Supporting Information

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SI Materials and Methods

Plasmids, Mutagenesis, siRNAs, Antibodies, and Fluorescent Compounds. The full-length Gadkin coding sequence or truncated versions thereof were cloned into pEGFP-N (Clontech) or pmRFP-N for mammalian expression of C-terminally eGFP- or mRFP-tagged proteins. Point mutants were generated using the QuikChange II kit (Stratagene). The presence of the mutations was verified by dsDNA sequencing. For bacterial expression of N-terminally GST-tagged proteins we used the pGEX 4T-1 vector (Amersham). Vectors for the mammalian expression of N-terminally HA-, FLAG-, and c-myc-tagged proteins were custom made based on the pcDNA3 backbone (Invitrogen). For the knockdown of human Gadkin, we used a 27-mer siRNA with the following sequence: CGA AGU AGU AGA CUC UCA UCA GAU GCU. Knockdown of human KIF5B was done with validated siRNAs provided by Qiagen (GeneSolution siRNA, cat. no. 1027416) with the sequences CGU UGC AAG CAG UUA GAA ATT and GGC CGA GUG CAA CAU CAA ATT. Knockdown of human clathrin heavy chain was done with siRNA with the sequence AUC CAA UUC GAA GAC CAA UTT. For controls, a nontargeting siRNA with the sequence AUC GUU GAC UUA CAA GAG ATT was used. We used the following antibodies for detection of proteins in immunoblots, immunofluorescence, and for immunoprecipitations: Monoclonal antibodies against AP-1-γ-adaptin, AP-3-μ3 (p47), GM130, TGN46, cytochrome *c* (clone 6H2.B4), and EEA1 (all from BD Biosciences), Golgin-97 (Invitrogen), TfR (clone H68.4; Zymed Laboratories), kinesin KIF5 heavy chain (H2; Chemicon), AP-2-α-adaptin (AP6), MPR46 (clone 10C6), CI-M6PR (Affinity Bioreagents), clathrin heavy chain (clones X22 for IF and TD.1 for IB), clathrin light chains (clone 57.1), tubulin (Sigma), synaptotagmin 1 (clone 41.1; Synaptic Systems), FLAG-tag (M2; Sigma), HA-tag (HA.11; Covance) and myc-tag (clone 9E19; Sigma), polyclonal antibodies against Rab11 (Zymed), KLC2 (a kind gift from Xiao-Jiang Li, Emory University, Atlanta, GA) and β- and δ-COP (a kind gift from F.T. Wieland, Heidelberg, Germany). Polyclonal antibodies against Gadkin were raised in rabbits by injecting a purified His-tagged Gadkin (amino acids 52–302). The serum was affinity-purified on crosslinked GST-tagged Gadkin (amino acids 52–302) and tested for specificity. Fluorescent dye conjugated secondary antibodies (Alexa^{488}, Alexa^{568}, Alexa^{594}, and Alexa^{647}) were purchased from Invitrogen. HRP-coupled secondary antibodies were from Dianova. Fluorescently labeled Tf and dextran (3,000 MW, lysine fixable) were purchased from Invitrogen. Cy3-labeled Shiga Toxin B (StxB) was a kind gift of Dr. Ludger Johannes (Institut Curie, Paris, France).

Floatation, Immunoisolation, and Electron Microscopy. Immunoisolation of Gadkin containing organelles was performed as described earlier (1) with minor modifications: We used brains from postnatal (P1) rats. The pellet P100 was resuspended in 2 M sucrose, homogenized, loaded underneath layers of 1.2, 0.8, and 0.3 M sucrose and centrifuged at 350,000 $\times$ *g* for 3 h. Eleven fractions were taken, and fraction 3 to 7 (light membrane fractions) were used for immunoisolation. Dynabeads M-280 sheep anti-rabbit IgG (Dynal Biotech) were coupled to affinity purified rabbit anti-Gadkin antibodies or nonspecific rabbit IgG (control) at a concentration of 10 μg/mg beads and incubated with the light membrane fractions overnight at 4 °C. Beads were extensively washed in PBS and eluted in sample buffer for immunoblotting or processed for electron microscopy. For EM, beads with isolated membranes were fixed in 3% glutaraldehyde, rinsed, and postfixed in 2% OsO_4. After washing, beads were embedded in agarose and dehydrated with increasing concentrations of ethanol (50%, 70% plus 1% uranylacetate, 80%, 90%, and 100%). Agarose blocks were embedded in Epon (Fluka), 60 nm thin sections were prepared and contrasted with 2% uranylaldehyde and lead citrate.

Shiga Toxin Trafficking Assay. After two rounds of siRNA transfection, HeLa cells were seeded on glass coverslips and starved overnight. On the next day, cells were washed once with ice-cold PBS and then incubated at 4 °C for 30 min with 1 μg/mL Cy3-StxB in DMEM supplemented with 2% BSA and 20 mM HEPES. After two washes with ice-cold PBS, cells were either fixed immediately with PFA (0 min time point) or incubated for 15 to 90 min in growth medium at 37 °C before fixation. Fixed cells were processed for indirect immunfluorescence microscopy using antibodies against GM130. For quantifications, the total fluorescence of Cy3-StxB was measured in the Golgi area delineated by accumulation of GM130 used as a mask.

Uptake Assays. For Tf uptake experiments, HeLa cells were starved for 1 h and then incubated for 20 min at 37 °C with 25 μg/mL Alexa568-Tf in medium without FCS. Cells were washed three times with ice-cold PBS, fixed with 4% PFA, and mounted on coverslips. For uptake of MPR-specific antibodies, HeLa cells were incubated with antibody in FCS containing medium for 90 min. Cells were washed three times with ice-cold PBS, fixed with 4% PFA, permeabilized, and blocked in PBS containing 0.3% Triton X-100 and 30% goat serum. Fluorescently labeled secondary antibody was added to the cells in the same buffer for 1 h at RT before cells were washed and mounted on coverslips. For dextran uptake experiments, HeLa cells were starved for 2 h in Optimem and then incubated with 250 μg/mL TxRed-Dextran (3,000 MW, lysine-fixable) in Optimem for 4 h at 37 °C. Cells were washed three times with ice-cold PBS, one time with ice-cold 500 mM NaCl containing PBS, two times with ice-cold PBS, before being fixed for 1 h with 4% PFA.

For dynasore-mediated inhibition experiments, HeLa cells were preincubated with 400 μM dynasore in Optimem for 6 h at 37 °C. Cells were either methanol-fixed immediately and processed for indirect immunofluorescence microscopy using TfR- and Gadkin-specific antibodies or subjected to Tf uptake assays.

Affinity Chromatography and Immunoprecipitation Experiments in Detail. GST-fusion proteins were expressed in *E. coli* and purified from benzonase-treated bacterial lysates (to remove possible nucleic acid contaminants) using GST-bind resin (Novagen) according to standard protocols. Adult rat brain tissue was homogenized in 320 mM sucrose, 4 mM HEPES, pH 7.4, supplemented with 1 mM PMSF and mammalian protease inhibitor mixture (Sigma), and the postnuclear supernatant was extracted with 1% Triton X-100. The buffer concentration in the extract was adjusted to 20 mM HEPES, pH 7.4, 2 mM $MgCl_2$, and 80–100 mM KCl or NaCl. Cleared protein extracts were prepared by ultracentrifugation at 180,000 $\times$ *g*. Protein extracts from cultured mammalian cells were prepared in lysis buffer containing 20 mM HEPES, pH 7.4, 2 mM $MgCl_2$, 100 mM NaCl, and 1% Triton X-100 supplemented with 1 mM PMSF and mammalian protease inhibitor mixture (Sigma). Cell extracts were cleared by ultracentrifugation. For affinity purifications, GST-fusion proteins were incubated with rat brain extracts with

a protein concentration of 4–6 mg/mL for 2 h at 4 °C. Alternatively, to demonstrate direct binding, proteins were synthesized in vitro by coupled transcription/translation (TNT Coupled Reticulocyte Lysate System; Promega) and offered to GST-fusion proteins. After extensive washes, bound proteins were eluted in sample buffer. For immunoprecipitation experiments, antibodies were immobilized on Protein A/G agarose beads (Santa Cruz Biotechnology) and incubated with either rat brain extracts as above or with cleared cell extracts (total protein concentration: 0.5–1 mg/mL) for 4 h at 4 °C under gentle agitation. Beads were washed extensively and eluted with sample buffer. Samples were analyzed by SDS-PAGE and immunoblotting.

