Supplemental Materials and Methods:

DNA constructs and Molecular cloning
The following expression constructs were kind gifts: HA-tagged ARF6Q67L, ARF6T27N and ARF6WT (Julie Donaldson, NIH, Bethesda); GFP-tagged EFA6A (Philippe Chavrier, Institut Curie, Paris); mRFP and mCherry (Roger Tsien, HHMI-USCD, California); RAB5Q79L (Marino Zerial, Max Planck Institute of Molecular Cell Biology and Genetics, Dresden); VSVG-tagged FERM (Janis Burkhardt, University of Pennsylvania, Philadelphia); GFP-tagged CD63 (Judith Klumperman, University Medical Center, Utrecht); GFP-tagged Flotillins (Lawrence Rajendran, ETH, Zurich); Mouse TLN (pBlue-mTLN, Yoshihiro Yoshihara, RIKEN Brain Science Institute, Japan; pSG5-mTLN: subclone d from pBlue-mTLN), HA-tagged ARF6T27N, ARF6WT, and ARF6Q67L in pXS expression vector were subcloned in pcDNA3.1+zeo. The fast cycling mutant ARF6T157A was constructed by site-directed mutagenesis of HA-tagged ARF6WT (Quickchange, Stratagene). Cerulean-tagged RAB5Q79L was constructed with the tag at the amino terminus. Cherry- or mRFP-tagged TLN (Fig. 4) has been characterized before (Nicolaï et al, 2010). TLN lacking the acidic cluster, EYNVQAESSGEAV, within the cytosolic domain was constructed using a variation of site-directed mutagenesis (Wang & Malcolm, 1999).

Antibodies and Reagents
The following mouse monoclonal antibodies were purchased: anti-N-Cadherin (32; Transduction Laboratories), anti-synaptophysin (SVP-38, Sigma), anti-CD63 (MEM-259; Abcam), anti-flotillin1 (18; Transduction Laboratories), anti-flotillin2 (29; Transduction Laboratories), anti-ARF6 (3A-1; Santa Cruz), anti-Transferrin receptor (H68.4, Invitrogen), anti-GFP (7.1 and 13.1, Roche Diagnostics), anti-tubulin (DM1A; Sigma), anti-MHC1 (W6/32; Abcam), anti-CD59 (MEM43; Millipore), anti-tau1 (PC1C6; Millipore), anti-VSVG (P5D4, Sigma) and anti-CD63 (AD1; Pharmingen). The only rat antibody used was: anti-HA (3F10; Roche Diagnostics). The rabbit polyclonal antibodies included anti-pERM (3141; Cell Signaling), anti-DsRed (632496; Clontech), anti-MAP2 (H-300, Santa Cruz), anti-CopGFP (AB513, Evrogen) and anti-GFP (A11122; Invitrogen). Other used antibodies include goat anti-ICAM5 ectodomain (AF1173; R&D Systems), chicken anti-GFP (1020, Aves Lab), and a bridging rabbit anti-mouse IgG (Jackson ImmunoResearch). The rabbit anti-Telencephalin antibody (B36.1) has been characterized by (Annaert et al, 2001), the rabbit anti-pan-Kalirin antibody (JH2580) by (Penzes et al, 2000), while the rabbit anti-ARF6 (1654) and guinea pig anti-EFA6A (1626) antibodies were described by (Choi et al, 2006). For co-localization of endogenous Kalirin and TLN we used biotinylated B36.1 antibody (Esselens et al., 2004) and a sequential staining where TLN was visualized using Alexa488-conjugated Streptavidin (Molecular Probes, Inc). Polystyrene microbeads (1 and 2 µm) were obtained from Polysciences Inc, while the dextran tetramethylrhodamine (70 kDa) beads were purchased from Invitrogen.

