Supporting Information

Maritzen et al. 10.1073/pnas.1206468109

SI Materials and Methods

Plasmids. For bacterial expression of N-terminally GST-tagged proteins, we used the pGEX 4T-1 vector (Amersham); for N-terminally His_6-tagged proteins, we used the pET-28a(+) vector (Novagen). For the bacterial expression of Gadkin, the N-terminally truncated variant Δ51 was used lacking amino acids 1–51 comprising the N-terminal palmitoylation site, the deletion of which is necessary to render the bacterially expressed protein soluble. Vectors for the mammalian expression of C-terminally EGFP-or mRFP tagged Gadkin are based on the pEGFP-N2 backbone (BD Biosciences Clontech). If not indicated otherwise, human Gadkin was expressed. EGFP-ARPC5B is described in ref. 1. The VCA domain of human N-WASP (amino acids 395–505) was amplified from HeLa cell cDNA and cloned into pGEX4T-1 or pET-28a(+) for bacterial expression.

Antibodies and Reagents. We used the following antibodies for detection of proteins in immunoblots, immunofluorescence, and for immunoprecipitations: monoclonal mouse antibodies against AP-1-γ-adaptin, ARP3 (both BD Biosciences), TfR (clone H68.4; Zymed Laboratories), kinesin KIF5 heavy chain (clone H2; Chemicon), clathrin heavy chain (clone TD.1), clathrin light chains (clone 57.1), synaptotagmin 1 (clone 41.1), and ARPC3 (both Synaptic Systems); polyclonal rabbit antibodies against ARPC2, ARP3 (both from Upstate), and WASH (Sigma-Aldrich). Polyclonal antibodies against human Gadkin were raised in rabbits by our group and are described in ref. 2. Fluorescent dye conjugated secondary antibodies (Alexa-488, Alexa-568, and Alexa-647) were purchased from Invitrogen, as well as Alexa-568-coupled phalloidin. HRP-coupled secondary antibodies were from Dianova. Purified ARP2/3 complex and GST-WASP-VCA were purchased from tebu-bio. The following siRNAs were used for knockdown experiments:

siRNA target	Species	siRNA sequence
scrambled no. 1	Mouse	GCU GAU ACG CUA UAC UGA U-TT
scrambled no. 2	Mouse	GUA ACU GUC GGC UCG UGG U-TT
scrambled no. 3	Human	AUC GUU GAC UUA CAA GAG A-TT
Gadkin no. 6	Mouse	GAG UUG CUC AGC ACU AUC A-TT
Gadkin no. 7	Mouse	UGA CGA CAG CAC CUC CUU A-TT
Gadkin no. 2	Human	CGA AGU AGU AGA CUC UCA UCA GAU GCU
Kinesin no. 8	Mouse	GGC GGA GUG CAA CAU CAA A-TT
AP-1 γ-adaptin no. 1	Mouse	ACC GAA UUA AGA AAG UGG U-TT
ARPC2	Mouse	AAG GAC UUG AUA GUU CCU AAG AAU UAA
ARPC2	Human	CAG GAG UUC AAA GAA GGA CGC AGA GCC

Cell Culture and Transfection. Mouse embryonic fibroblasts (MEFs), mouse melanoma cells (B16F1), epidermoid carcinoma cells (A431), HeLa, and Cos7 cells were cultured at 37 °C in the presence of 5% CO_2 in high glucose (4,500 mg/L)-containing DMEM (Gibco BRL) supplemented with 10% (vol/vol) FCS and antibiotics. Transfections were done using Lipofectamine 2000 (Gibco BRL) for plasmid DNA or Oligofectamine (Gibco BRL) for siRNA oligonucleotides according to the protocol of the manufacturer. For knockdown experiments, cells were transfected on the day after seeding at *ca.* 30% confluency. The next day, the cells were split to low confluency to be transfected a second time with siRNA on the following day. The day after the second siRNA transfection, cells were either split onto coverslips for immunofluorescence at low density (5,000–20,000 cells per 12well) or left in their well and used for experiments on the following day. Cells were sometimes seeded on fibronectin-coated (BD Biosciences) coverslips (coverslips were incubated over night at 4 °C in 10 μg/mL fibronectin in PBS, washed with PBS and used without allowing to dry), on laminin-coated (Invitrogen Gibco) coverslips (coverslips were incubated for 6 h at room temperature in 25 μg/mL laminin in PBS, washed with PBS, and used without allowing to dry), on Matrigel-coated (BD Biosciences) coverslips (coverslips were incubated for 30 min at 37 °C in 1:50 Matrigel in Opti-MEM), and treated with aluminum fluoride to induce lamellipodia (cells were incubated for 15 min at 37° in medium containing 50 μM $AlCl_3$ and 30 mM NaF in the presence of FCS) (3) or treated with the Rac1 inhibitor NSC23766 (Tocris) to inhibit the formation of lamellipodia (cells were incubated at 37 °C in medium containing 50 μM NSC23766 either for 15 min in the absence of FCS or for 12 h in the presence of FCS).