For direct binding assays immobilized GST fusion proteins (20 μg) were incubated for 1 h at 4 °C with 20 μg of recombinant His_6-tagged Gadkin proteins and washed extensively, complexes were analyzed by SDS-PAGE with Coomassie blue staining.

For immunoprecipitation from V1 fraction, eight P0–P3 rat brains were homogenized in 5 volumes ice-cold homogenization buffer (0.32 M sucrose, 10 mM HEPES, pH 7.4, 5 mM NEM, 5 mM EDTA). The homogenate was centrifuged for 8 min at 12,000 $\times$ *g*. The postnuclear-supernatant was centrifuged for 40 min at 40,000 $\times$ *g*. The resulting supernatant was centrifuged for 1 h at 100,000$\times$ *g*. The resulting V1 pellet was solubilized in 900 μL IP buffer [75 mM NaCl, 5 mM EDTA, 5 mM NEM, 10 mM CHAPS, 50 mM Tris-HCl, pH 7.5, supplemented with 1 mM PMSF and mammalian protease inhibitor mixture (Sigma)] for 1 h at 4 °C. The solubilized V1 fraction was centrifuged for 15 min at 180,000 $\times$ *g* to pellet unsolubilized material. Cleared V1 fraction (1.5 mg, 2 μg/μL) were incubated with Protein A/G agarose beads (Santa Cruz Biotechnology) coupled to Gadkin-specific antibodies, KHC-specific antibodies, or nonspecific rabbit immunoglobulins (control IP) for 3 h at 4 °C on a rotator. Beads were washed extensively and eluted with sample buffer. Samples were analyzed by SDS-PAGE and immunoblotting.

Biotinylation. On day 5 twice siRNA-transfected HeLa cells (days 1 and 3) were washed two times with ice-cold PBS and incubated with 0.25 mg/mL biotin in PBS for 30 min at 4 °C. Cells were washed two times for 5 min with ice-cold 50 mM glycin 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 $MgCl_2$, 1% Triton X-100, supplemented with 1 mM PMSF and mammalian protease inhibitor mixture (Sigma). Lysates were cleared by centrifugation. Streptavidin-beads were incubated with cell lysates for 60 min at 4 °C on a rotator. Beads were washed extensively, and bound protein was eluted with sample buffer. Samples were analyzed by SDS-PAGE and immunoblotting.

1. Zhai RG, et al. (2001) Assembling the presynaptic active zone: A characterization of an active one precursor vesicle. *Neuron* 29:131–143.

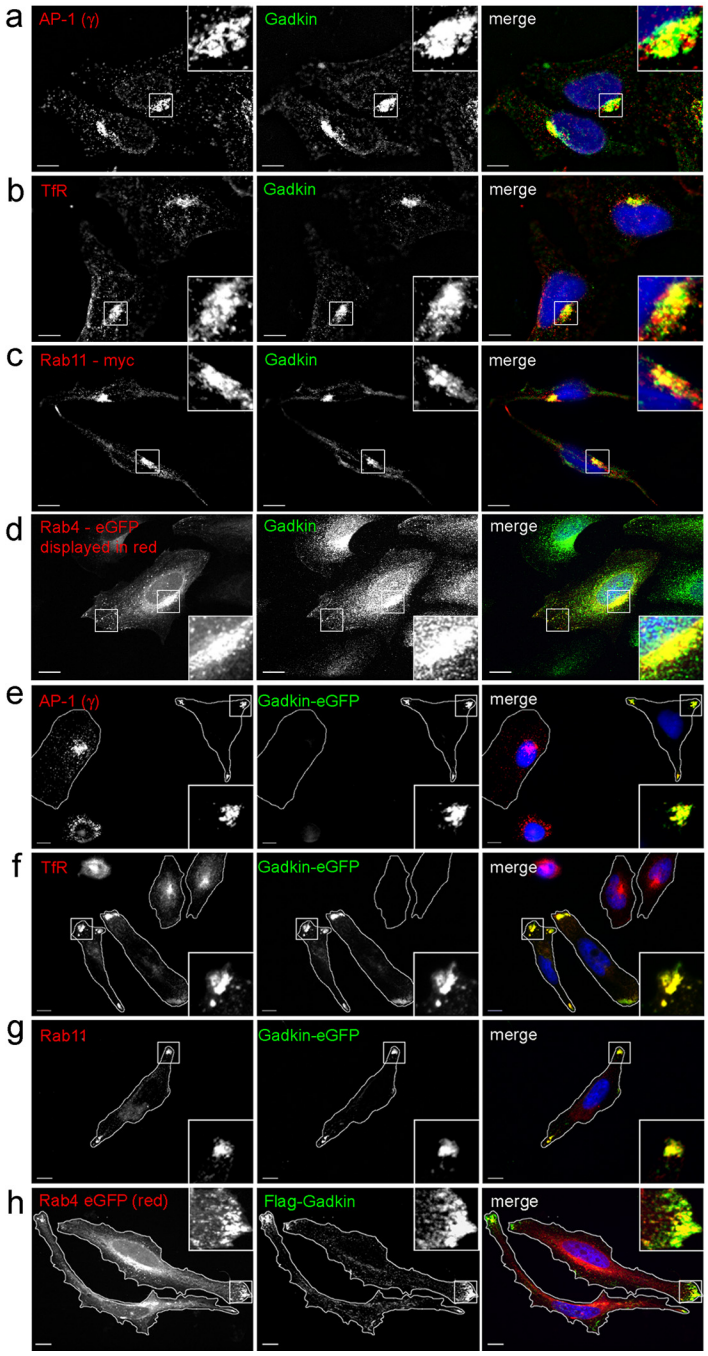


Fig. S1. Gadkin is associated with AP-1- and TfR-containing endosomal organelles and redistributes them to the periphery upon overexpression. (*a*–*d*) Colocalization of endogenous Gadkin with AP-1, TfR, Rab11-c-myc, and Rab4-eGFP in HeLa cells. Nontransfected (*a* and *b*), Rab11-c-myc (*c*), or Rab4-eGFP (*d*) expressing HeLa cells were fixed and analyzed by indirect immunofluorescence microscopy with antibodies against AP-1γ (*a*, red), TfR (*b*, red), c-myc (*c*, red), and Gadkin (green). DAPI-stained nuclei are shown in blue. Images *a*–*c* were processed by deconvolution and are shown at low overall intensity to be able to discern structures at the intensely stained TGN area, whereas image *d* is depicted at higher intensity to visualize peripheral vesicles. *Insets*, 3× magnified views of boxed areas. (Scale bar, 10 μm.) For line profiles of fluorescence intensities see Fig. 2*A*. For enlargement of second *Inset* in (*d*) see Fig. 2*B*. (*e*–*h*) Overexpression of Gadkin-eGFP or Flag-Gadkin leads to relocalization of AP-1- and TfR-containing Rab11- and Rab4-positive endosomal vesicles toward the cell periphery. HeLa cells expressing Gadkin-eGFP or Flag-Gadkin were fixed 24 h posttransfection and analyzed by indirect immunofluorescence microscopy with antibodies against AP-1γ (*e*, red), TfR (*f*, red), and Rab11 (*g*, red), whereas Rab4 was detected via its eGFP-tag (*h*, displayed in red). Cell boundaries are outlined in white. DAPI-stained nuclei are shown in blue. Images *e*–*g* were processed by deconvolution. *Insets*, 3× magnified views of boxed areas. (Scale bar, 10 μm.) Merged images are also shown in Fig. 3*A*.

