EARP is a multisubunit tethering complex involved in endocytic recycling

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Recycling of endocytic receptors to the cell surface involves passage through a series of membrane-bound compartments by mechanisms that are poorly understood. In particular, it is unknown if endocytic recycling requires the function of multisubunit tethering complexes, as is the case for other intracellular trafficking pathways. Herein we describe a tethering complex named endosome-associated recycling protein (EARP) that is structurally related to the previously described Golgi-associated retrograde protein (GARP) complex. The two complexes share the Ang2, Vps52 and Vps53 subunits, but EARP contains an uncharacterized protein, syndetin, in place of the Vps54 subunit of GARP. This change determines differential localization of EARP to recycling endosomes and GARP to the Golgi complex. EARP interacts with the target SNARE syntaxin 6 and various cognate SNAREs. Depletion of syndetin or syntaxin 6 delays recycling of internalized transferrin to the cell surface. These findings implicate EARP in canonical membrane-fusion events in the process of endocytic recycling.

Plasma membrane proteins are internalized through vesicular carriers that transport cargo to early endosomes. Once in endosomes, the fates of internalized proteins diverge. Some proteins are transported to lysosomes by either staying within the limiting membrane or budding into intraluminal vesicles while early endosomes mature to late endosomes1. Other internalized proteins exit endosomes by way of tubular–vesicular carriers that deliver proteins to the trans-Golgi network (TGN; ref. 2), to specialized storage organelles such as the insulin-responsive glucose transporter type 4 (GLUT4) compartment3 and melanosomes4, or back to the plasma membrane (‘endocytic recycling’)5. This latter pathway is complex, involving various recycling intermediates and, in polarized cells, sorting to different plasma membrane domains.

The ability of proteins to navigate this maze of post-endocytic transport pathways depends on an array of molecular machineries that function at different stages of the endomembrane system. In recent years, substantial progress has been made in the elucidation of the mechanisms that mediate retrograde transport from endosomes to the TGN (refs 6,7). A key player in these mechanisms is a multisubunit complex named ‘retromer’, which promotes cargo sorting into membrane tubules that bud from endosomes8-10. The resulting transport intermediates fuse with the TGN by virtue of a soluble N-ethylmaleimide-sensitive fusion protein attachment protein receptor (SNARE) complex formed by the vesicle SNARE (or R-SNARE) VAMP4 and the target SNAREs (or Q-SNAREs) syntaxin 6 (Stx6), StxX16 and Vti1A (ref. 11). Assembly of these SNAREs is regulated by the TGN-associated, multisubunit tethering complex GARP (for Golgi-associated retrograde protein), which is composed of four subunits named Ang2 (another new gene 2, also known as Vps51), Vps52, Vps53 and Vps54 (vacuolar protein sorting proteins)12.

Recycling of internalized proteins from endosomes to the plasma membrane also involves segregation into tubular intermediates13, but the mechanisms responsible for cargo sorting as well as formation and consumption of the tubules are less well understood14. These tubules participate in fast and slow recycling pathways that are characterized by the association with the small GTPases Rab4 (refs 15,16) and Rab11 (refs 16–18), respectively. The slow pathway includes passage through a pericentriolar organelle referred to as the ‘endocytic recycling compartment’19. Endocytic recycling is probably a dissociative process involving budding and fusion of membrane-enclosed transport intermediates, as suggested by the implication of several SNARES, including VAMP4 (ref. 20), Stx6 (refs 20–23), Stx16 (refs 24,25) and Vti1a (refs 26,27), as well as VAMP3 (also known as cellubrevin)22,28-30 and Stx13 (refs 31–34), in this pathway. The exact composition of SNARE complexes and the specific steps in which they function in this pathway, however, remain to be determined. Furthermore, SNARE-mediated fusion generally involves tethering complexes, but so far none have been shown to function in endocytic recycling.