Immunoblot Analysis of Cell and Tissue Lysates. Cells were trypsinized using 0.2 ml Trypsin and washed off the culture plate in 1 mL medium. The cell suspension was centrifuged at 800 × *g* for 5 min at 4 °C, washed with PBS, and centrifuged again. The resulting pellet was resuspended in 20 mM Hepes (pH 7.4), 100 mM KCl, 2 mM $MgCl_2$, and 1% Triton X-100 supplemented with 1 mM PMSF and mammalian protease inhibitor mixture (Sigma). Alternatively, organs dissected from C57BL/6 mice were homogenized in the same buffer. After 15–30 min solubilization on ice, the suspension was centrifuged at 20,800 × g for 10 min at 4 °C to remove debris. After determination of protein concentration by bicinchoninic acid (BCA) assay lysates were diluted into sample buffer [50 mM Tris, 2% (wt/vol) SDS, 0.1% bromophenol blue, 10% (vol/vol) glycerol] to a final concentration of 1–2 μg/μL and analyzed by SDS/PAGE and Western blotting.

Calculation of ARP2/3 and Gadkin Molarity by Quantitative Immunoblotting. B16F1 cell extracts were prepared as described above after counting the cells. His_6-Δ51-Δexon6-mouse Gadkin was purified. Protein concentrations of lysates and purified proteins were determined in parallel by BCA assay. A concentration range of recombinant His_6-Δ51-Δexon6-ms-Gadkin respectively purified ARP2/3 complex was blotted together with a concentration range of B16F1 cell lysate. Membranes were incubated with Gadkin respectively ARPC2 specific antibodies. Immunoreactive bands were revealed by chemiluminescence detection after incubation with secondary horseradish peroxidase coupled antibodies. Band intensities were quantified using ImageJ. The linear range of the integrated signal density of the Gadkin respectively ARPC2 detection was used to determine the amount of Gadkin respectively ARPC2 in a certain amount of lysate. The cellular concentration of the proteins was determined assuming a mean volume for B16F1 cells of 2,780 μm^3 as published in (4).

Purification of Native Untagged Δ51-Gadkin-WT. Wild-type His_6-tagged Δ51-Gadkin containing a thrombin cleavage site after the His_6-tag (see ref. 2 for details) was expressed in *E. coli* and puri-

fied using Ni-NTA agarose (Qiagen) according to the instructions of the manufacturer. Ni-NTA agarose bound His_6-tagged Δ51-Gadkin was incubated with 2 U of thrombin (Sigma) per mg protein in PBS containing 200 mM NaCl and 10 mM imidazole for 18 h at 4 °C on a rotating wheel. The supernatant was collected and dialyzed into PBS. Native untagged Δ51-Gadkin was shock-frozen in liquid nitrogen and stored at −80 °C until further use for direct binding experiments or in vitro actin polymerization assays.

In Vitro Actin Polymerization Assay. In vitro actin polymerization assays were performed using the actin polymerization biochem kit (no. BK003) from tebu-bio in conjunction with purified ARP2/3 (no. RP01; tebu-bio), purified GST-WASP-VCA (no. VCG03; tebu-bio), and purified GST-Δ51-Gadkin-WT according to the instructions of the manufacturer. In short, 9 μM pyrene-labeled actin (no. BK003) were incubated in 1× actin polymerization buffer (no. BK003) alone or together with the proteins indicated in Fig. S4 at the following concentrations: 20 nM ARP2/3, 500 nM GST-WASP-VCA (for experiments shown in Fig. S4*Ci*), or 50 nM respectively 20 nM GST-WASP-VCA (for experiments shown in Fig. S4*Cii*), 500 nM or 5 μM GST-Δ51-Gadkin-WT (in Fig. S4*Ci*), respectively 500 nM or 5 μM untagged Δ51-Gadkin-WT (in Fig. S4*Cii*). For the experiment in Fig. S4*Cii*, ARP2/3 and Gadkin were preincubated at 4 °C for 30 min before the addition of GST-WASP-VCA and pyrene-labeled actin. Samples were measured every 60 s for 15 min in a spectrofluorometer (Fluoro-Max-3) at 350-nm excitation and 406-nm emission at room temperature.

Binding and Competition Experiments. GST-fusion proteins were purified using GST-bind resin (Novagen) according to the instructions of the manufacturer and remained bound to the resin. His_6-fusion proteins were purified using Ni-NTA agarose (Qiagen) and were eluted in 20 mM Hepes (pH 7.4), 100 mM NaCl, 300 mM imidazol. Lyophilized ARP2/3 complex (tebu-bio) was resuspended according to the instructions of the manufacturer and diluted to 0.1 μg/μL in 10 mM Hepes (pH 7.4), 100mM KCl, 1 mM $MgCl_2$, 0.1 mM EDTA, and 0.1% Tween-20 supplemented with 1 mM PMSF and mammalian protease inhibitor mixture (Sigma). GST-fusion protein (7 μg) was incubated with 4.6 μg of ARP2/3 and varying amounts of His_6-fusion proteins (0-50× molar amount of GST-fusion protein). For the experiment in Fig. 2*G*, GST-Δ51-Gadkin (WT or W210A mutant), 0–10× His-N-WASP-VCA or His-EGFP and ARP2/3 were added at the same time and incubated for 2 h at 4 °C on a rotating wheel. For the experiment in Fig. S4*Di*, His-Δ51-Gadkin (WT or W210A mutant) was preincubated with ARP2/3 for 30 min at 4 °C while rotating. GST-N-WASP-VCA was added and samples were rotated for an additional 30 min at 4 °C.