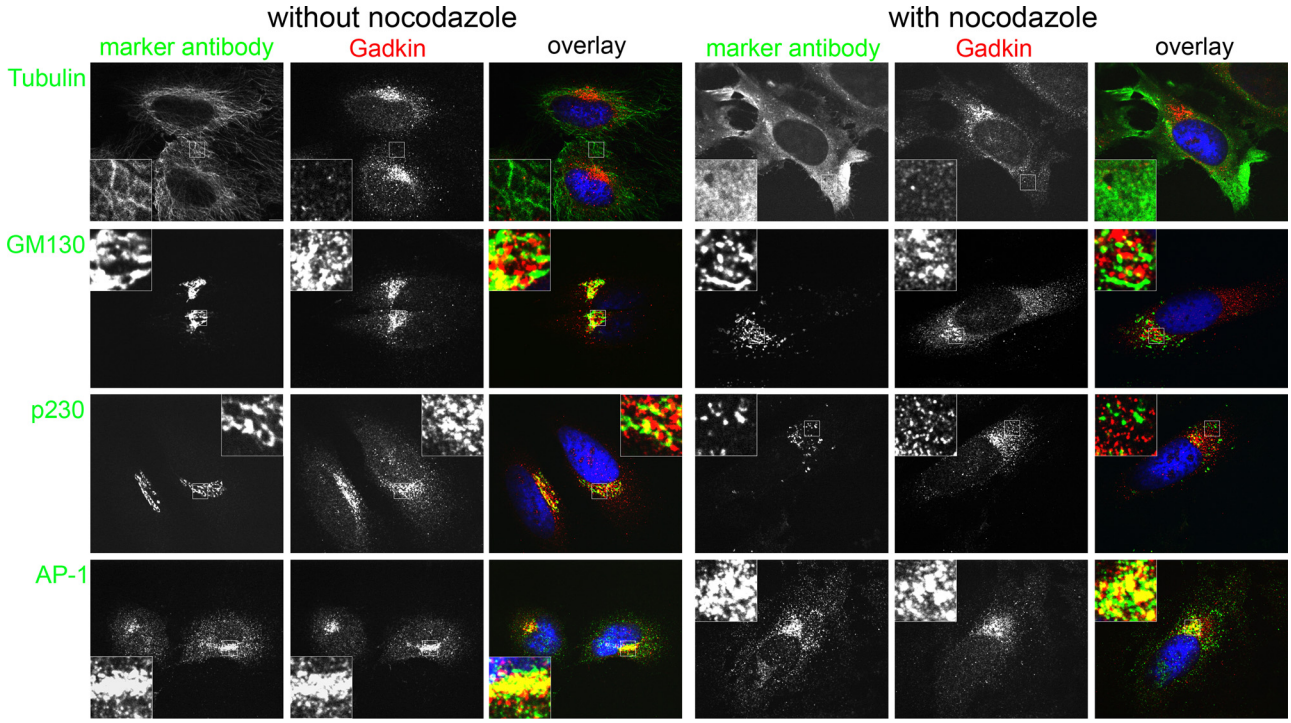


Fig. S2. Gadkin staining segregates from Golgi and TGN markers upon microtubule depolymerization by nocodazole. HeLa cells were treated for 1 h with 30 μM nocodazole or left untreated, fixed, and processed for indirect immunofluorescence microscopy using antibodies against endogenous Gadkin in conjunction with antibodies against tubulin, GM130, p230, or AP-1. Images were taken by a spinning disc confocal microscope.

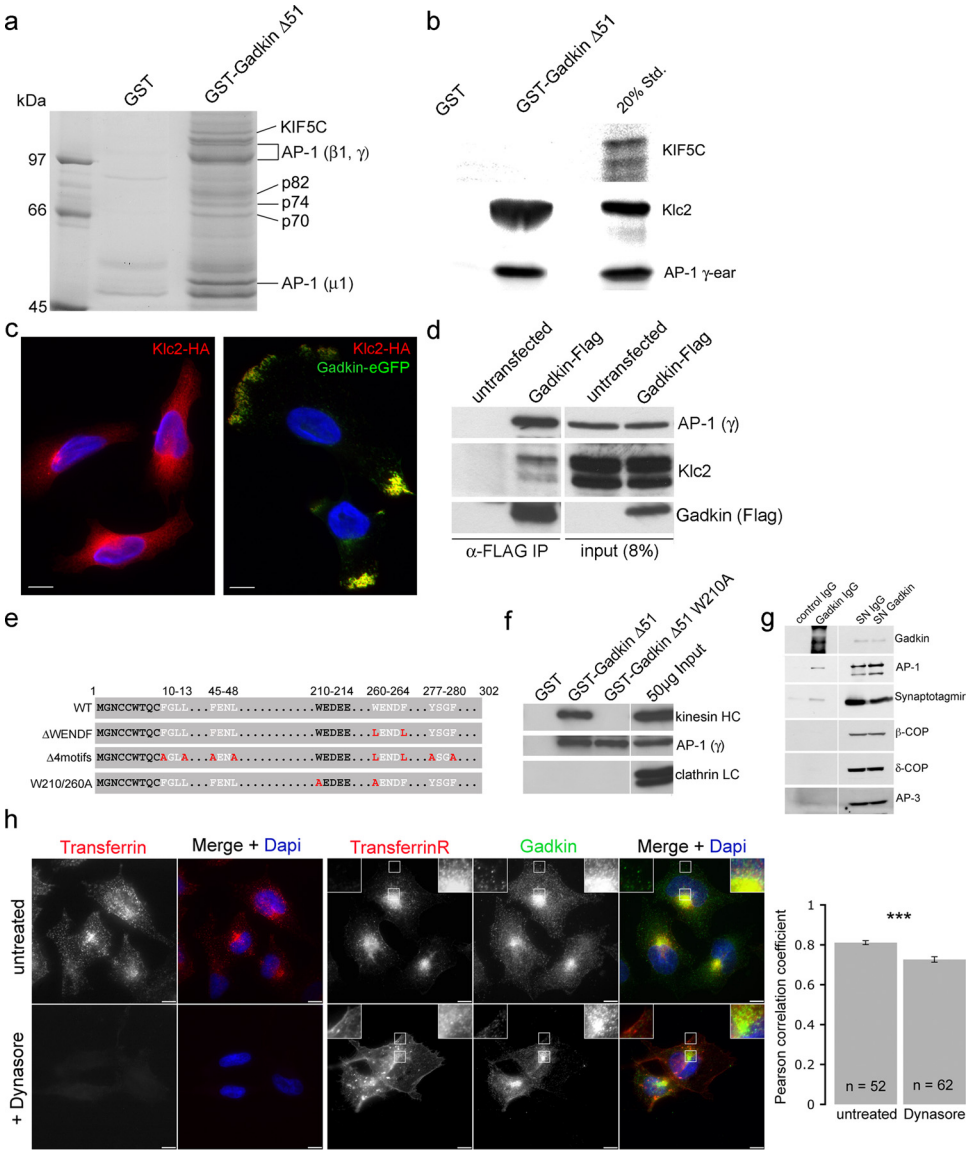


Fig. S3. Association of Gadkin with kinesin KIF5 and TfR positive EVs. (*a*) Coomassie blue-stained gel of material isolated by affinity-chromatography from 12.5 mg rat brain extract using 1.5 mg GST or GST-fused Gadkin Δ51 cross-linked to glutathione Sepharose beads as baits. The bands labeled KIF5C and AP-1 (γ, β1, μ1) were identified by MALDI-TOF mass spectrometry. (*b*) Gadkin directly binds to kinesin light chain (Klc2). Kinesin heavy (KIF5C) or light chains (Klc2) or the ear domain of AP-1γ (as positive control) were synthesized by transcription/translation in vitro and offered to 12.5 μg GST or GST-Gadkin immobilized on beads. Twenty percent Std, one-fifth of the total amount of in vitro translated protein used for affinity chromatography. Samples were analyzed by SDS-PAGE followed by autoradiography. (*c*) Klc2-HA is recruited to peripherally accumulated endosomal compartments in Gadkin-eGFP transfected HeLa cells. HeLa cells were transfected with expression plasmids for Klc2-HA either alone or together with Gadkin-eGFP. Twenty-four hours posttransfection, cells were fixed and analyzed by indirect immunofluorescence microscopy with monoclonal antibodies against the HA epitope tag (red). DAPI-stained nuclei are shown in blue. (Scale bar, 10 μm.) Images were deconvolved. (*d*) Endogenous AP-1 and Klc2 associate with Gadkin in transfected fibroblasts. Triton X-100 extracted lysates (300 μg total protein) prepared from FLAG-Gadkin-expressing Cos7 cells were subjected to anti-FLAG immunoprecipitation (α-FLAG IP). Input, 25 μg cell lysate used for immunoprecipitation. (*e*) Schematic diagram of the primary sequence of WT Gadkin and mutants used in this study. AP-1 binding motifs are printed in white, mutations indicated in red. (*f*) Immunoblot analysis of proteins isolated by affinity chromatography from 1.6 mg rat brain extract using 20 μg GST-tagged Gadkin (Δ51) and point mutants thereof. Samples were analyzed by immunoblotting for kinesin heavy chain (HC), AP-1, and clathrin light chains (LC). (*g*) Immunoblot analysis of immunoisolated Gadkin-containing organelles analogous to the ones shown in Figs. 2*E* and 5C. Input, 20 μg starting material. Samples were analyzed by SDS-PAGE and immunoblotting for Gadkin, AP-1, Synaptotagmin-1, β- and δ-COP, and AP-3-μ3. Whereas AP-1 is clearly enriched on the Gadkin-containing organelles (as shown in Fig. 2*E*), other coat proteins like β- and δ-COP, as well as AP-3, could not be shown to be enriched. Synaptotagmin-1, a vesicular cargo, is detected on the Gadkin-containing organelles. (*h*) Dynasore-mediated inhibition of TfR endoctosis. HeLa cells were incubated with the small molecule dynamin inhibitor dynasore for 6 h. Afterward, cells were either allowed to internalize Tf (left four images) or immunostained with antibodies against TfR and Gadkin (right six images). DAPI-stained nuclei are shown in blue. *Insets*, 3× boxed area. (Scale bars, 10 μm.) (*Right*) Pearson correlation coefficient depicting the degree of colocalization between Gadkin and TfR in mock- or dynasore-treated cells. Data are depicted as mean values ± SEM (two independent experiments).