In the course of studies on GARP, we obtained insights into tethering complexes that participate in endocytic recycling. A search
**RESULTS**

**Identification of syndetin as a GARP-subunit interactor**

In experiments aimed at identifying proteins that interact with GARP, we carried out tandem affinity purification and mass spectrometry using detergent extracts from H4 human neuroglioma cells stably expressing One-STrEp-Flag (OSF)-tagged human GARP subunits. We set a threshold of 10 unique peptides per protein in the mass spectrometry results to limit our analyses to the most abundant tethering proteins (see later). Likewise, analyses using Vps52–OSF and Vps53–OSF as baits yielded syndetin and the four GARP subunits as the top five hits (Fig. 1a). Similar analyses using syndetin–OSF resulted in the co-isolation of Ang2, Vps52 and Vps53, but, remarkably, not Vps54 (Fig. 1a). These observations raised the possibility that syndetin was a component of a GARP complex variant lacking Vps54. The remaining five proteins that co-isolated with Ang2–OSF did not co-purify with Vps52–OSF, Vps53–OSF or syndetin–OSF (Fig. 1a), and were thus deemed unlikely to represent specific GARP interactors.

Protein sequence analysis using the HHpred tool showed homology of syndetin to Vps54 (significance value $E = 5.5 \times 10^{-62}$) and, to a lesser extent, Vps53 ($E = 4.9 \times 10^{-10}$), as well as the Sec8 ($E = 1 \times 10^{-10}$) and Sec15 ($E = 2 \times 10^{-11}$) subunits of the exocyst complex, and the Cog2 ($E = 1.4 \times 10^{-10}$) and Cog5 ($E = 1.4 \times 10^{-10}$) subunits of the conserved oligomeric Golgi (C OG) complex. All of these proteins are subunits of multisubunit tethering complexes that are structured as tandem repeats of an $\alpha$-helical amino- and carboxy-terminal regions (corresponding to domains of unknown function DUF2450 and DUF2451, respectively), connected by a less structured middle region (Fig. 1b and Supplementary Fig. 1). Moreover, the Phyre2 protein fold recognition server predicted that both syndetin and Vps54 are structured as five or six tandemly arranged CATCHR modules, and the Cog2 (E = 10) and Sec15 (E = 10) subunits of the exocyst showed homology to these syndetin and Vps54 according to the Phyre2 server. Individual CATCHR modules are identified by different colours. Syndetin Vps54


deduced evolutionary history of syndetin according to Walker and colleagues.

