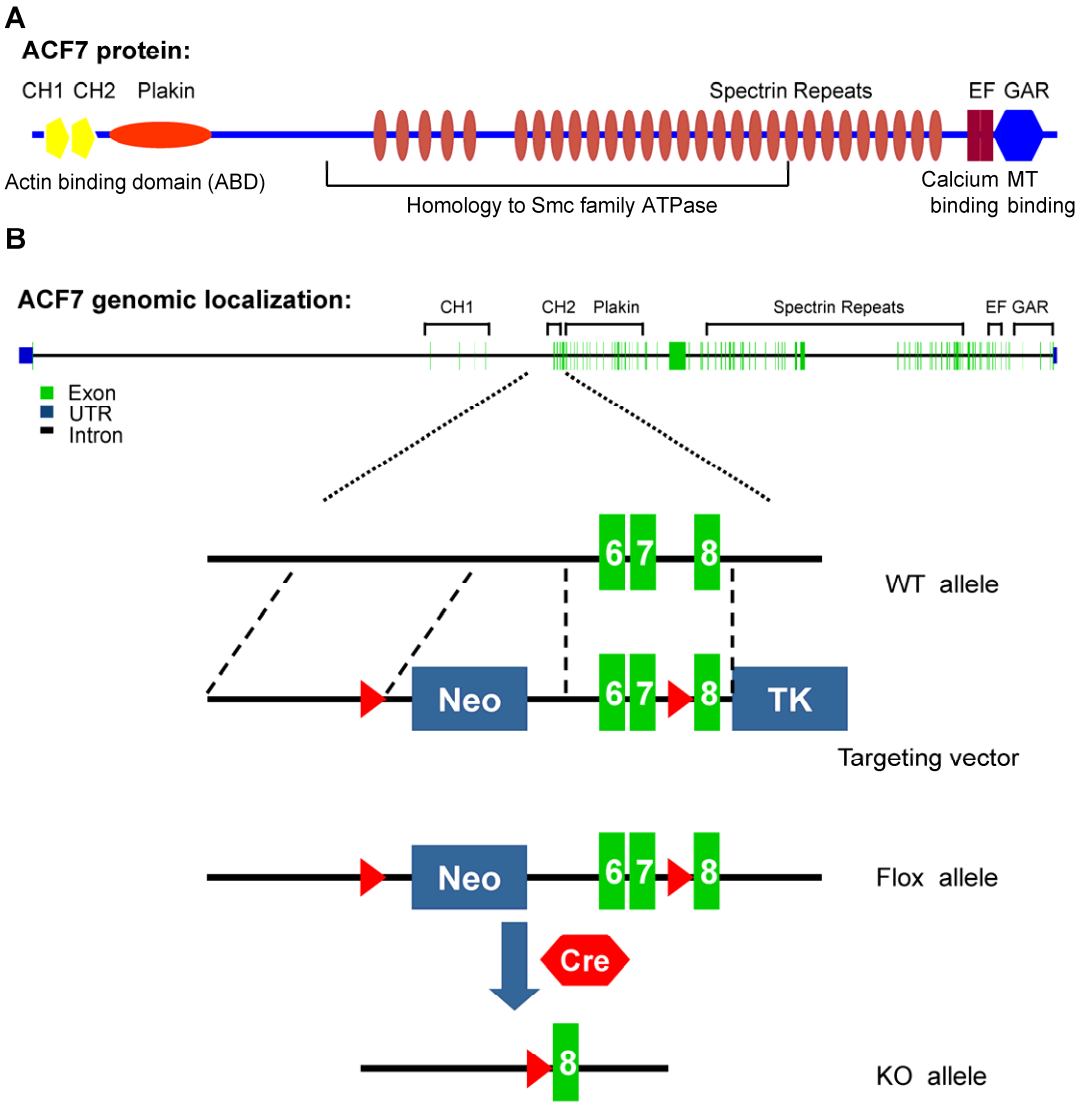

Figure S1: Schematic diagrams of ACF7 domain structures, genomic structures and targeting strategy. (A) Diagram showing domain structure of ACF7. CH1, CH2: calponin homology domain 1 and 2, mediating F-actin binding. EF: EF hand motif mediating potential calcium binding. GAR: Gas2-related domain mediating microtubule binding. **(B)** Diagrams showed the genomic strucuture of mouse ACF7 gene and our conditional targeting strategy. Red triangles represent loxP sites. Neo: neomycin resistance cassette. TK: thymidine kinase cassette.