Cell based assays and quantifications
The antibody uptake assays were done using anti-DsRed antibody. After a 30 minutes long incubation at 37°C, the unbound antibodies were removed by several washes in PBS, after which their surface bound pool was left for an extra 2 hours to internalize. The cells were finally fixed and stained with appropriate secondary antibodies. In HeLa cells, this was done first under non-permeabilizing conditions to label non-internalized primary antibodies, followed by the co-labeling of their internalized pool and the endogenous CD63. In neurons the Cerulean signal of tagged Rab5Q79L was enhanced using anti-GFP (Aves) antibody, while the total anti-DsRed antibody pool (surface and internal), was stained with the appropriate secondary antibody.

The detergent-based lipid extraction assay was carried out using live neurons that were incubated in ice cold microtubule stabilizing buffer (MSB; 2 mM MgCl₂, 10 mM EGTA, 60 mM...
Pipes buffer, pH7.0) for 1 minute followed by a 5 minutes extraction in 1% TX-100 (in MSB) as described by (Ledesma et al, 1998). Neurons were subsequently fixed and stained.

Knock down of ARF6 in hippocampal neurons was carried out using SMART vector lentiviral shRNA particles (SH-096951-03-10; Thermo Fisher Scientific), which include the ARF6-targeting sequence: 5’-TATCCAAGATCTTGGGAA-3’. Effects were compared to non-targeting control particles. Neurons were infected at DIV6 and processed at DIV16 for western blot analysis or immunostaining. The acquired images were analysed using ImageJ software. The length of TLN-positive dendritic protrusions (DIV16) was measured within randomly selected dendritic stretches. The sum of measured lengths (TLN-positive dendritic protrusions) was divided by the length of a given dendritic stretch. Three stretches were analyzed per neuron and the acquired relative values were averaged. The data from 18 randomly selected neurons from three independent experiments were used to obtain population mean. The final result was expressed as a relative increase where the control sample was defined as 100%. In a similar way we calculated the average length of TLN-positive protrusions. A total of 1978 and 1931 TLN-positive protrusions were measured in control and ARF6-downregulated neurons, respectively. The two-tailed Student’s t-test was used to compare the mean ± SEM values.

**Immunoelectron microscopy (EM)**
Labeling of cells for EM was carried out according to the method of Tokuyasu (Tokuyasu, 1973) with modifications (Slot & Geuze, 2007). Antigen detection using anti-TLN (B36.1) and anti-CD63 (MEM-259) antibodies was followed by a sequential labeling with respectively 5 nm (TLN) and 15 nm (CD63) protein A-gold (CMC, Utrecht). For the mouse-raised anti-CD63 antibody, a bridging rabbit anti-mouse antibody was used. After each protein A-gold labeling step, grids were fixed in 1% glutaraldehyde. Finally, sections were visualized using JEOL JEM1400 transmission EM at 80kV and micrographs were acquired on either a SIS Quemesa or Veleta camera.

**References**


Supplemental Figure legends

Figure S1. Polystyrene microbeads bind specifically to Telencephalin-expressing dendrites.
Primary hippocampal neurons (DIV 9) were incubated with polystyrene microbeads (1 µm) overnight and stained for Telencephalin (TLN; red) and the axonal marker, tau1 (green). Phase contrast images of microbeads were colour-coded (blue). Arrows indicate the microbead attachment sites that are enriched for TLN, but not tau1. Note that microbeads align along dendrites and are absent from axons (see the axonal stretch that does not run along dendrites).

Figure S2. Binding of polystyrene microbeads to neurons requires expression of Telencephalin (presence at the cell surface).
Primary hippocampal neurons at an early (DIV 4) and later (DIV 7) stage of development were incubated with microbeads (1 µm) overnight and subsequently stained for Telencephalin (TLN; red) and actin (green). Phase contrast images of microbeads were colour-coded (blue). Arrows indicate the microbead attachment sites, and show binding only at the later stage of neuronal development when TLN is expressed and is present at the cell surface. Bar: 10 µm.