**Figure 1** Identification of syndetin as a GARP-subunit interactor. (a) Table listing the most abundant proteins in order of unique peptide number identified by tandem affinity purification and mass spectrometry from Ang2–OSF-expressing H4 cells, and their presence in similar isolates from Vps52–, Vps53– and syndetin–OSF-expressing cells. (b) Consensus secondary structure prediction for human syndetin using the NPS@ server. Regions predicted to be $\alpha$-helix, $\beta$-sheet and random coil (C) are indicated. (c) Predicted tertiary structures of syndetin and Vps54 according to the Phyre2 server. Individual CATCHR modules are identified by different colours. (d) Deduced evolutionary history of syndetin according to Walker and colleagues.
**Figure 2** Biochemical characterization of syndetin. (a) Immunoblot analysis of syndetin, Vps52, Vps53, actin and clathrin heavy chain (CHC) in different mouse tissues and HeLa cells resolved by SDS–PAGE (polyacrylamide gel electrophoresis). (b) HeLa cell lysates were fractionated into 13,000g supernatant (S13), 100,000g supernatant (S100) and 100,000g pellet (P100) fractions; P100 was extracted with 1 M NaCl or 1% Triton X-100 (TX-100) and further separated into S100 and P100 fractions. (c,d) The supernatant from the 1 M NaCl extraction was analysed by sedimentation velocity on 5–20% glycerol gradients (c) or size-exclusion chromatography on Superose 6 (d). The positions of standard proteins on the gradients (sedimentation coefficient $S_{20,w}$ values in Svedberg units) or the column (Stokes radii $Rh$ in Ångströms) are indicated. Vo, void volume. In b–d, fractions were analysed by SDS–PAGE and IB for the indicated proteins. (e) Detergent extracts of HeLa cells (left panel) or rat cortical neurons (right panel) were subjected to immunoprecipitation (IP) with antibody to syndetin followed by SDS–PAGE and IB with antibodies to the indicated proteins. (f,g) HeLa cells expressing V5-tagged versions of Ang2, Vps52, Vps53 and Vps54 were extracted in lysis buffer and subjected to immunoprecipitation with antibodies to syndetin (f) or the V5 epitope (g), followed by SDS–PAGE and IB with antibodies to the V5 epitope (f) or syndetin (g). In a–g, molecular mass markers (in kilodaltons) are indicated on the left. (h) Yeast two-hybrid interaction of syndetin and Vps54 with Vps53. Growth in the absence of histidine (−His) and presence of 0.2 mM 3-amino-1,2,4-triazole (3-AT) is indicative of interactions. T-Ag, T antigen; AD, activation domain; BD, binding domain. Uncropped images of the blots are shown in Supplementary Figs 5a and b.
Figure 3 Localization of syndetin in rat hippocampal neurons and H4 cells. (a,b) Rat hippocampal neurons were transfected with plasmids encoding Vps54–eGFP (a) or syndetin–eGFP (b) on day in vitro 3 (DIV-3) and processed for confocal microscopy on DIV-7. Cells were fixed and localization was determined by immunostaining with mouse antibody to GFP, rabbit antibody to p230 (TGN marker) and chicken antibody to MAP2 (somatodendritic marker) followed by Alexa Fluor 488 donkey anti-mouse, Alexa Fluor 555 donkey anti-rabbit and Alexa Fluor 647 goat anti-chicken antibodies. (c) Rat hippocampal neurons were transfected with plasmids encoding syndetin–eGFP and Vps54–13Myc and immunostained with rabbit antibody to GFP, mouse antibody to the Myc epitope and chicken antibody to MAP2, followed by Alexa Fluor 488 donkey anti-rabbit, Alexa Fluor 555 donkey anti-mouse and Alexa Fluor 647 goat anti-chicken antibodies. (d) H4 cells stably expressing Vps54–eGFP were transfected with a plasmid encoding syndetin–mCherry and imaged 48 h later by live-cell confocal microscopy. Images show the first frame from Supplementary Video 1. (e,f) Rat hippocampal neurons were transfected with plasmids encoding Vps54–eGFP and Vps53–13Myc (e) or syndetin–eGFP and Vps53–13Myc (f) and processed as described in a and b. In a–c and e,f, bars, 10 μm; insets show ×2 magnifications of the boxed areas. In d, bar, 6 μm; inset bar, 1.5 μm.
Figure 4  Analysis of the co-localization of syndetin with endosomal Rabs. (a) Rat hippocampal neurons were co-transfected with plasmids encoding syndetin–eGFP plus TagRFP–Rab4A (upper row), TagRFP–Rab11A (middle row) or TagRFP–Rab5A (lower row) on DIV-3 and examined by TIRF microscopy on DIV-7. Shown is the first frame from live-cell imaging movies. Bars, 5 μm. Insets show magnifications of the areas boxed with solid lines. Bar, 1 μm. (b) Selected frames from the area boxed with dashed lines in a (upper row) taken from Supplementary Video 2 showing a moving structure containing syndetin–eGFP and TagRFP–Rab4A (arrows). Bar, 2.5 μm (c) Quantification of syndetin co-localization with endosomal Rabs. Pearson’s correlation coefficients for syndetin–eGFP and TagRFP–Rab4A, TagRFP–Rab5A and TagRFP–Rab11A were calculated using the ImageJ plugin JACoP. For each group, image pairs from \( n = 9 \) cells pooled from three independent experiments were used in the calculations. Values are the mean ± s.e.m. *** \( P < 0.0002 \) (Rab4A versus Rab11A) and \( P < 0.0011 \) (Rab11A versus Rab5A).

supergroups (categorized according to ref. 38), indicating that the two proteins arose from gene duplication in a common eukaryotic ancestor. A few groups, however, seem to have lost expression of syndetin (for example fungi and entamoebida), or both syndetin and Vps54 (for example rhizaria and non-bony fish), over the course of evolution (Fig. 1d). The presence of both syndetin and Vps54 in
such a wide range of eukaryotes suggests that they fulfil critical, yet distinct, functions.