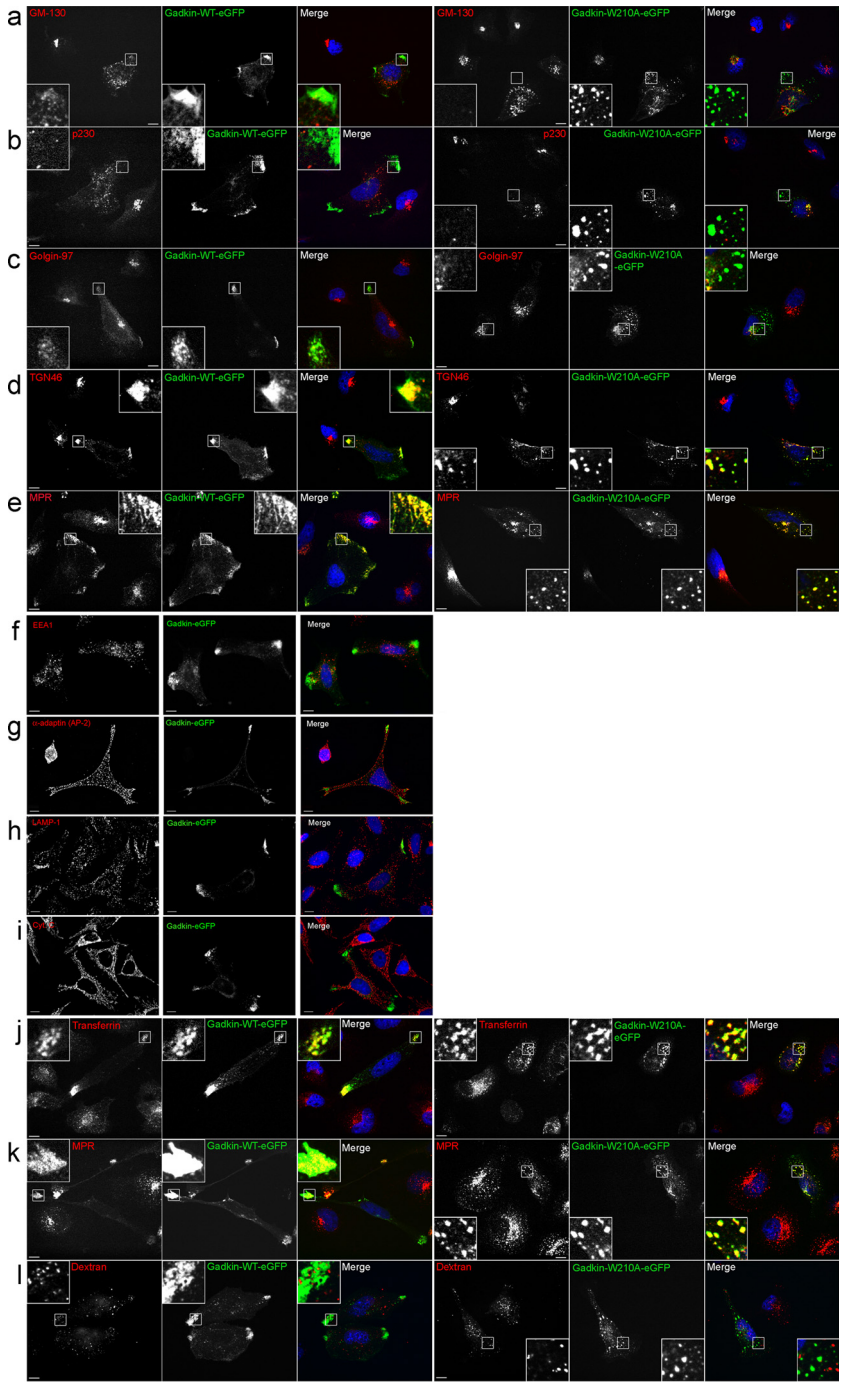


Fig. S4. Effect of Gadkin-eGFP WT and its kinesin binding mutant (W210A) expression on the localization of different organelle marker proteins and endocytosed material. (*a*–*i*) HeLa cells expressing Gadkin-eGFP WT or W210A (in green) were fixed 24 h posttransfection and analyzed by indirect immunofluorescence microscopy with antibodies against different marker proteins (in red). Gadkin-eGFP WT and W210A substantially overlap with proteins that cycle through the TGN such as TGN46 and the mannose-6-phosphate receptor MPR (*d* and *e*), but hardly with Golgi markers like GM-130 (*a*) or noncycling TGN proteins like p230 and Golgin-97 (*b* and *c*). There is also no substanial colocalization of Gadkin-eGFP WT with marker proteins for early endosomes (EEA1; *f*), clathrin-coated pits (AP-2; *g*), late endosomes/lysosomes (LAMP-1; *h*), or mitochondria (Cytochrom C; *i*). (*j* and *k*) HeLa cells expressing Gadkin-eGFP WT or W210A (in green) for 24 h were allowed to internalize Alexa^{568}-Tf (*j*), MPR specific antibodies (*k*), or TxRed-dextran (*l*). Whereas endocytosed Tf and MPR-specific antibodies colocalize to a large degree with Gadkin-eGFP WT or W210A, the fluid phase marker dextran is hardly found in Gadkin-positive structures. Pictures were acquired on a spinning disc confocal microscope. DAPI-stained nuclei are shown in blue. *Insets*, 3× boxed area. (Scale bars, 10 μm.)