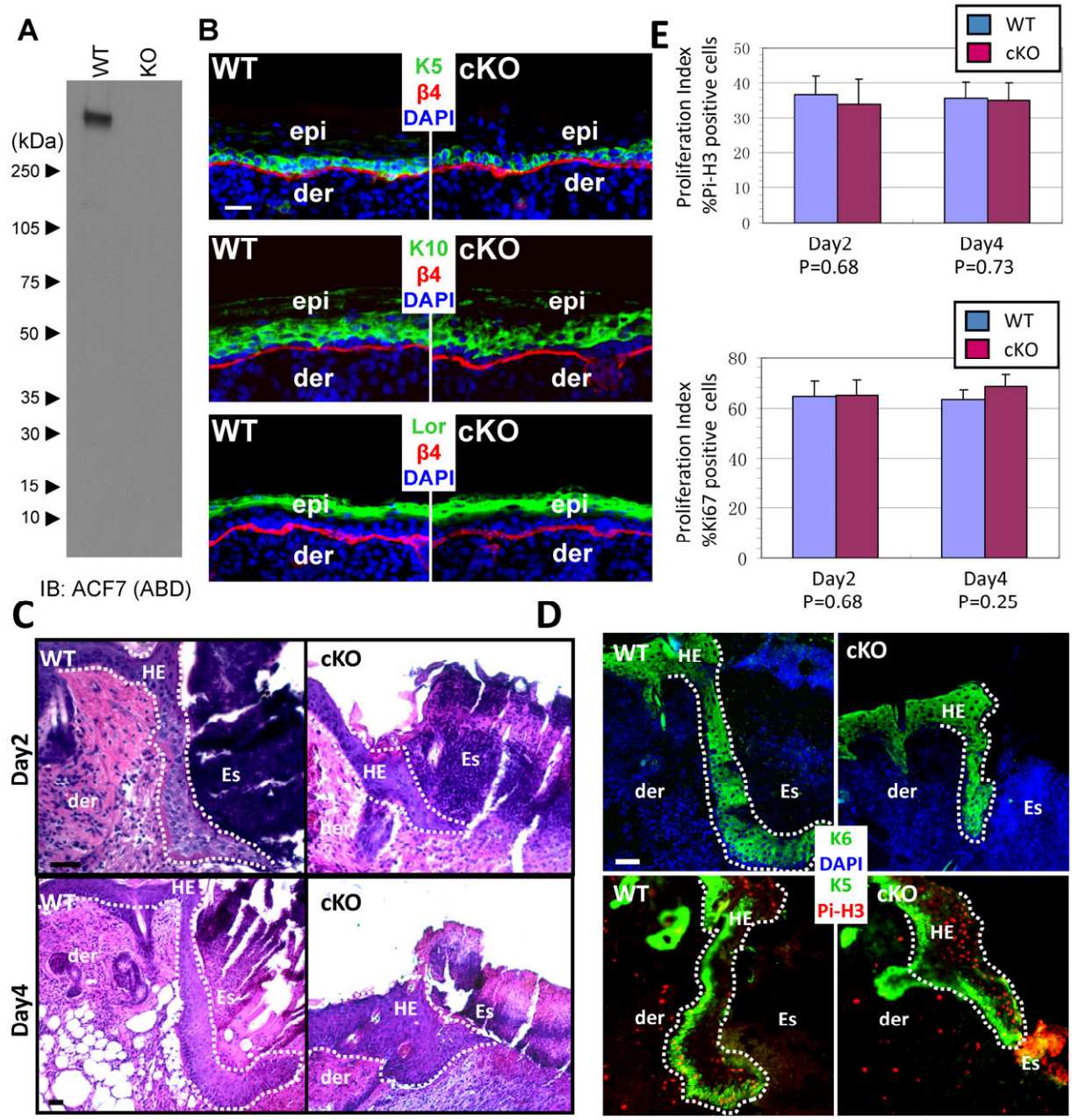

Figure S2: ACF7 conditional knockout caused defects in wound healing but not in cell proliferation or epidermal differentiation. (A) Immunoblot (IB) analysis using an antibody recognizing the N-terminal actin binding domain (ABD) of ACF7 reveals absence of full-length or any truncated ACF7 in KO keratinocytes. **(B)** Dorsal skins of neonatal mice were processed for immunofluorescence microscopy as described in Figure 1C, using different antibodies as indicated (K5: keratin 5, K10: keratin 10, Lor: Loricrin, β4: β4-integrin, CD104). Scale bar represents 20 μm. **(C)** Wound healing as monitored by hematoxylin/eosin staining of skin sections at the wound edges 2d and 4d after injury. Halves of wound sections are shown. HE: hyperproliferative epithelium, Es: eschar. Dotted