For the experiment in Fig. S4*Diii*, untagged Δ51-Gadkin-WT was preincubated with ARP2/3 for 1 h at 4 °C on a rotating wheel. GST-N-WASP-VCA was added, followed by an additional incubation period for 30 min at 4 °C while rotating. In all cases, beads were washed extensively and eluted with sample buffer. Samples were analyzed by SDS/PAGE and immunoblotting.

Generation of Gadkin KO Mice. To abrogate protein expression from the Gadkin locus, we designed a targeting vector containing a neomycin cassette flanked on both sites by loxP sequences and preceded on the one side by 5-kb genomic sequence homologous to the upstream region of exon 1, which contains the start ATG and on the other side by 2 kb of genomic sequence homologous to the downstream region of exon 1. This construct was electroporated into R1 mouse embryonic stem cells. ES cell clones in which exon1 had been replaced by the loxP flanked neomycin cassette were injected into C57BL/6 blastocysts, which were subsequently implanted into pseudopregnant mice. The resulting chimeric males were mated with C57BL/6 females to yield heterozygous Gadkin KO mice. These mice were crossed with EIIa Cre Deleter mice to remove the neomycin cassette. Heterozygous Gadkin KO mice without neomycin cassette did not show any phenotypic differences to WT animals and were interbred to obtain Gadkin WT and KO litter mates for the generation of mouse embryonic fibroblasts. Animals were housed on a 12 h light/dark cycle with food and water available ad libitum. All experiments in the present study were conducted in accordance with the guidelines of the Landesamt für Gesundheit und Soziales (LAGeSo) Berlin.

Generation of Gadkin WT and KO Mouse Embryonic Fibroblasts. WT and KO MEFs were prepared from litter mate embryonic day (E) 13.5 embryos of heterozygous parents according to the protocol of Matthew Fero (Fred Hutchinson Cancer Research Center, Seattle, WA). Two independent Gadkin WT (no. 3, no. 5) and Gadkin KO (no. 2, no. 6) MEF cell lines were established. After the second passage, cells were transfected with SV40 large T antigen for immortalization. Spreading assays were performed with primary cells (passages 0 and 4). Biotinylation was performed with immortalized cells.

SILAC, Surface Biotinylation, and Affinity Purification. Gadkin MEFs were cultured for 6 d in L-lysine- and L-arginine-free DMEM/10% (vol/vol) FCS (Thermo Fisher Scientific) supplemented either with $^{13}C_6$-L-lysine and $^{13}C_6$,$^{15}N_4$-L-arginine (Silantes) in case of KO MEFs or normal amino acids in case of WT MEFs. Cells were washed 2× with ice-cold PBS and incubated with 0.5 mg/mL biotin in PBS for 30 min at 4 °C. Cells were washed 2× for 5 min with ice-cold 50 mM glycine in PBS to quench remaining biotin. After two additional washes in ice-cold PBS, cells were harvested and lysed in 20 mM Hepes (pH 7.4), 100 mM KCl, 2 mM $MgCl_2$, and 1% Triton-X100, supplemented with 1 mM PMSF and mammalian protease inhibitor mixture (Sigma). Lysates were cleared by centrifugation, and protein concentration was determined by BCA. Equal amounts of protein from WT and KO lysate were mixed (700 μg each to a final concentration of 1.6 μg/μL) and incubated with Streptavidin beads for 90 min at 4 °C on a rotator. Beads were washed extensively and bound protein was eluted with sample buffer and separated by SDS/PAGE. For LC-MS/MS analysis, Coomassie-stained lanes were cut into slices and proteins were digested with trypsin as described (5). In brief, gel slices were washed with 50% (vol/vol) acetonitrile in 50 mM ammonium bicarbonate, dehydrated in acetonitrile, and dried in a vacuum centrifuge. The dried gel pieces were reswollen in 15 μL of 50 mM ammonium bicarbonate containing 60 ng of trypsin (sequencing-grade; Promega). After 17 h of incubation at 37 °C, 15 μL of 0.3% trifluoroacetic acid in acetonitrile was added, and the separated supernatant was dried under vacuum and redissolved in 6 μL of 0.1% trifluoroacetic acid, 5% (vol/vol) acetonitrile in water.

Mass Spectrometry and Data Analysis. Tryptic peptides were analyzed by a reversed-phase capillary liquid chromatography system (Eksigent NanoLC Ultra; Axel Semrau) connected to an LTQ-Orbitrap XL mass spectrometer (Thermo Scientific). LC separations were performed on a capillary column (PepMap100; C18; 3 μm; 100 Å; 250 mm × 75 μm i.d.; Dionex) at an eluent flow rate of 250 nL/min using a linear gradient of 0–40% B in 90 min. Mobile phase A contained 0.1% formic acid (vol/vol) in water, and mobile phase B contained 0.1% formic acid in acetonitrile. Mass spectra were acquired in a data-dependent mode with one MS survey scan (with a resolution of 30,000) in the Orbitrap and MS/MS scans of the four most intense precursor ions in the linear trap quadrupole. Identification and quantification of proteins were carried out with version 1.2.0.18 of the MaxQuant software package as described (6). Data were searched against an International Protein Index mouse protein database (version 3.68). The mass tolerance of precursor and sequence ions was set to

7 ppm and 0.35 Da, respectively. Methionine oxidation and the acrylamide modification of cysteine were used as variable modifications. False discovery rates were <1% based on matches to reversed sequences in the concatenated target-decoy database. Proteins were considered if at least two sequenced peptides were identified.