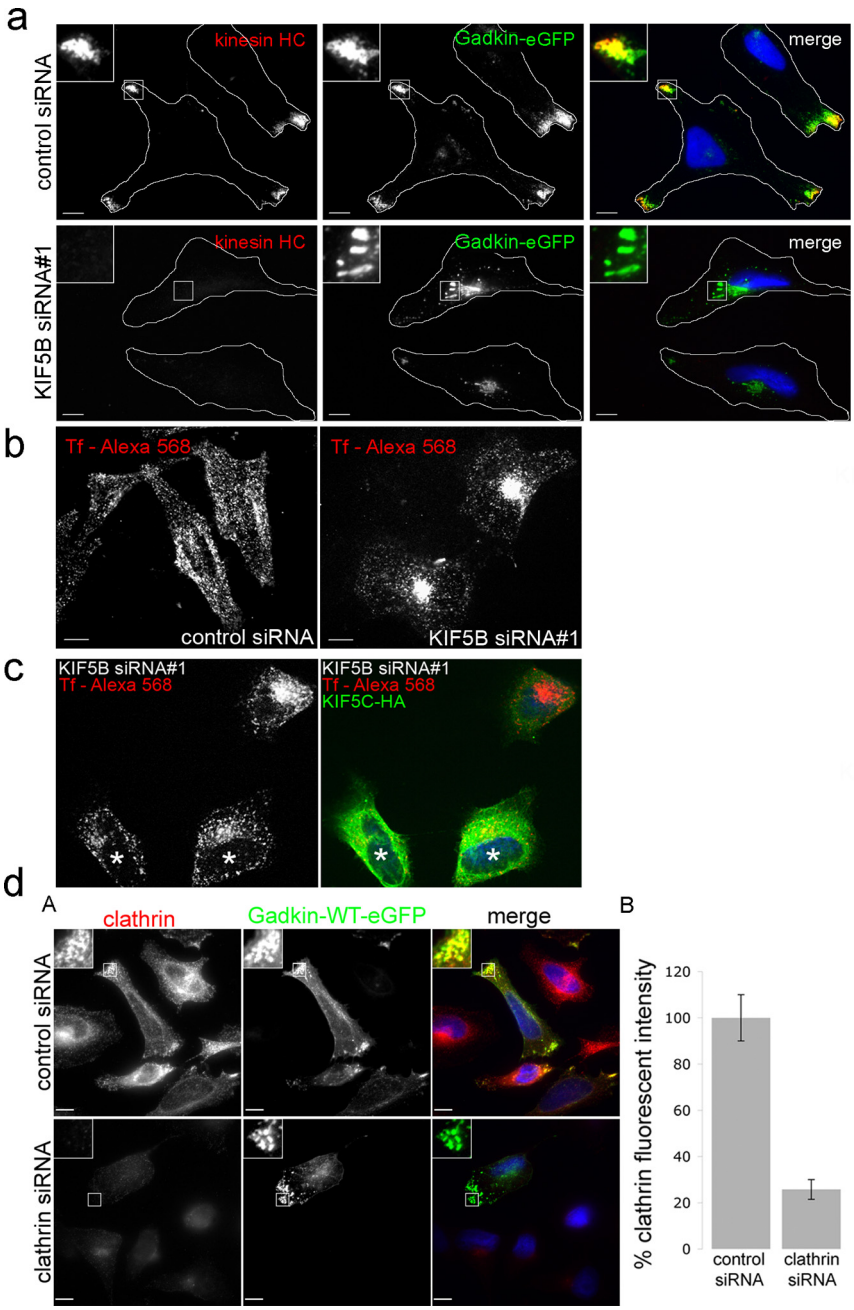


Fig. S5. Kinesin KIF5B but not clathrin is required for Gadkin-mediated translocation of EVs. (*a*) Localization of Gadkin-eGFP-containing endosomes in control siRNA or anti-KIF5B siRNA-treated HeLa cells. HeLa cells were transfected twice (days 1 and 4) with control siRNA *(Top)* or siRNA against kinesin heavy chain KIF5B (*Bottom*). Expression plasmid for Gadkin-eGFP was cotransfected on day 4. On day 5, cells were fixed and analyzed by indirect immunofluorescence microscopy using antibodies against KIF5 (red). Cell boundaries are outlined in white. *Inset*, 3-fold magnified image of boxed area. DAPI-stained nuclei are shown in blue. (Scale bar, 10 μm.) Images were processed by deconvolution. For control of knockdown efficiency and quantification of Gadkin distribution, see Fig. 4 *C* and *D*. (*b*) Distribution of Tf-containing perinuclear endosomes in HeLa cells after RNAi-mediated knockdown of kinesin KIF5B heavy chain (as described in *a*). Shown are still images extracted from live-cell imaging of REVs labeled with Alexa^{568}-Tf by spinning disc confocal microscopy. (Scale bar, 10 μm.) For line profiles of Tf intensity see Fig. 4*E*. (*c*) Spinning disc confocal images of the distribution of Alexa^{568}-Tf containing perinuclear endosomes in HeLa cells after RNAi-mediated knockdown of kinesin KIF5B heavy chain (as described in *a*) and concomitant transfection of siRNA-resistant KIF5C heavy chain (green) to rescue kinesin 1. (*d*) Gadkin-WT-eGFP causes peripheral accumulations in the absence of clathrin. (*d Left*) HeLa cells were transfected twice (days 1 and 4) with control siRNA *(Top)* or siRNA against clathrin heavy chain (*Bottom*). Expression plasmid for Gadkin-eGFP was cotransfected on day 4. On day 5, cells were fixed and analyzed by indirect immunofluorescence microscopy using antibodies against clathrin heavy chain (red). *Inset*, 3-fold magnified image of boxed area. DAPI-stained nuclei are shown in blue. (Scale bar, 10 μm.) Images were acquired on a spinning disc confocal microscope. (*d Right*) The efficiency of clathrin siRNA was quantified by measuring the intensity of clathrin staining in knockdown versus control cells. Data are depicted as normalized mean values ± SEM (control $n = 42$ cells, clathrin siRNA $n = 50$ cells; two independent experiments).

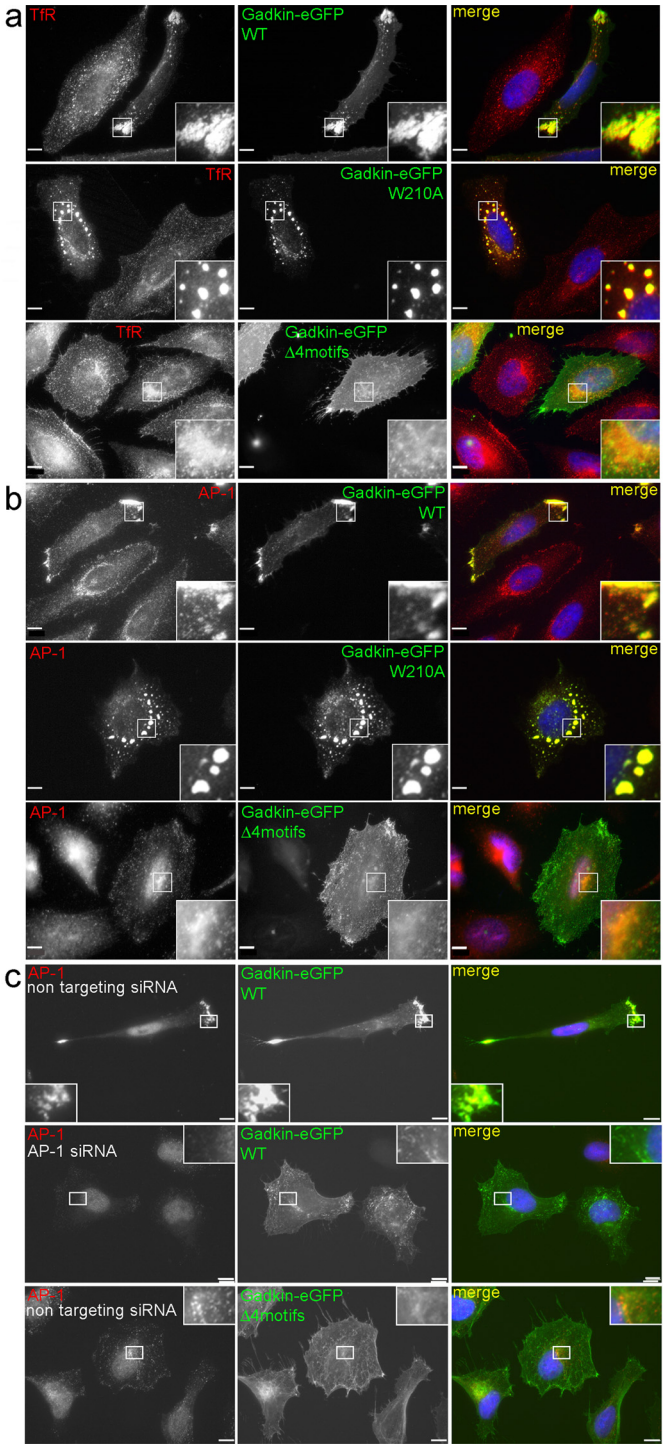


Fig. S6. Gadkin induced peripheral accumulation of TfR depends on the ability of Gadkin to directly associate with both AP-1 and kinesin. (*a* and *b*) HeLa cells expressing Gadkin-eGFP WT, the kinesin-binding-deficient (W210A), or the AP-1-binding-deficient (Δ4motifs) mutant were fixed 24 h posttransfection and analyzed by indirect immunofluorescence microscopy with antibodies against endogenous TfR (*a*, red) or AP-1γ (*b*, red). Blue, DAPI-stained nuclei. *Insets*, 4× magnified view of boxed areas. (Scale bar, 10 μm.) For quantifications of TfR and Gadkin distribution see Fig. 4*A*. (*c*) Knockdown of AP-1 prevents the peripheral accumulation of Gadkin. HeLa cells treated with siRNA against AP-1γ or a nontargeting siRNA were transfected with expression plasmids encoding Gadkin-eGFP WT or Δ4motifs. Cells were fixed 24 h posttransfection and analyzed by indirect immunofluorescence microscopy with antibodies against AP-1γ (red). Blue, DAPI-stained nuclei. *Insets*, 4× magnified view of boxed areas. (Scale bar, 10 μm.)