Figure S3. The Cherry tag of Telencephalin does not interfere with its recruitment to microbead attachment sites.
Primary hippocampal neurons (DIV 5) expressing internally Cherry-tagged Telencephalin (TLN-Cherry) were incubated overnight with polystyrene microbeads (2 µm) and subsequently fixed and analyzed. Note that TLN-Cherry displays a strong recruitment to microbead attachment sites, much like endogenous TLN. Endogenous TLN is normally not expressed at this early stage of neuronal development. Binding of beads to TLN-Cherry expressing neurons therefore also demonstrates a specific role of TLN in this process. Bar: 10 µm.

Figure S4. At the cell surface Telencephalin and EFA6A co-localize within distinct membrane subdomains.
Top panel: Primary hippocampal neurons (DIV 14) were stained for endogenous Telencephalin (TLN; red), EFA6A (green), and N-Cadherin (blue). Note the similar distribution pattern of TLN and EFA6A both being confined to distinct membrane subdomains while N-Cadherin is more uniformly distributed throughout the cell body. Middle and bottom panels: HeLa cells expressing TLN-Cherry, were stained for surface-associated molecules involved in adhesion, namely α-actinin (middle panel) and vimentin (bottom panel). Note that as in neurons, TLN localizes to distinct surface microdomains, which clearly differ from α-actinin- and vimentin-containing domains. Bars: 10 µm.

Figure S5. Surface Telencephalin localizes to flotillin2-positive membrane subdomains.
HeLa cells co-expressing TLN-Cherry and flotillin2-GFP were fixed and analyzed. Arrowheads indicate basal cell surface microdomains devoid of TLN and flotillin2. Note that both proteins show a similar distribution pattern in other areas of plasma membrane. Bar: 10 µm.

Figure S6. Telencephalin and flotillin2 co-enrich at microbead attachment sites.
HeLa cells co-expressing TLN-Cherry and flotillin2-GFP were incubated with polystyrene microbeads (1 µm) overnight, and subsequently analyzed. The arrow indicates the evident co-recruitment of both proteins to the microbead attachment site. Bar: 10 µm.
Figure S7. Telencephalin and flotillin2 but not N-Cadherin resist detergent extraction in neurons.

Hippocampal neurons co-expressing TLN-Cherry and flotillin2-GFP were subjected to detergent extraction in 1% TX-100 for 5 minutes, and subsequently fixed and stained for N-Cadherin (blue). Arrowheads indicate overlap of TLN and flotillin2 in punctae where N-Cadherin is no longer present. This indicates an association of TLN and flotillin2 with detergent-resistant membrane microdomains. Bar: 10 µm.

Figure S8. Telencephalin and EFA6A interact in vitro.

Cell lysates obtained from HeLa cells co-expressing TLN-mRFP and/or EFA6A-GFP, with or without ARF6T27N-HA, as indicated on the top, were subjected to co-immunoprecipitation experiments with anti-TLN Ab. Note that binding occurred in both the presence/absence of the GDP-bound ARF6 mutant (ARF6T27N), which we expressed to potentially enhance binding of EFA6A to TLN, as described in an earlier study (Decressac et al, 2004).

Figure S9. EFA6A expression induces Telencephalin internalization.

HeLa cells co-expressing TLN-Cherry and EFA6A-GFP were stained for surface TLN using an ectodomain antibody in non-permeabilized cells (blue). Arrowheads indicate internal vesicles of TLN-Cherry, which do not overlap with the staining pattern of surface-localized TLN-Cherry. Bar: 10 µm.

Figure S10. Telencephalin but not transferrin receptor gets entrapped in the ARF6Q67L grape-like vacuoles.

HeLa cells co-expressing TLN-Cherry and ARF6Q67L-HA were stained for HA (green) and the transferrin receptor (TfR; blue), which is a well-described clathrin-dependent cargo protein, and was used as a negative control. Note that TLN, but not TfR, gets entrapped in the ARF6Q67L vacuoles. Bar: 10 µm.