Syndetin is a component of a GARP complex variant lacking Vps54

Immunoblot analysis using antibodies to syndetin, Vps52 and Vps53 showed expression of the three proteins in all mouse tissues examined, with highest levels in different areas of the brain, as well as kidney and testis (Fig. 2a). They were also expressed in human cell lines such as HeLa (Fig. 2a). Subcellular fractionation of HeLa cells revealed that syndetin, Vps52 and Vps53 were mainly associated with membranes, from which they could be extracted by treatment with 1 M NaCl or 1% Triton X-100 (Fig. 2b). Thus, these proteins behaved as peripheral membrane components. Further analyses of the NaCl-extracted proteins by sedimentation velocity on glycerol gradients (Fig. 2c) and size-exclusion chromatography on Superose 6 (Fig. 2d) showed that syndetin behaved as a monodisperse species with hydrodynamic properties similar to those of GARP (sedimentation coefficient $S_{20,w} = 9.3\, S$; Stokes radius $R_h = 91\, Å$; calculated molecular mass $M_i = 360 ± 20\, kDa$; ref. 39).

To directly assess the relationship of syndetin to GARP, we analysed cell lysates by immunoprecipitation with antibody to syndetin followed by immunoblotting (IB) with antibodies to Ang2, Vps52 and Vps53. We found that syndetin co-precipitated with Ang2, Vps52 and Vps53 in both HeLa cells (Fig. 2e, left panel) and rat cortical neurons (Fig. 2e, right panel). We could not test if syndetin assembles with Vps54 using this protocol because of the lack of a suitable antibody to Vps54. To overcome this limitation, we applied the same immunoprecipitation–IB protocol to HeLa cells transiently expressing Ang2, Vps52, Vps53 and Vps54, all tagged with the V5 epitope29. We observed that the antibody to endogenous syndetin brought down V5-tagged Ang2, Vps52 and Vps53, but not Vps54 (Fig. 2f). Reciprocally, the antibody to the V5 epitope brought down endogenous syndetin from cells expressing V5-tagged Ang2, Vps52 and Vps53, but not Vps54 (Fig. 2g). Finally, we examined pairwise interactions using the yeast two-hybrid system and found preferential interactions of both syndetin and Vps54 with Vps53 (Fig. 2h). Taken together, these experiments indicated that syndetin is a subunit of a complex consisting of Ang2, Vps52 and Vps53, but not Vps54. The sum of the predicted molecular masses of syndetin (111 kDa), Ang2 (86 kDa), Vps52 (82 kDa) and Vps53 (80 kDa) is 359 kDa, similar to the molecular mass calculated from hydrodynamic measurements of the syndetin-containing complex, consistent with it being a 1:1:1:1 heterotrimer. This complex differs from the previously characterized human GARP (ref. 39) in that it has syndetin in place of Vps54. For reasons that will become apparent in the following sections, we refer to this complex as ‘EARP’.