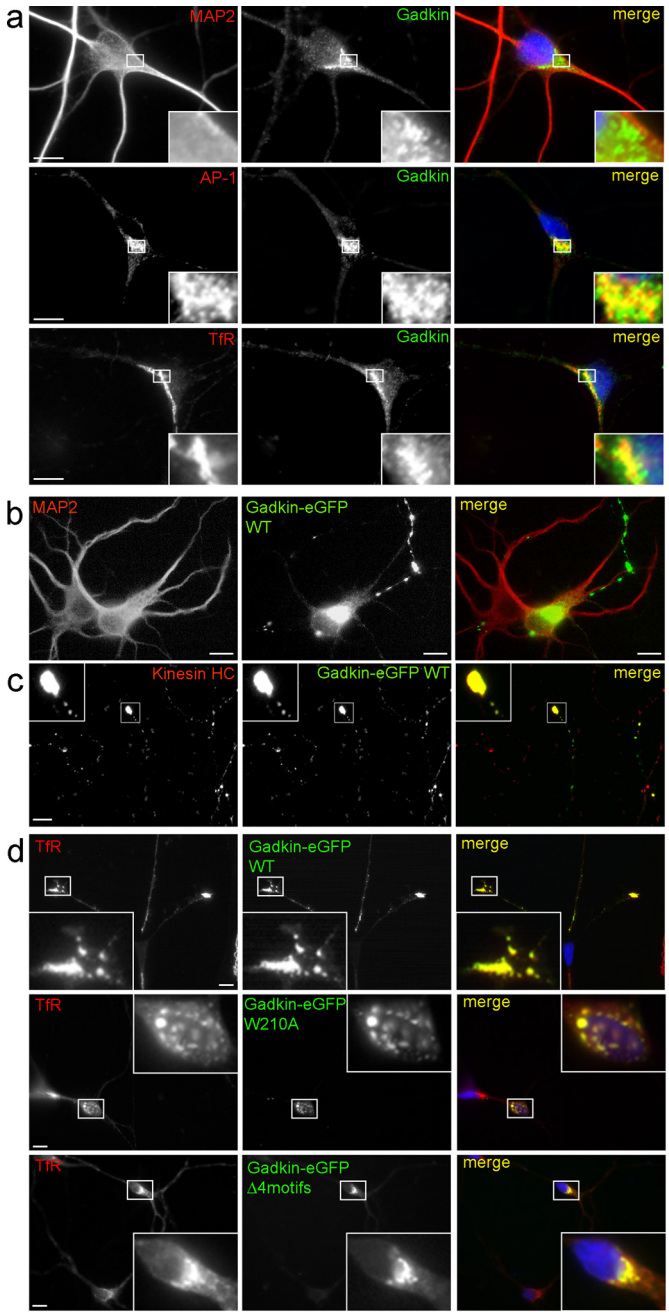


Fig. S7. Gadkin redirects TfR and AP-1 to peripheral structures in primary hippocampal neurons. (*a*) Endogenous Gadkin (green) localizes to the soma (identified by costaining with the somatodendritic marker MAP2) where it colocalizes with AP-1γ (red) and TfR (red) in perinuclear endosomes. Primary hippocampal neurons prepared from E18 embryonic rats (8 DIV) were fixed with 4% PFA and analyzed by indirect immunofluorescence microscopy using antibodies against MAP2, AP-1γ, TfR, and Gadkin. Blue, DAPI-stained nuclei. *Insets* show 4$\times$ magnified views of boxed areas. (Scale bar, 10 μm.) Images were processed by deconvolution. (*b*) Primary hippocampal neurons prepared from E18 embryonic rats (10 DIV) were transfected with Gadkin-eGFP, fixed, and analyzed by indirect immunofluorescence microscopy. Gadkin-eGFP (green) localizes to MAP2 (red) negative axonal processes. (Scale bar, 10 μm.) Images were processed by deconvolution. (*c*) Primary hippocampal neurons prepared from E18 embryonic rats (10 DIV) were transfected with Gadkin-eGFP (neuronal splice variant) 24 h before fixation with 4% PFA and analyzed by indirect immunofluorescence microscopy. Overexpressed Gadkin-eGFP (green) redirects endogenous kinesin HC (red) (as well as TfR and AP-1) to endosomal structures within axons and axonal growth cones. *Insets*, 3$\times$ magnified view of boxed areas. (Scale bar, 10 μm.) Images were deconvolved. (*d*) Mutating the kinesin or AP-1 binding sites of Gadkin abolishes its ability to redirect TfR to peripheral structures. Primary hippocampal neurons prepared from E18 embryonic rats (8 DIV) expressing Gadkin eGFP WT, the kinesin- (W210A), or the AP-1-binding-deficient mutant (Δ4motifs) were fixed 24 h posttransfection and processed for indirect immunofluorescence microscopy using antibodies against TfR (red). Plasmalemmal mistargeting of Gadkin eGFP (Δ4motifs) is less pronounced compared with the phenotype seen in HeLa cells. Blue, DAPI-stained nuclei. *Insets*, 4$\times$ magnified view of boxed areas. (Scale bar, 10 μm.) White arrows point to cell body staining of Gadkin, yellow arrows point to axonal staining.