Figure S11. Telencephalin gets entrapped in the ARF6T27N-positive perinuclear recycling compartment in HeLa cells.

HeLa cells co-expressing TLN-Cherry and ARF6T27N-HA were stained for HA (green) and CD59 (blue), which is a well-described ARF6 cargo protein and was used as a positive control. Arrowheads indicate that TLN and CD59 get entrapped in an ARF6T27N-positive perinuclear recycling compartment. Bar: 10 µm.

Figure S12. Telencephalin associates with ARF6T27N-positive endosomes in primary hippocampal neurons.

Primary hippocampal neurons (DIV 14) co-expressing TLN-Cherry and ARF6T27N-HA were stained for HA (green). Arrowheads indicate the close association of TLN with ARF6T27N-positive punctae along the dendrite and at the cell body. Bar: 10 µm.

Figure S13. The acidic cluster-deleted mutant of Telencephalin (TLNΔAC) gets trapped in ARF6Q67L vacuoles.

HeLa cells co-expressing TLNΔAC and ARF6Q67L-HA were stained for HA (blue) and MHC1 (green), which is a well-described ARF6 cargo protein, and was used as a positive control. Note that as with full length TLN (Fig. 2D), both TLNΔAC and MHC1 are clearly entrapped within the ARF6Q67L grape-like vacuoles. This indicates that deletion of the acidic cluster does not affect ARF6-dependent trafficking of TLN. Bar: 10 µm.

Figure S14. The acidic cluster-deletion mutant of Telencephalin (TLNΔAC) reaches the cell surface before its internalization into RAB5Q79L-enlarged endosomes.

Top panel: HeLa cells co-expressing TLNΔAC-Cherry and RAB5Q79L-Cerulean were subjected to an antibody uptake assay using anti-Cherry (anti-DsRed) antibodies. Surface-bound antibodies (green) were labeled before cell permeabilization. The internal antibody pool was labeled after permeabilization (colour coded: red). Arrowheads indicate enlarged
RAB5Q79L (blue) endosomes. After 30 minutes these endosomes were devoid of internalized antibodies, suggesting very slow internalization kinetics of TLNΔAC. Middle panel: Like (top panel) but after an additional 2 hours of antibody internalization. Only then we could detect appreciable amounts of internalized antibody (colour coded: red). Antibodies bound to the cell surface, were stained (green) as in the top panel. Lower panel: Like (middle panel) only in hippocampal neurons (DIV 14). Arrowheads indicate taken up antibody confined to RAB5Q79L endosomes (green). Arrows indicate antibodies bound at the filopodia surface from where TLNΔAC likely internalizes.

Figure S15. Characterization of Telencephalin-deficient neurons.
Wild-type (+/+) and TLN-deficient (-/-) neurons (DIV 16) stained for endogenous TLN and the dendritic marker MAP2. Also shown is the western blot analysis of total cell lysates derived from primary neuronal cultures of both genotypes after probing for TLN and the loading control, tubulin. The data confirm the absence of TLN in neurons derived from knock-out mice. Bar: 10 µm.

Figure S16. Downregulation of ARF6 results in more prominent Telencephalin-positive dendritic protrusions.
(A) Primary hippocampal neurons (DIV 6) were infected with lentiviral particles delivering control-(non-targeting) or ARF6-targeting shRNAs. Western blot analysis of total cell lysates (DIV 16) shows reduced ARF6 protein levels: 30% relative to the control, defined as 100% (bands were normalized to tubulin signal). Note also the relative increase in total TLN levels after ARF6 knockdown (145% of control). Detection of GFP (copGFP) confirms successful infection. (B) Primary hippocampal neuron expressing control or ARF6-targeting shRNA were stained for endogenous TLN (red) and GFP (green). Note the more prominent TLN-positive dendritic protrusions in neurons expressing ARF6-targeting shRNA. The accompanying quantifications demonstrate that ARF6 knockdown increases the average (top graph) and relative total length (bottom graph) of TLN-positive dendritic protrusions. The obtained values and SEM for the average protrusion length are: 2.5±0.1 µm (control) and 3.1±0.1 µm (ARF6 shRNA). For the relative total length of TLN protrusions the values are expressed relative to the control, defined as 100%. The obtained values are: 100±4.4% (control) and 140.4±5.2% (ARF6 shRNA). p-value (Student’s t-test): **p<0.01,***p<0.001. Bars: 10 µm.