Syndetin and Vps54 confer distinct intracellular localizations on their corresponding complexes

Next we examined the intracellular localization of syndetin in comparison to Vps54. Because antibodies to these proteins do not work for immunostaining, we carried out confocal immunofluorescence microscopy on cells expressing tagged proteins. Initial experiments were carried out with rat hippocampal neurons because of the high expression levels of these proteins in the brain (Fig. 2a), which ensured an abundance of the cognate subunits. We observed that Vps54–eGFP (enhanced green fluorescent protein) co-localized with the TGN marker p230 (Fig. 3a). In contrast, syndetin–eGFP was found in punctate foci scattered throughout the cytoplasm of the soma and dendrites, and did not significantly co-localize with p230 (Fig. 3b) or Vps54–13Myc (Fig. 3c). Live-cell imaging of H4 neuroglioma cells co-expressing Vps54–eGFP and syndetin–mCherry also showed predominant localization of these proteins to the TGN and cytoplasmic puncta, respectively (Fig. 3d and Supplementary Video 1). The lower background in these cells enabled us to observe a population of Vps54–eGFP in cytoplasmic puncta containing syndetin–mCherry (Fig. 3d and Supplementary Video 1). From these experiments, we concluded that syndetin localizes to punctate cytoplasmic structures, whereas Vps54 localizes largely to the TGN and, to a lesser extent, to the same punctate structures that contain syndetin.

We also compared the localizations of tagged Vps54 and syndetin with those of tagged Ang2 and Vps53, co-expressed in a pairwise manner in rat hippocampal neurons. Interestingly, we observed that in Vps54–eGFP-expressing cells Vps53–13Myc (Fig. 3e) and Ang2–13Myc (Supplementary Fig. 2a) co-localized with Vps54–eGFP at the TGN, whereas in syndetin–eGFP-expressing cells Vps53–13Myc (Fig. 3f) and Ang2–13Myc (Supplementary Fig. 2a) co-localized with syndetin–eGFP on punctate structures. Therefore, Vps54 and syndetin are the subunits that specify the localization of their corresponding complexes to the TGN and to punctate structures, respectively.

Syndetin localizes to Rab4-containing endosomes involved in TIR recycling

To identify the punctate structures containing syndetin, we compared the localization of syndetin–eGFP with that of various endosomal markers in rat hippocampal neurons (Fig. 4). The best co-localization was observed for syndetin–eGFP with TagRFP–Rab4A, where TagRFP is a red fluorescent protein, by live-cell, total internal reflection fluorescence (TIRF) microscopy, which enabled low-background visualization of cytoplasmic particles within a ~200 nm evanescent field (Fig. 4a–c and Supplementary Video 2). Because TIRF could under-report perinuclear pools of these proteins, we additionally carried out confocal microscopy of live HeLa cells expressing syndetin–eGFP with TagRFP–Rab4A; under these conditions, we could also observe co-localization of these two proteins on punctate structures distributed throughout the cytoplasm (Supplementary Video 3). Rab4A is associated with an endosomal domain that mediates fast endocytic recycling15. We also observed significant, albeit less extensive, co-localization of syndetin-GFP with TagRFP–Rab11A (Fig. 4a,c), a marker for a different endosomal domain involved in slow endocytic recycling17,18. Less co-localization was observed for syndetin–eGFP and TagRFP–Rab5A (Fig. 4a,c and Supplementary Video 4), a marker for early endosomes40,41.

Endosomal domains containing Rab5 and Rab4 represent consecutive stages in the recycling of endocytic receptors such as TIR back to the cell surface42. Consistent with the placement of syndetin in this pathway, live-cell imaging using HeLa cells showed that Tf–Alexa568 accessed a syndetin–eGFP compartment as early as 3 min after internalization, and its presence in this compartment increased for up to 20 min (Fig. 5 and Supplementary Video 5).
From these experiments, we concluded that syndetin localizes mainly to Rab4-containing endosomes involved in TfR recycling to the cell surface.