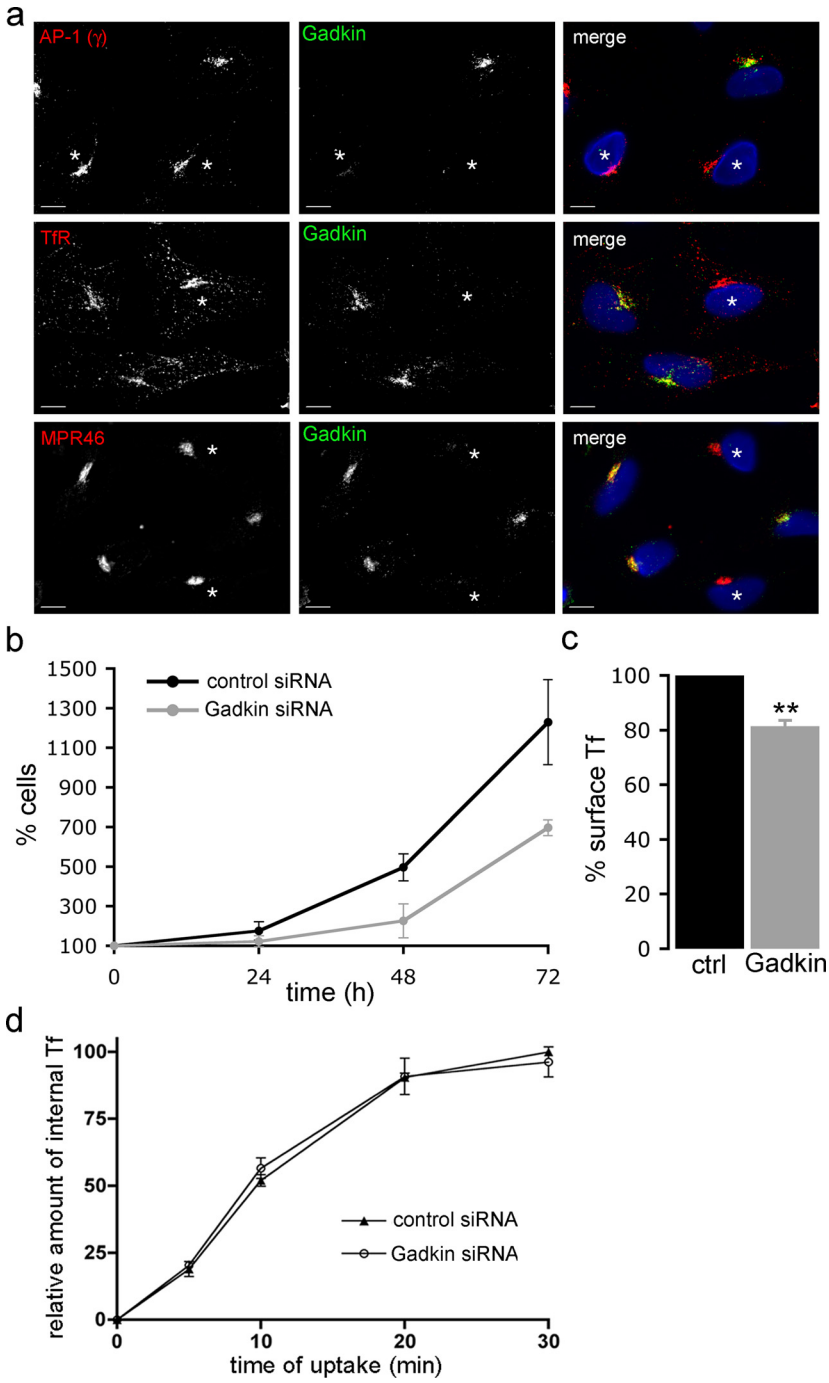


Fig. S8. Gadkin depleted cells display reduced rates of cell division and mildly reduced levels of cell surface TfRs. (*a*) Steady state distribution of AP-1, TfR, and MPR46 in Gadkin-deficient HeLa cells. HeLa cells were transfected twice (days 1 and 3) with either control siRNA or siRNA oligonucleotides directed against Gadkin. The two pools of cells were mixed before fixation on day 5 and analyzed by indirect immunofluorescence microscopy and deconvolution. Shown is the localization of Gadkin (green) and AP-1 (*c*, red), TfR (*d*, red), or MPR46 (*e*, red) in Gadkin knockdown (identified by lack of Gadkin staining and marked with an asterisk) or nontransfected control cells. DAPI-stained nuclei are shown in blue. (Scale bar, 10 μm.) (*b*) RNAi-mediated knockdown of endogenous Gadkin impairs growth of Cos7 cells. Cos7 cells were transfected twice (days 1 and 3) with either control siRNA (a nontargeting mutant version of the Gadkin-specific siRNA harboring four nucleotide exchanges) or siRNA oligonucleotides directed against Gadkin. On day 5, equal cell numbers of control or Gadkin siRNA-transfected cells were plated for counting after 24, 48, and 72 h. (c) RNAi-mediated knockdown of endogenous Gadkin reduces surface-bound Tf levels by ≈20%. HeLa cells were transfected on days 1 and 3 with either control siRNA or siRNA oligonucleotides directed against Gadkin. On day 5, cells were incubated with ^{125}I-Tf for 30 min at 4 °C. After extensive washing, ^{125}I-Tf remaining bound at the cell surface was analyzed by liquid scintillation counting. (*d*) RNAi-mediated knockdown of endogenous Gadkin does not affect the rate of Tf internalization. HeLa cells were transfected on days 1 and 3 with control siRNA (mutant version of Gadkin-specific siRNA with four mismatches) or anti-Gadkin siRNA oligonucleotides. On day 5, cells were analyzed for their ability to internalize ^{125}I-Tf. The amount of ^{125}I-Tf internalized into control cells after 30 min was set to 100%. Data represent mean values ± SEM (n = 4 values for each data point; two independent experiments). For control of knockdown efficiency see *Inset* in Fig. 1*C*.

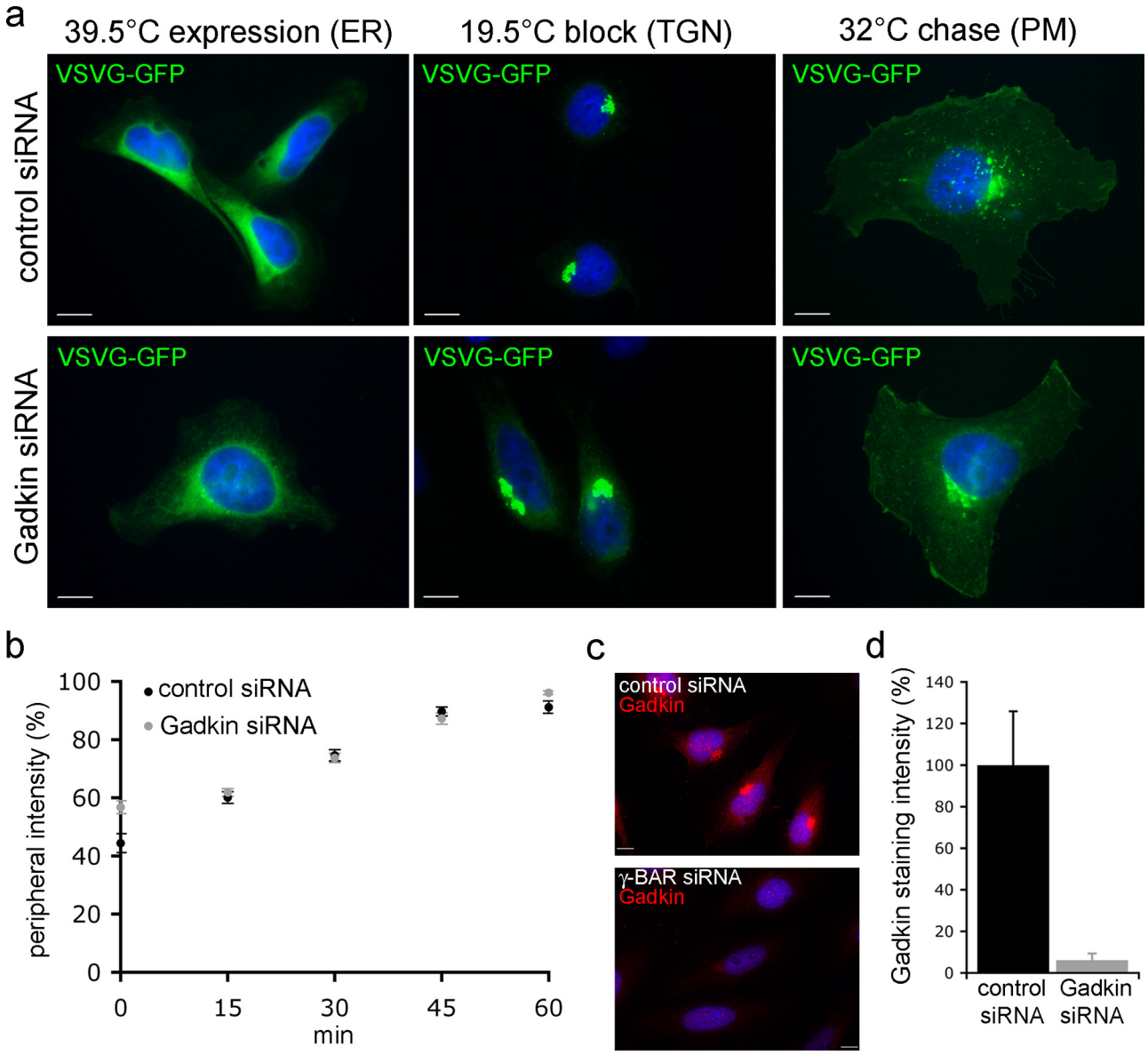


Fig. S9. Gadkin depletion does not affect vesicular stomatitis virus (VSVG) export. (*a*) HeLa cells were transfected twice with siRNAs against Gadkin or nontargeting siRNAs (control). At the time of the second transfection, a plasmid encoding the glycoprotein of the ts045 mutant of VSVG containing a hydrophilic spacer region (denoted SP) fused to GFP (VSVG-SP-GFP) was cotransfected. Cells were incubated overnight at 39.5 °C to drive expression and accumulation of VSVG in the endoplasmic reticulum (ER). Cells were treated with 10 μg/mL cycloheximide and shifted to 19.5 °C for 60 min to block protein exit from the Golgi and induce accumulation at the TGN. Afterward, cells were shifted to the permissive temperature of 32 °C (chase) for up to 60 min to allow Golgi exit and trafficking to the plasma membrane (PM). After fixation, VSVG-SP-GFP localization was analyzed by fluorescence microscopy (green). DAPI-stained nuclei are shown in blue. (Scale bar, 10 μm.) (*b*) Quantification of VSVG fluorescence in the cell periphery of control and Gadkin knockdown cells before the shift to 32 °C (0 min) and after 15 min, 30 min, 45 min, and 60 min chase. No overt differences in VSVG trafficking were seen between Gadkin-depleted vs. control siRNA-treated cells. (*c*) Control siRNA and Gadkin siRNA-transfected cells were stained with antibodies against Gadkin to control for the knockdown efficiency. (*d*) Quantification of the Gadkin staining intensity in control and Gadkin knockdown cells. Given are mean values ± SEM.