1. Rottner K, Kaverina IN, Stradal TEB (2006) Cytoskeleton proteins. *Cell Biology, A Laboratory Handbook*, ed Celis JE (Elsevier Academic Press, London), Vol 3, pp 111–119.
2. Schmidt MR, et al. (2009) Regulation of endosomal membrane traffic by a Gadkin/AP-1/kinesin KIF5 complex. *Proc Natl Acad Sci USA* 106:15344–15349.
3. Hahne P, Sechi A, Benesch S, Small JV (2001) Scar/WAVE is localised at the tips of protruding lamellipodia in living cells. *FEBS Lett* 492:215–220.
4. McMillan TJ, Rao J, Hart IR (1986) Enhancement of experimental metastasis by pretreatment of tumour cells with hydroxyurea. *Int J Cancer* 38:61–65.
5. Lange S, Sylvester M, Schümann M, Freund C, Krause E (2010) Identification of phosphorylation-dependent interaction partners of the adapter protein ADAP using quantitative mass spectrometry: SILAC vs (18)O-labeling. *J Proteome Res* 9:4113–4122.
6. Cox J, Mann M (2008) MaxQuant enables high peptide identification rates, individualized p.p.b.-range mass accuracies and proteome-wide protein quantification. *Nat Biotechnol* 26:1367–1372.

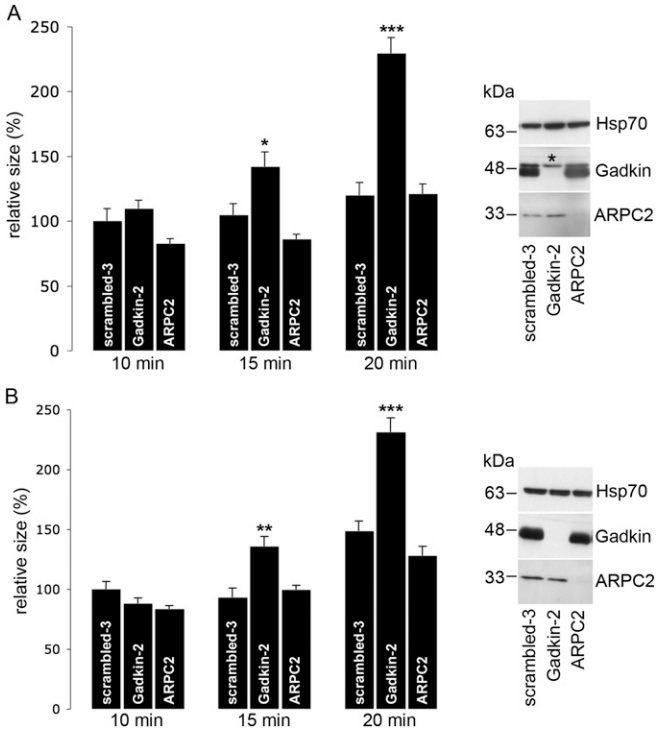

Fig. S1. Gadkin modulates cell size also in HeLa and Cos7 cells. Cos7 (*A*) respectively HeLa (*B*) cells treated with the indicated siRNAs were trypsinized and seeded onto glass coverslips. After the indicated time intervals cells were rapidly washed, fixed, and stained with phalloidin to visualize their morphology. The cell area of individual cells was quantified and is depicted normalized to the size of cells treated with scrambled-3 siRNA (representative experiments; $n = 84$–215 cells per condition; $^{*}P < 0.05$; $^{**}P < 0.001$; $^{***}P < 0.0001$ for Gadkin siRNA-treated cells vs. scrambled siRNA treated cells in unpaired Student's t test). (*Right*) Representative images of immunoblots of lysates from cells treated with the indicated siRNAs incubated with antibodies against Hsp70, Gadkin and ARPC2 are shown. Error bars always depict SEM.