white lines denote dermal–epidermal boundaries. Scale bar represents 50μm. **(D)** Sections of wound edges (2d after wounding) were immunostained with different Abs as indicated (K6: keratin 6, Pi-H3: phospho-Histone 3). Phospho-Histone 3 staining labeled cells at M, G2 and late S phase, and was used here as a proliferation marker. Scale bar represents 50μm. **(E)** Quantification of phospho-histone3 positive cells and Ki67-positive cells present in wound HE. Error bars represent standard deviations (SD).

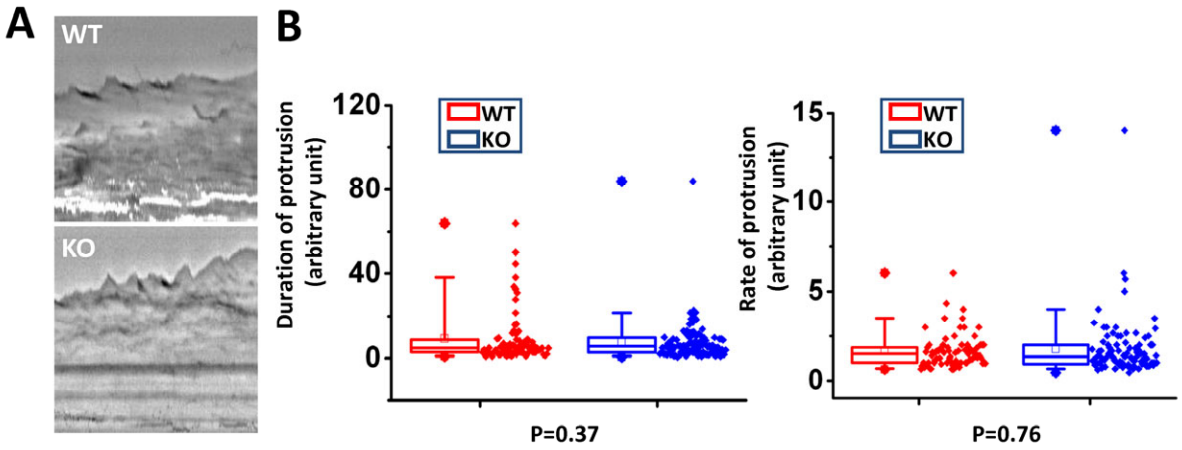

Figure S3: Membrane protrusive activity was unperturbed in ACF7 KO cells. (A) Representative kymographies of keratinocytes. **(B)** Box and whisker plots of membrane protrusion parameters (protrusion duration and protrusion rate). Scale bar represents 50 μm.