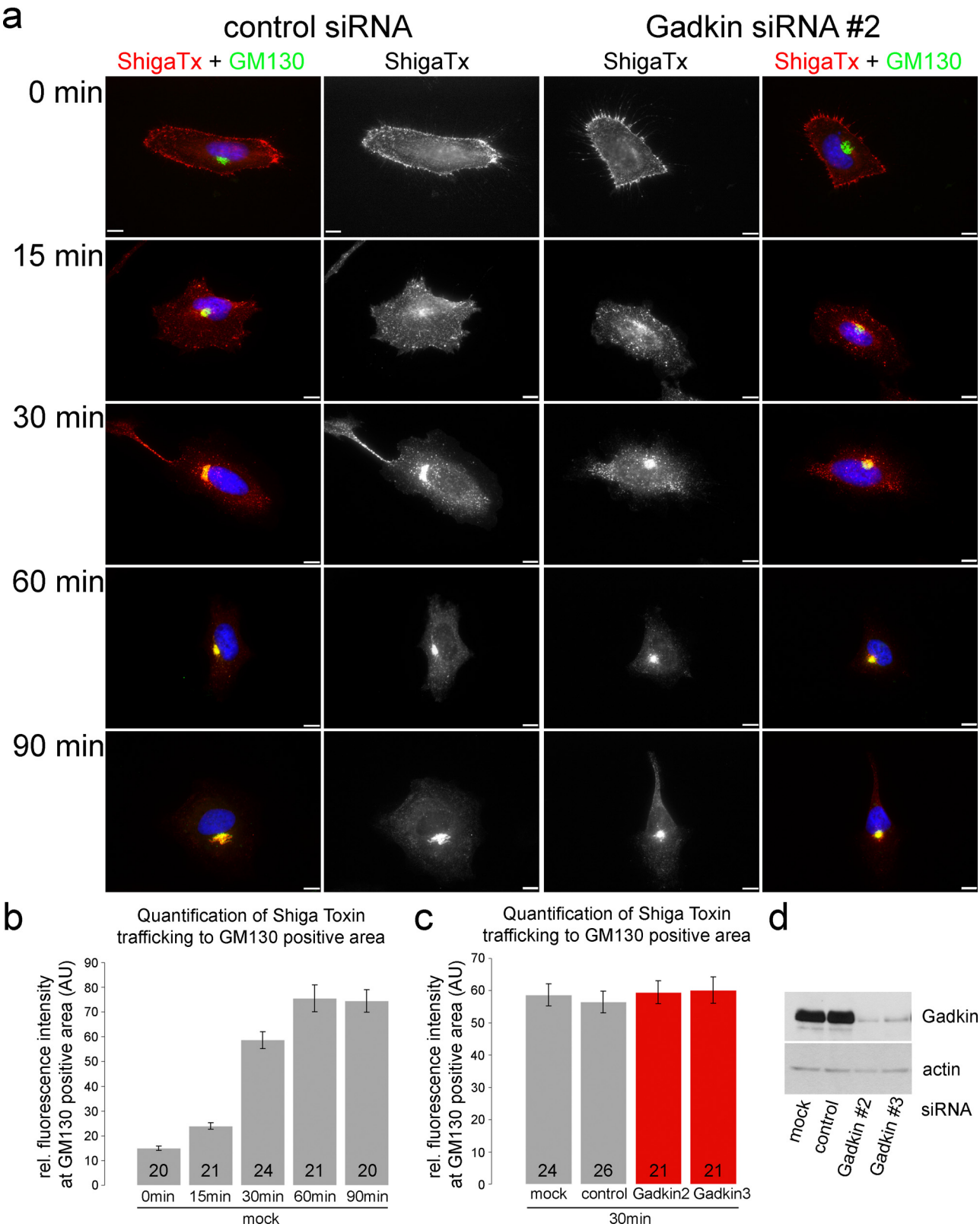
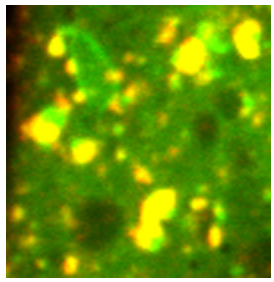
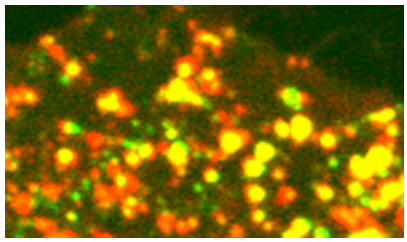


Fig. S10. Retrograde trafficking of Shiga toxin B is not perturbed in Gadkin knockdown cells. (*a*) Control or Gadkin siRNA treated cells were incubated with the Cy3-labeled retrograde cargo Shiga toxin B (StxB). Trafficking of Cy3-StxB from the plasma membrane via an endosomal compartment to the Golgi was monitored by fluorescence microscopy at the indicated time points. To delineate the Golgi area, cells were co-stained with an antibody against GM130. DAPI-stained nuclei are shown in blue. (Scale bars: 10 μM.) (*b*) Quantification of Cy3-StxB fluorescence intensity at the Golgi at different time points. Further quantifications (*c*) were done using the 30 min time point. (*c*) Quantification of Cy3-StxB accumulation within the Golgi in mock (no siRNA), control (scrambled siRNA), or Gadkin knockdown cells (Gadkin siRNA #2, #3). Retrograde trafficking of Cy3-StxB is not perturbed in the absence of Gadkin. (*d*) Knockdown of Gadkin was monitored by immunoblotting using Gadkin specific antibodies.



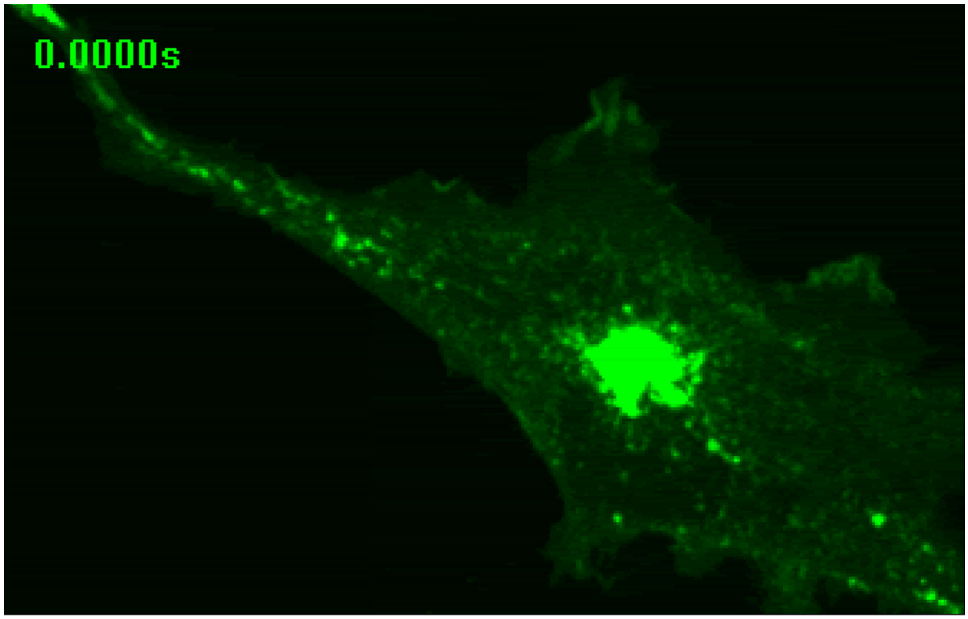
Movie S1. Time-lapse movie recorded by live-cell spinning disc confocal microscopy illustrating colocalization of motile Gadkin-eGFP-containing organelles with Tf. HeLa cells were transfected with Gadkin-eGFP. Twenty hours posttransfection, cells were starved for 1 h at 37 °C in serum-free medium. Cells were then incubated for 1 h at 37 °C in 50 μg/mL Alexa^{568}-Tf containing starving medium. Tf-loaded cells were washed once and imaged at 37 °C and 5% CO_2. Images of the green channel (488 nm, Gadkin-eGFP) and the red channel (568 nm, Alexa^{568}-Tf) were taken every 1.30 s. Fourteen consecutive images are displayed as merge between the two channels with colocalization appearing in yellow. Because of the high exposure time needed for image acquisition and the speed of vesicular movement, the two colors do not move completely synchronously. Images were acquired on a Perkin-Elmer spinning disc confocal microscope and processed by ImageJ. Selected still images are shown in Fig. 3*C*.

Movie S1 (AVI)



Movie S2. Time-lapse movie recorded by live-cell spinning disc confocal microscopy illustrating colocalization of motile Gadkin-mRFP-containing organelles with Rab4-eGFP. HeLa cells were transfected with Gadkin-mRFP and Rab4-eGFP. Twenty hours posttransfection, cells were imaged at 37 °C and 5% CO_2. Images of the green channel (488 nm, Rab4-eGFP) and the red channel (568 nm, Gadkin-mRFP) were taken every 1.30 s. Fourteen consecutive images are displayed as merge between the two channels with colocalization appearing in yellow as described in the legend to Movie S1. Selected still images are shown in Fig. 3*B*.

Movie S2 (AVI)



Movie S3. Time-lapse movie recorded by live-cell spinning disc confocal microscopy illustrating the dynamics of Gadkin-eGFP-containing organelles in transfected Cos7 cells. Images were acquired at 10 Hz on a Perkin-Elmer spinning disc confocal microscope.

Movie S3 (MOV)