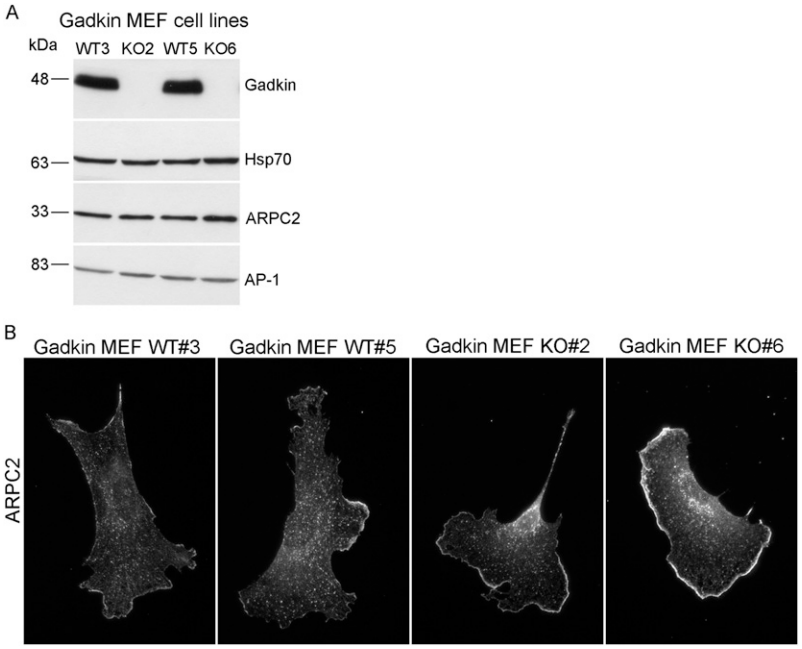

Fig. S2. Characterization of Gadkin WT and KO MEFs. (*A*) Samples of cell lysates derived from Gadkin WT and KO MEF cell lines were analyzed by immunoblotting for Gadkin, heat-shock protein (Hsp)70, ARPC2, and AP-1. (*B*) Two independent lines of Gadkin WT (no. 3, no. 5) and KO MEFs (no. 2, no. 6) were seeded onto coverslips, fixed 24 h postplating, and stained with antibodies specific for ARPC2.