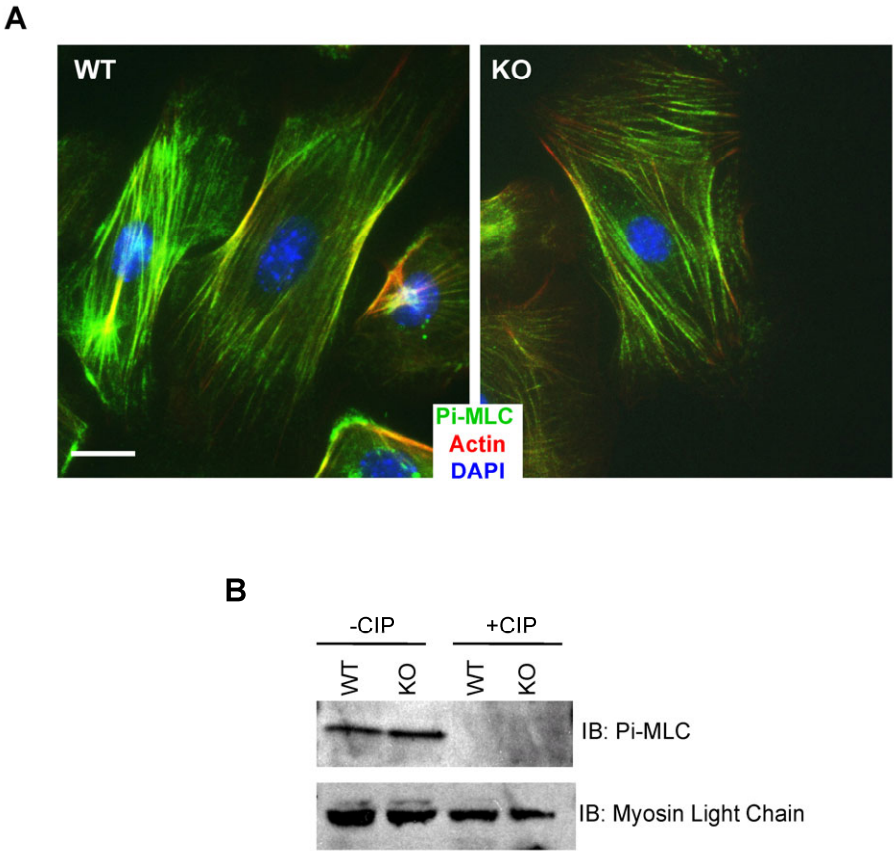

Figure S4: Levels of Myosin light chain Ser^{19} phosphorylation were comparable in WT and KO keratinocytes. (A) WT and KO keratinocytes were immunostained with different antibodies as indicated. Pi-MLC: Myosin light chain Ser^{19} phosphorylation. **(B)** WT and KO cells lysate treated with or without calf alkaline phosphatase (CIP, 30 minutes at 37°C) were subjected to western blotting with different antibodies as indicated.

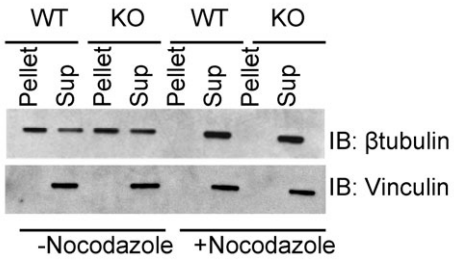

Figure S5: Levels of polymerized microtubule were comparable in WT and KO keratinocytes. To examine the amount of polymerized tubulin versus free tubulin in WT and KO cells, lysate from cells treated with or without nocodazole (25 μm) were subjected to ultracentrifugation. Pellets and supernatants (sup) were analyzed by western blotting with different antibodies as indicated.