**Syndetin regulates endocytic recycling of the transferrin receptor**

To investigate the function of syndetin, we carried out short interfering RNA (siRNA)-mediated knockdown (KD) in HeLa cells and examined the cells for various trafficking phenotypes. Syndetin KD was quite efficient (>90% depletion) and also caused large reductions in the amounts of Vps52 and Vps53 (Fig. 6a), indicating that most of the latter proteins exist as subunits of EARP rather than GARP. Moreover, KD of Ang2, Vps52 or Vps53 depleted not only the primary targets of the siRNAs but also the other proteins, including syndetin (Fig. 6a). Syndetin KD had no effect on the levels of the autophagy marker LC3-II, in contrast to Ang2, Vps52, Vps53 or Vps54 KD, which greatly increased them (Fig. 6a). Treatment with the lysosome acidification inhibitor bafilomycin A1 abolished these differences in LC3-II levels (Supplementary Fig. 4b), suggesting that they were due to impaired lysosomal degradation of LC3-II in GARP-deficient but not EARP-deficient cells. These findings are consistent with the known requirement of GARP for sorting of acid hydrolases to lysosomes, and suggest that EARP is not involved in this process. Confocal microscopy showed that syndetin KD had no effect on retrograde transport of internalized Shiga toxin B subunit to the Golgi complex and steady-state localization of TGN46 to the plasma membrane.
Figure 6 Syndetin KD delays Tf recycling. (a) HeLa cells were treated with siRNAs targeting the proteins indicated at the top and analysed by SDS–PAGE IB for the proteins indicated on the right. Molecular mass markers (in kilodaltons) are indicated on the left. Uncropped images of the blots are shown in Supplementary Fig. 5b. (b) HeLa cells transfected with the indicated siRNAs and plasmids encoding human Vps53 or mouse syndetin (mSyndetin) were left to internalize Tf–Alexa568 for 20 min and then chased for different times at 37°C. Tf–Alexa568 fluorescence was monitored by live-cell confocal microscopy imaging. Representative maximum projection images acquired at the indicated times are shown. Bars, 6 μm. (c) Quantification of Tf–Alexa568 fluorescence intensity over time. Values are the mean ± s.e.m. (n=3 independent experiments). The numbers of cells quantified were: mock, 301; Vps54 KD, 297; syndetin KD, 250; syndetin KD plus Vps53, 73; syndetin KD plus mSyndetin, 61. (d) HeLa cells were treated with siRNA to syndetin and transfected with a plasmid encoding eGFP–Rab4A. At 72 h after transfection, cells were left to internalize Tf–Alexa568 for 20 min and imaged by live-cell confocal microscopy in chase medium at the indicated times. Bars, 6 μm; inset bar, 1.5 μm.

the TGN (Supplementary Fig. 2b), in contrast to Ang2 or Vps54 KD, which caused accumulation of these proteins in endosomal intermediates (Supplementary Fig. 2b)19,42–44. These experiments thus demonstrated that, unlike GARP, the syndetin-containing complex is not involved in retrograde transport from endosomes to the TGN.
Figure 7 EARP functions in association with Stx6 and cognate SNAREs. (a) H4 cells, untransfected or stably transfected with syndetin–OSF, were crosslinked with dithiobis(succinimidylpropionate) and lysed. Syndetin–OSF and associated proteins were pulled down on Strep-Tactin beads (STREP-PD) and analysed by SDS–PAGE and IB with antibodies against the indicated proteins. Molecular mass markers (in kilodaltons) are indicated on the left. Uncropped images of the blots are shown in Supplementary Fig. 5c. (b) Endosomal localization of Stx6 and cognate SNAREs. HeLa cells were co-transfected with TagRFP–Rab4A and different eGFP-tagged SNAREs as indicated, and examined 24 h after transfection. Bars, 6 μm. (c) Mock-treated or Stx6 siRNA-treated HeLa cells were left to internalize Tf–Alexa568 for 20 min and then chased at 37 °C. Representative maximum projection images acquired at the indicated times are shown. Values are the mean ± s.e.m. (n = 3 independent experiments). The numbers of cells quantified were: mock, 162; Stx6 KD, 143. Bars, 6 μm.