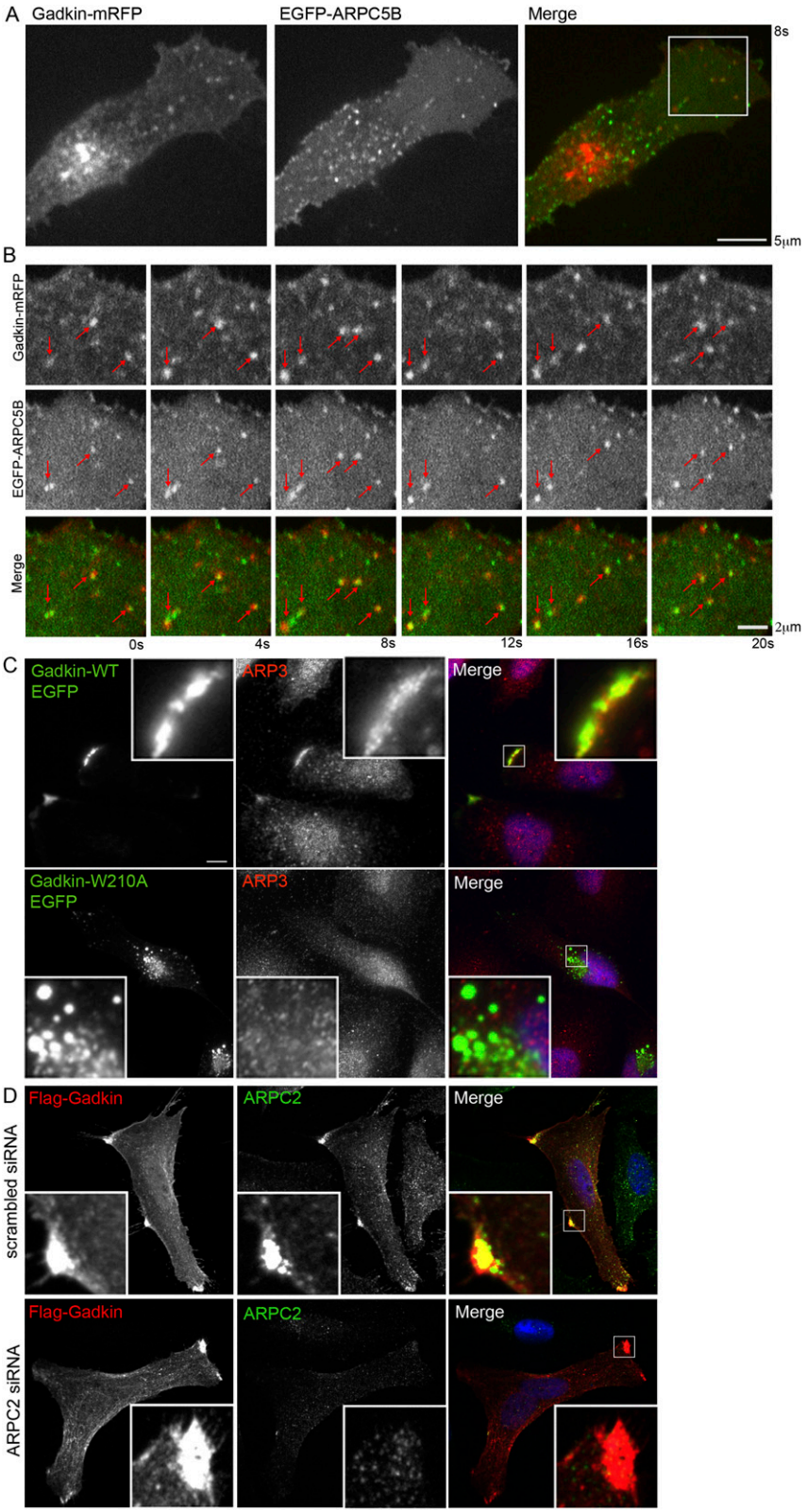

Fig. S3. Subpool of motile Gadkin vesicles is positive for ARP2/3. (*A* and *B*) B16F1 cells cotransfected with Gadkin-mRFP and EGFP-ARPC5B were analyzed by live-cell confocal imaging at 37 °C. Still images (enlargements of the boxed area of the cell depicted in *A*) extracted at the indicated time points are shown in (B). Examples of vesicles positive for both markers are pointed at with arrows. (Scale bars: 5 μm in *A*; 2 μm in *B*). (C) HeLa cells transfected with EGFP-tagged Gadkin-WT or W210A mutant were fixed 24 h posttransfection and stained with ARP3-specific antibodies. DAPI-stained nuclei depicted in blue. (Scale bar: 10 μm.) (*Inset*) Magnification: 5× of boxed area. (*D*) HeLa cells treated with ARPC2-targeting or scrambled siRNAs were transfected with Flag-tagged Gadkin-WT, fixed 24 h posttransfection, and stained with ARPC2-specific antibodies. DAPI-stained nuclei depicted in blue. (*Inset*) Magnification of boxed area.