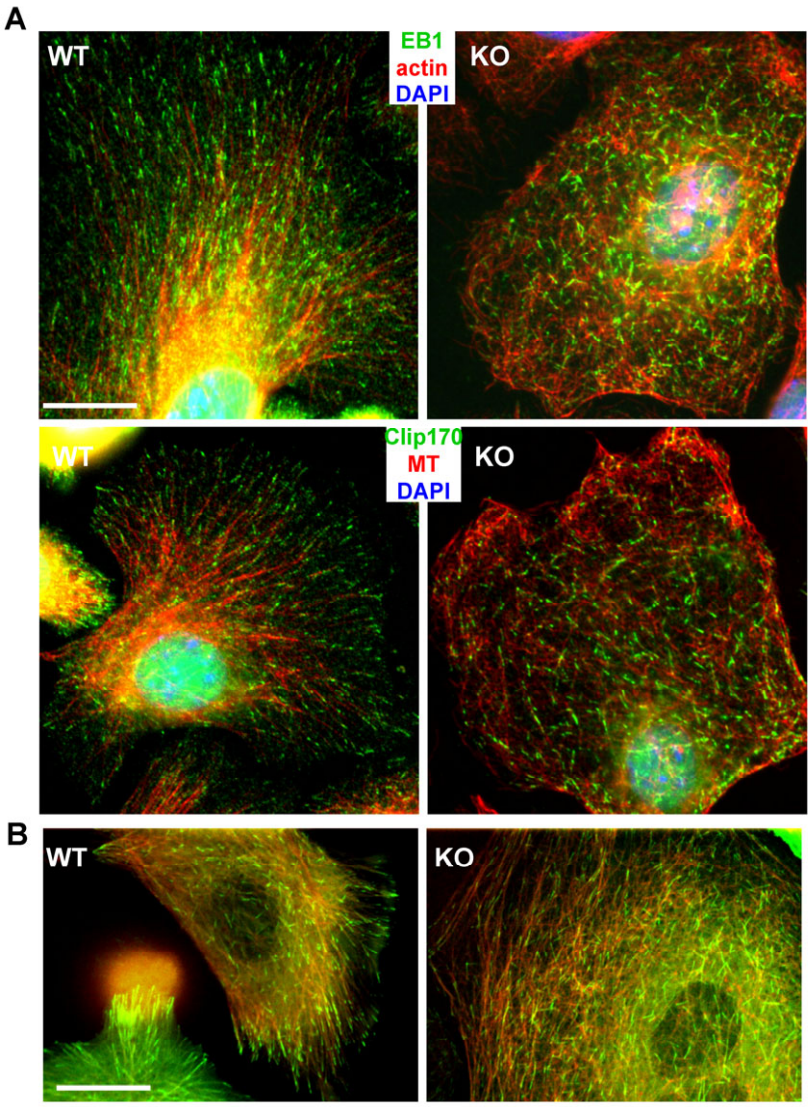

Figure S6: Microtubule +tip localization of Clip170 and EB1 was retained in ACF7 KO keratinocytes. (A) WT and KO keratinocytes were immunostained with different antibodies as indicated. Localization of Clip170 and EB1 at the tips of microtubules was detected in both WT and KO cells. **(B)** WT and KO keratinocytes were cotransfected with plasmids encoding mCherry-α-tubulin (red) and GFP-EB1 (green), and localization of exogenous EB1 at the tips of microtubule in both WT and KO cells were examined by confocal microscopy in live cells. Scale bars represent 10 μm.