The localization of syndetin to Rab4A-containing endosomes prompted us to test its requirement for endocytic recycling of the TIR. The initial internalization rate of Tf–Alexa568 after 3 min of uptake was similar in mock-, syndetin- and Vps54-KD cells, indicating that these proteins are not involved in Tf endocytosis (Supplementary Fig. 3a,b). At later times, however, syndetin-KD
cells, and to a lesser extent Vps54-KD cells, accumulated more intracellular Tf-Alexa568 than mock-treated cells (Supplementary Fig. 3c). To determine if this increased accumulation was due to decreased recycling, mock-, syndetin- and Vps54-KD HeLa cells were left to internalize Tf-Alexa568 for 20 min, after which the cells were chased for different times and intracellular fluorescence was quantified by live-cell imaging. We observed that the intracellular fluorescence was lost more rapidly in mock-treated ($t_{1/2} = 6\text{ min}$) than in syndetin-KD cells ($t_{1/2} = 15\text{ min}$; Fig. 6b,c). Expression of siRNA-resistant mouse syndetin, but not Vps54, rescued Tf-Alexa568 recycling (Fig. 6b,c). The retained Tf-Alexa568 in syndetin-KD cells partly accumulated in a Rab4A-containing compartment as observed by fluorescence microscopy (Fig. 6d). Subcellular fractionation of syndetin-KD cells also showed partial accumulation of Tf-biotin in a light fraction that co-sedimented with Rab4, although the majority of Tf-biotin was found in a heavier fraction that partly overlapped with Lamp1 (Supplementary Fig. 4a). This latter fraction might reflect the rerouting of the retained Tf-biotin to lysosomes. These experiments thus demonstrated that syndetin is required for efficient recycling of the TfR. Vps54 KD also delayed Tf-Alexa568 recycling, albeit to a lesser extent ($t_{1/2} = 11\text{ min}$; Fig. 6b,c). This may indicate a recycling role for the smaller population of Vps54 that co-localizes with syndetin on endosomes (Fig. 3d).

**EARP interacts and co-localizes with endosomal SNAREs**

GARP interacts with the Stx6–Stx16–Vti1a–VAMP4 SNARE complex involved in fusion of endosome-derived transport carriers to the TGN (refs 43,45). Interactions are mainly mediated by binding of the regulatory Habc domain of Stx6 to an N-terminal motif in the Ang2 subunit of GARP (refs 43,45). As EARP also contains Ang2 as one of its subunits, we expected it to interact with a similar set of SNAREs. Indeed, syndetin–OSF pulldowns from H4 cells resulted in the co-isolation of endogenous Stx6, Stx16, Vti1a and VAMP4, as well as the Stx6-interacting, recycling endosomal SNAREs Stx13 (ref. 20) and VAMP3 (refs 11,22,46; Fig. 7a). In contrast, syndetin–OSF did not pull down two other SNAREs, Stx2 and Stx4, that are involved in exocytosis at the plasma membrane$^{47,48}$ (Fig. 7a).

Moreover, Stx6, Stx16, Vti1a, VAMP4, Stx13 and VAMP3 exhibited varying degrees of co-localization with Rab4A on cytoplasmic puncta in HeLa cells (Fig. 7b). Finally, Stx6 KD also delayed recycling of the TfR (Fig. 7c). These findings suggest that EARP acts on Stx6, probably in conjunction with a set of endosomal SNAREs, to promote TfR recycling to the plasma membrane.

**DISCUSSION**

Our search for interactors of the GARP complex resulted in the unexpected discovery of a structurally related complex, EARP, in which Vps54 is substituted by a previously uncharacterized protein herein named syndetin. Vps54 and syndetin are the key subunits that determine distinct localization of GARP to the TGN and EARP to recycling endosomes. Most importantly, whereas GARP is mainly involved in retrograde transport from endosomes to the TGN (refs 39, 42–44), EARP is required for recycling of internalized TIRs to the cell surface (Fig. 8). A small population of GARP also localizes to recycling endosomes and contributes to TfR recycling. Because multisubunit tethering complexes generally promote SNARE-mediated fusion$^{36,49}$,
the requirement of EARP (and to a lesser extent GARP) in endocytic recycling supports the notion that this process involves transfer between discontinuous membrane-bound compartments.