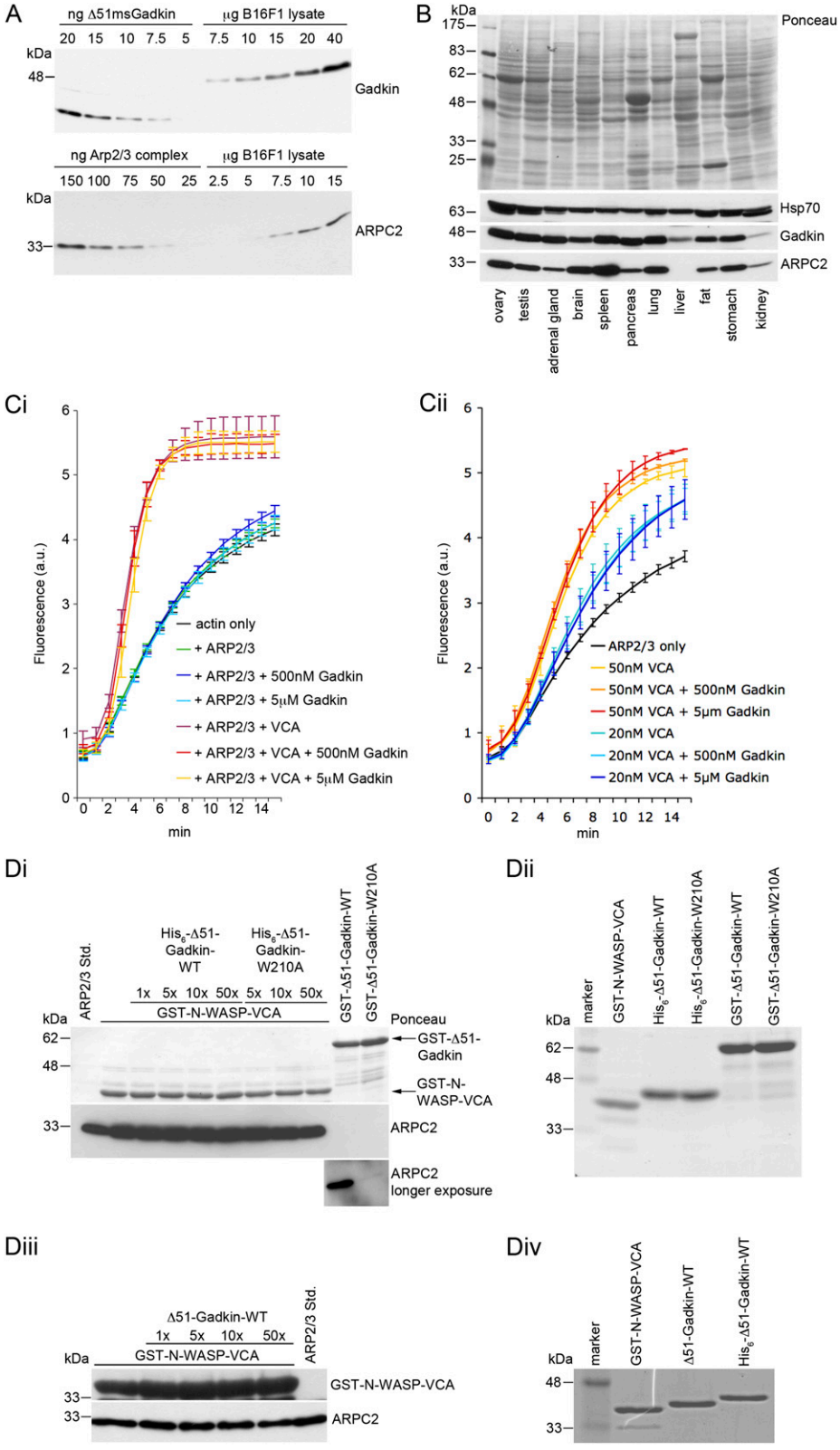

Fig. S4. Gadkin is an abundant ubiquitously expressed protein that fails to outcompete VCA domain for binding to ARP2/3. (*A*) Determination of the Gadkin and ARPC2 amount in B16F1 cells by quantitative immunoblotting. A concentration range of recombinant His_6-Δ51-Δexon6-ms-Gadkin (*Upper*) respectively purified ARP2/3 complex (*Lower*) was blotted together with a concentration range of B16F1 cell lysate. The linear range of the integrated signal density of the Gadkin respectively ARPC2 detection was used for the calculation of the Gadkin and ARPC2 molarity in B16F1 cells. (*B*) Broad expression pattern of Gadkin and ARPC2. Lysates of the indicated organs were immunoblotted and probed with antibodies specific for Hsp70, Gadkin and ARPC2. (*C*) Addition of Gadkin does not inhibit VCA-stimulated ARP2/3-mediated actin polymerization in vitro. In vitro actin polymerization assays were done by incubating pyrene-actin (9 μM) with purified ARP2/3 (20 nM), GST-WASP-VCA (500 nM), and GST-Δ51-Gadkin-WT (500 nM or 5 μM) (*Ci*). Native untagged Gadkin also failed to inhibit ARP2/3-

Legend continued on following page

mediated actin polymerization at low concentrations of GST-WASP-VCA (20 nM or 50 nM), even if added at a more than 200-fold molar excess (500 nM or 5 μM of native recombinant untagged Δ51-Gadkin-WT) (*Cii*). Fluorescence increase is plotted over time. Representative experiments are shown that were performed in triplicate (*Ci*) or in duplicate (*Cii*), respectively. Error bars represent SEM. (*D*) Direct binding of purified ARP2/3 complex to GST-N-WASP-VCA cannot be outcompeted by the addition of 1–50× the molar amount of purified His_6-Δ51-Gadkin-WT or untagged Δ51-Gadkin-WT. Immobilized GST-N-WASP-VCA was added for 30 min to purified ARP2/3 complex, which had been preincubated with varying amounts of His_6-Δ51-Gadkin-WT or W210A mutant for 30 min. Immobilized GST-Δ51-Gadkin (WT and W210A mutant) was incubated with ARP2/3 for 60 min. After extensive washing, proteins were eluted in sample buffer and analyzed by immunoblotting for ARPC2 (*Di*). The integrity of the purified proteins was verified by SDS/PAGE and Coomassie staining (*Dii*). Varying amounts of untagged Δ51-Gadkin-WT were preincubated with purified ARP2/3 complex for 1 h. Afterward, immobilized GST-N-WASP-VCA was added for 30 min. After extensive washing, proteins were eluted in sample buffer and analyzed by immunoblotting for ARPC2 and GST (*Diii*). The integrity of the purified proteins and the effectiveness of the thrombin cleavage of His_6-Δ51-Gadkin-WT used to obtain the untagged Δ51-Gadkin-WT was verified by SDS/PAGE and Coomassie staining (*Div*).

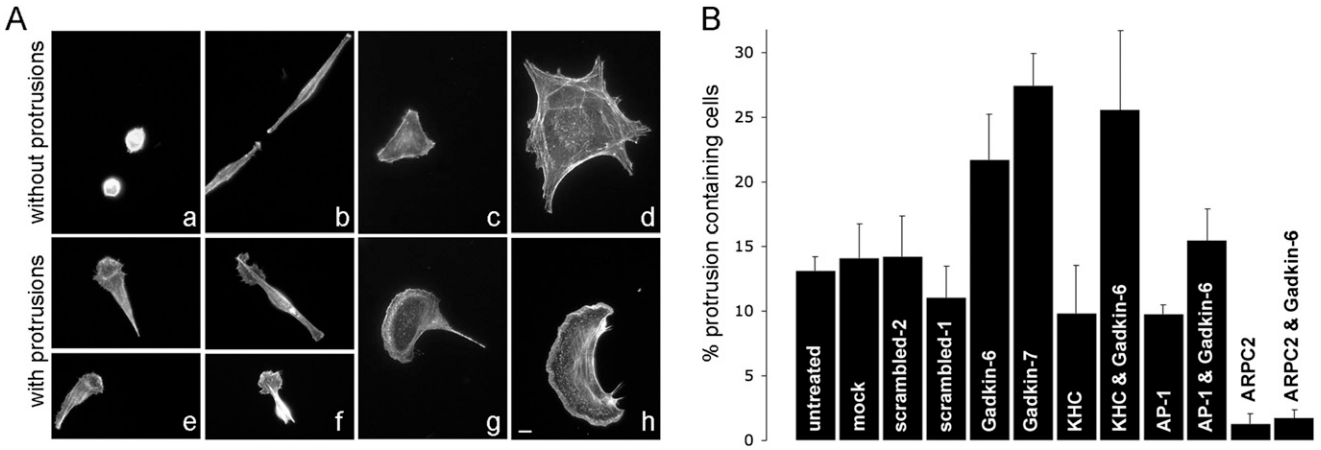

Fig. S5. Gadkin modulates cell shape and size. (*A*) siRNA treated B16F1 cells were fixed 24 h after plating and stained with phalloidin to visualize their morphology. Cells were grouped in either protrusion-free cells (examples *a*–*d*) or in smooth protrusion containing cells (examples *e*–*h*). The percentage of protrusion-containing cells for cells treated with different siRNAs is illustrated in *B*. Error bars represent SEM.