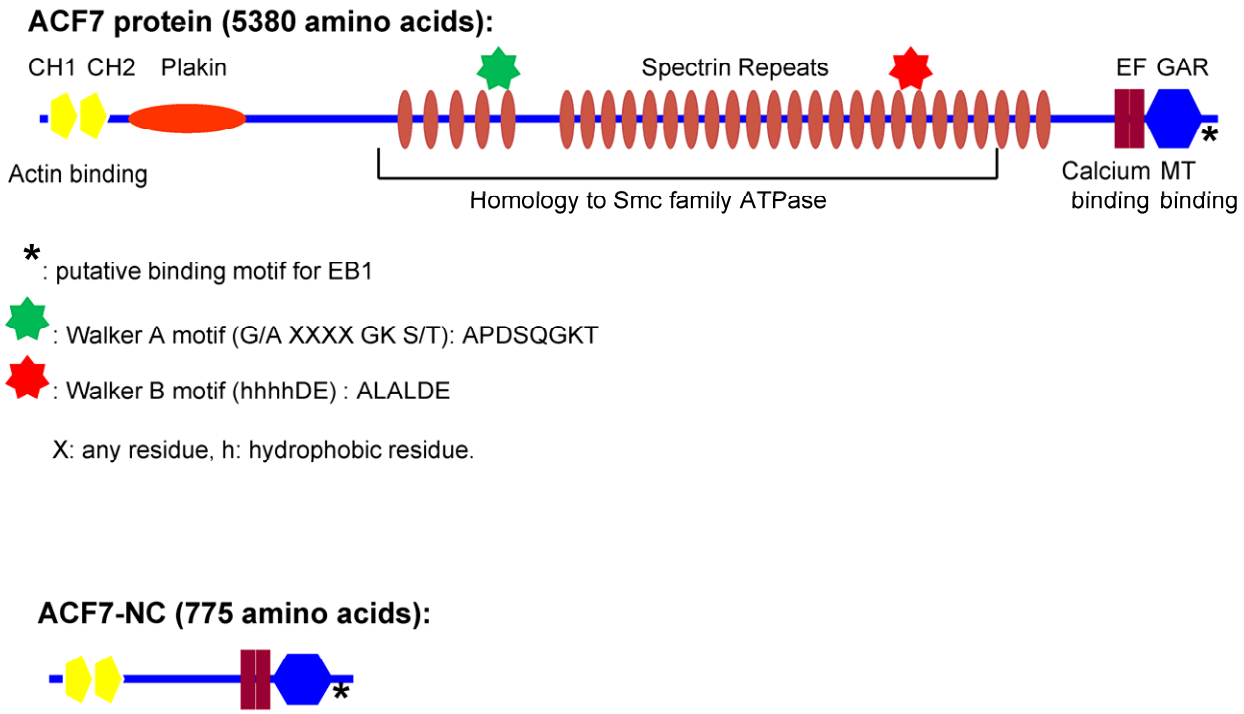

Figure S7: Schematic diagram indicating the domain structure of full-length ACF7 and the truncated mutant, ACF7-NC used in rescue experiments. CH: calponin homology domain, responsible for actin binding. EF: EF hand motif, responsible for Ca^{2+} binding. GAR: GAS2 related domain, responsible for microtubule binding. The putative nucleotide binding motifs (Walker A and Walker B) were also shown.