Figure S17. The Telencephalin acidic cluster-deletion mutant (TLNΔAC) co-localizes with CD63 in intralumenal vesicles of RAB5Q79L endosomes.
Top panel: HeLa cells co-expressing TLNΔAC-Cherry (red) and RAB5Q79L-Cerulean (blue) were stained for endogenous CD63 (green). Arrowheads indicate an evident co-localization between TLNΔAC and CD63. Middle panel: like (top panel) but in primary hippocampal neurons (DIV 16). Arrowheads indicate co-localization of TLNΔAC-Cherry (red) and endogenous CD63 (green). Bar: 10 µm. Bottom panel: Immuno-EM micrographs of enlarged Rab5Q79L endosomes, acquired in HeLa cells co-expressing TLNΔAC-Cherry and Rab5Q79L-Cerulean. The cells were stained for TLNΔAC (5 nm) and CD63 (15 nm) and the enlarged endosomes, induced by the expression of Rab5Q79L mutant, were identified on the ultrastructural level based on their characteristic/distinct large spherical shape and a strong CD63 immuno-labelling of these structures (in line with the confocal data shown in the top and the middle panel). Arrows show intralumenal vesicles, where TLN and CD63 are closely apposed. Bar: 200 nm.

Figure S18. Internalized Telencephalin co-trafficks with Flotillin1.
Top panel: HeLa cells co-expressing TLNΔAC-Cherry and flotillin1-GFP. Arrowheads indicate the juxta-positioning of both proteins at internal vesicles. Shown in the graph is the percentage of flotillin1-GFP endosomes that contain TLNΔAC, quantified from multiple, randomly selected cells. The obtained percentage and SEM is: 68.8±2.2. Bottom panel: Primary hippocampal neuron (DIV 13) co-expressing TLN-Cherry and flotillin1-GFP.
Arrowheads indicate internal co-localization of TLN and flotilin1 at the cell body (top panel), and along the dendrites (bottom panel). Shown in the graph is the percentage of flotilin1-GFP endosomes that contain TLN-Cherry, quantified from multiple, randomly selected neurons. The obtained percentages are: 80.9±7.6 (cell body) and 71.2±5.9 (dendrites). Bars: 10 µm.

**Figure S19. Telencephalin and Kalirin can co-localize in maturing dendritic protrusions of primary hippocampal neurons.**

Mature primary hippocampal neurons (DIV 32) were fixed (methanol) and stained for endogenous TLN (colour coded: red), Kalirin (colour coded: green) and postsynaptic density protein 95 (PSD95; blue). Note that the analysis at this late stage of development allows for evident detection of endogenous Kalirin. Top panel: Kalirin and TLN can be found within the same dendritic protrusions. Note that the more prominent co-localization of both proteins coincides with the presence of PSD95 (filled arrowheads; unfilled arrowheads indicate absence of PSD95 and less prominent co-localization of TLN and Kalirin). Arrows point to less mature TLN-positive protrusions (filopodia) where no evident co-localization with Kalirin is observed. This suggests that Kalirin requires appropriate triggering for its recruitment to maturing, TLN-containing dendritic protrusions. Such partial co-localization of TLN and Kalirin may thus reflect a transient moment before the exclusion of TLN from the maturing spines (synapses) occurs. In agreement, the bottom panel shows a strong Kalirin and PSD95 co-localization in punctae where TLN is no longer present (arrowheads). Bar: 10 µm.

**References**