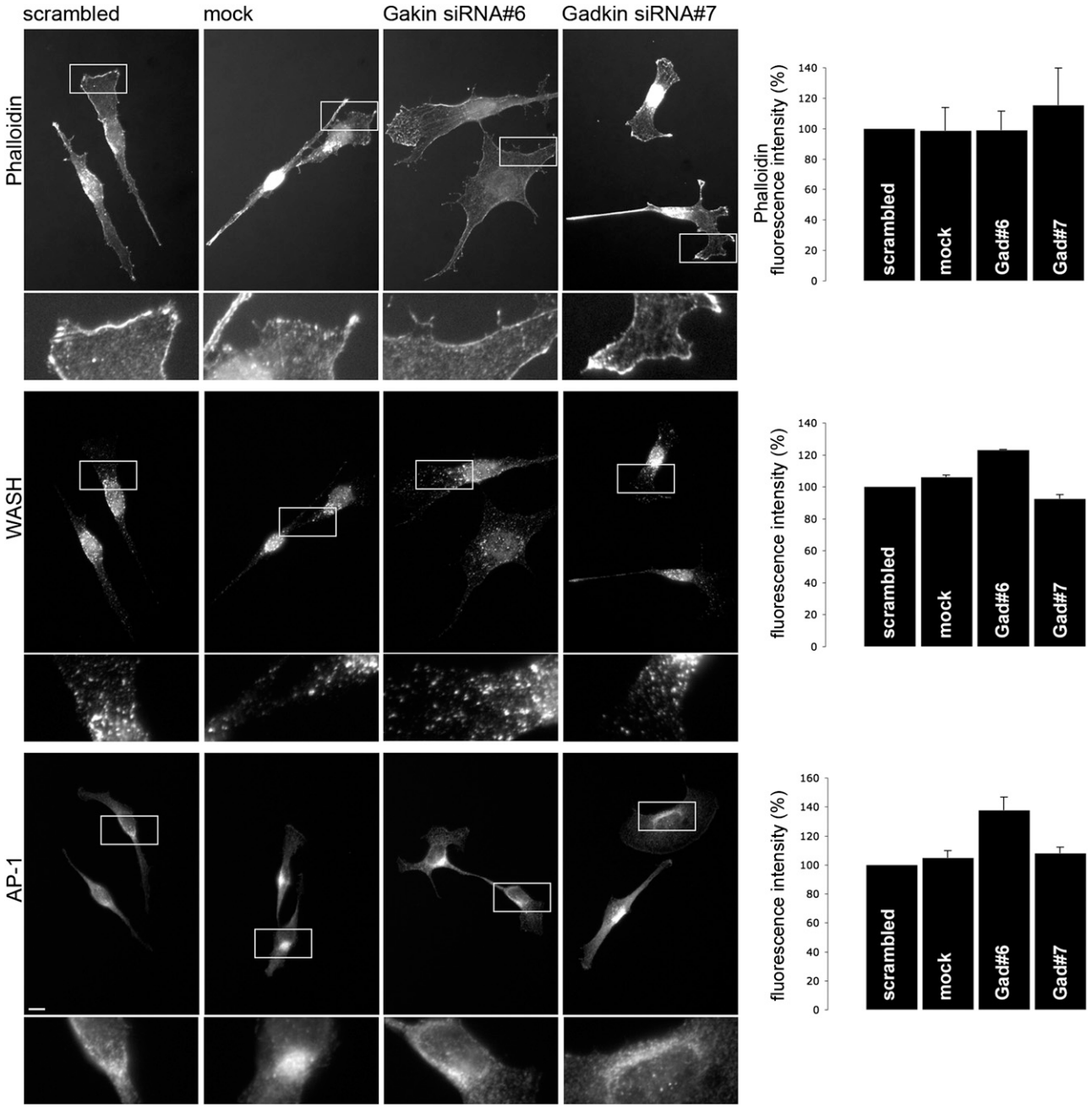

Fig. S6. Total levels of F-actin, WAVE, and AP-1 are not altered. B16F1 cells were seeded onto coverslips after two rounds of transfection either without siRNA (mock), with nontargeting scrambled siRNA, or with Gadkin-targeting siRNAs. Cells were fixed and processed for immunocytochemistry using fluorescently labeled phalloidin to label F-actin, as well as antibodies specific for the ARP2/3 interactor WASH and the Gadkin interactor AP-1. The quantification of the overall sum fluorescence intensity per cell normalized to the cells treated with scrambled siRNA is shown on the right (n = 2–4; n = 47–107 per condition per experiment). Error bars represent SEM.

Table S1. Enrichment of ARP2/3 subunits in biotinylated surface fraction of Gadkin KO MEFs determined by SILAC-based quantitative MS/MS analysis

Gene name	Normalized increase
Arpc5l	2.21
Arpc5	1.91
Arpc1b	1.67
Arp2	1.62
Arpc2	1.56
Arp3	1.52
Arpc3	1.50
Arpc4	1.41
PDGFR	0.89