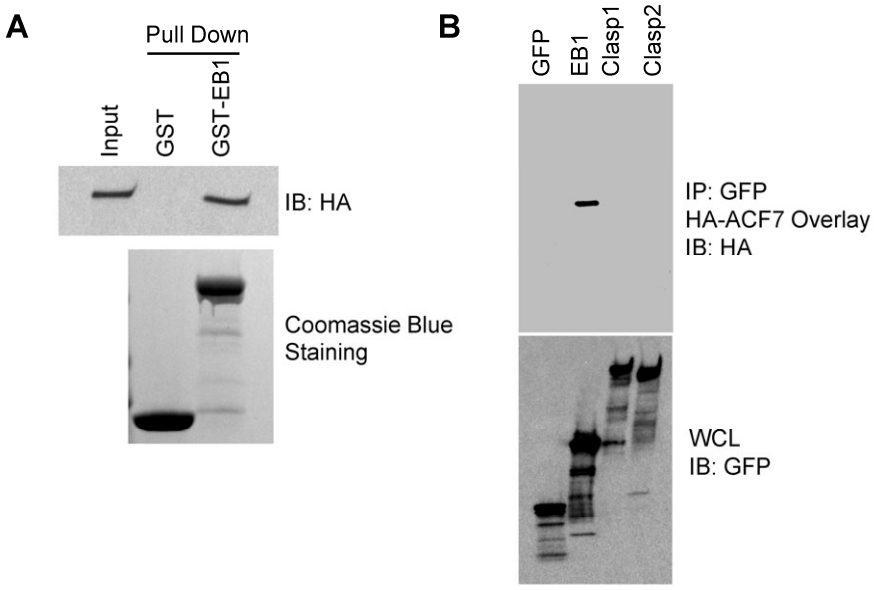

Figure S8: ACF7 directly associates with EB1 but not Clasp1 or Clasp2. (A) Purified HA-ACF7 was subjected to pull down assays with purified GST or GST-EB1 fusion proteins. Precipitated proteins together with an aliquot of input were analyzed by western blotting with HA antibody. Coomassie blue staining of an aliquot of GST or GST-EB1 was also shown. **(B)** Gel overlay assay was performed to examine the direct interactions between ACF7 with EB1, Clasp1 and Clasp2. GFP alone or GFP-tagged EB1 and Clasp1/2 were immunoprecipitated from transfected cell lysate. Immunoprecipitates were resolved by SDS-PAGE, transferred to nitrocellulose membrane, and overlay assay was performed using purified HA-tagged ACF7. The membrane was then subjected to western blotting with HA antibody. Aliquots of whole cell lysate (WCL) were examined by immunoblot with GFP antibody as well.

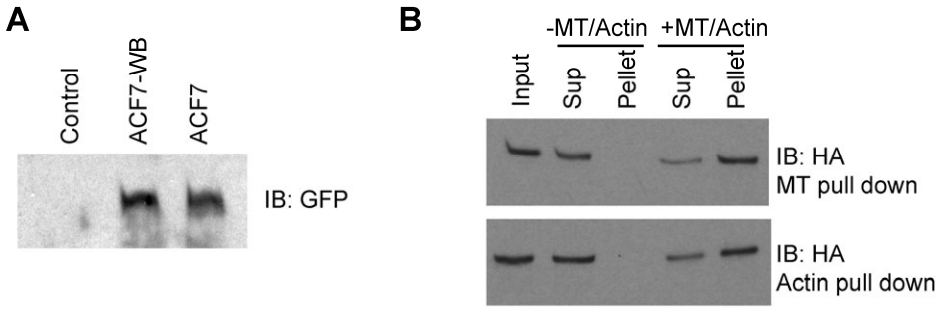

Figure S9: Deletion of walker B motif in ACF7 does not affect MT or F-actin interaction. (A) Lysate of untransfected cells (control) or cells transfected with plasmids encoding GFP-tagged ACF7-walker B (WB) deletion mutant or wild-type ACF7 was examined by immunoblot with GFP antibody. **(B)** Purified HA-tagged ACF7-WB mutant was incubated with or without microtubules (MT) or F-actin, and bound and unbound proteins were subsequently separated by ultracentrifugation. Pellets and supernatants (Sup) together with aliquots of input proteins were subjected to SDS-PAGE followed by western blotting with HA antibody.

Supplemental References

Guasch, G., Schober, M., Pasolli, H. A., Conn, E. B., Polak, L., and Fuchs, E. (2007). Loss of TGFbeta signaling destabilizes homeostasis and promotes squamous cell carcinomas in stratified epithelia. Cancer Cell *12*, 313-327.

Karakesisoglou, I., Yang, Y., and Fuchs, E. (2000). An epidermal plakin that integrates actin and microtubule networks at cellular junctions. J Cell Biol *149*, 195-208.

Kodama, A., Karakesisoglou, I., Wong, E., Vaezi, A., and Fuchs, E. (2003). ACF7: an essential integrator of microtubule dynamics. Cell *115*, 343-354.

Perez-Moreno, M., Davis, M. A., Wong, E., Pasolli, H. A., Reynolds, A. B., and Fuchs, E. (2006). p120-catenin mediates inflammatory responses in the skin. Cell *124*, 631-644.

Schober, M., Raghavan, S., Nikolova, M., Polak, L., Pasolli, H. A., Beggs, H. E., Reichardt, L. F., and Fuchs, E. (2007). Focal adhesion kinase modulates tension signaling to control actin and focal adhesion dynamics. J Cell Biol *176*, 667-680.

Wu, X., Suetsugu, S., Cooper, L. A., Takenawa, T., and Guan, J. L. (2004). Focal adhesion kinase regulation of N-WASP subcellular localization and function. J Biol Chem *279*, 9565-9576.
Wu, Y., Li, Q., and Chen, X. Z. (2007). Detecting protein-protein interactions by Far western blotting. Nat Protoc *2*, 3278-3284.