The implication of EARP in endocytic recycling poses the question of what SNAREs participate in this process. The Ang2, Vps52 and Vps53 subunits shared by GARP and EARP engage in multiple interactions with components of the Stx6–Stx16–Vti1a–VAMP4 complex23,43,45, which functions in fusion of endosome-derived intermediates with the TGN (ref. 11). Among these interactions, the strongest and most specific occurs between Ang2 and Stx6 (refs 39,45). As Ang2 is also a component of EARP, it is logical to find co-isolation of syndetin with Stx6 in affinity-purification experiments. Moreover, Stx6 partially co-localizes with Rab4A on endosomal structures, and Stx6 KD delays TIR recycling. These observations are consistent with Stx6 being one of the SNAREs that are regulated by EARP in the process of endocytic recycling (Fig. 8). This conclusion is in line with the notion that Stx6 is a promiscuous SNARE (ref. 50) that functions not only in endosome-to-TGN transport but also in recycling of integrins to the cell surface22,23 and insulin-regulated aminopeptidase to the GLUT4 compartment23. The identity of the other SNAREs involved in EARP and Stx6-dependent endocytic recycling remains to be determined. Stx16 (refs 24,25), Vti1a (refs 26,27), Stx13 (refs 31–34) and VAMP3 (refs 22,28–30) have all been partly localized to recycling endosomes and/or shown to promote endosomal transport events, and we find that they also co-isolate with syndetin. Furthermore, a SNARE complex composed of Stx6, Stx13, Vti1a and VAMP4 has been shown to mediate homotypic fusion of early endosomes in vitro20, although it remains to be determined if this complex also plays a role in recycling. Genome-wide screens identified GARP subunits among various factors required for ricin21,22 and Pseudomonas exotoxin23 toxicity. Remarkably, in one of these screens syndetin was identified as a protein whose depletion increased susceptibility to ricin52. These findings can now be explained on the basis of the different trafficking events mediated by GARP and EARP. Endosome-to-TGN transport dependent on GARP is an essential step in the itinerary of ricin to its eventual site of action in the cytosol. Recycling from endosomes to the plasma membrane promoted by EARP, on the other hand, is not just dispensable for retrograde transport of the toxin, but probably diminishes the flow of toxin to the TGN by redirecting some of it to the cell surface.

Of the many intercompartmental transport processes that take place in the endomembrane system, transport through recycling endosomes was one for which no multisubunit tethering complexes had been implicated. Our findings now demonstrate a role for EARP, and to some extent GARP, in this process. This discovery should enable future studies on the precise nature of the membrane-bound compartments through which cargos traffic along the endocytic recycling pathway, the detailed molecular mechanisms of budding and fusion events in this pathway and the range of physiological processes (for example, receptor recycling, integrin-mediated cell adhesion and motility, synaptic plasticity) that are dependent on EARP-dependent transport. Our findings should also contribute to the elucidation of the pathogenesis of progressive cerebello-cerebral atrophy type 2 caused by mutations in Vps53 (ref. 53), which in light of our results could result from impairment of both GARP-mediated retrograde transport to the TGN- and EARP-mediated recycling to the plasma membrane. □

METHODS

Methods and any associated references are available in the online version of the paper.

Note: Supplementary Information is available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

C.S., Y.C. and J.S.B. conceived the project. C.S. and Y.C. carried out most of the experiments. J.P. contributed to SNARE pulldowns and X.G. to subcellular fractionation experiments. C.S., Y.C., J.P., X.G. and J.S.B. analysed the data. J.S.B., C.S. and Y.C. wrote the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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Recombinant DNA procedures. Plasmids encoding OSF-tagged and V5-tagged Ang2, Vps52, Vps53 and Vps54, as well as the same GARP subunits cloned into the yeast two-hybrid vectors pGADT7 and pGBK7, have been described before.28,30 The plasmid encoding Vps54-GFP (ref. 1) was used for subcloning the Vps54 coding region of syndetin and 5'-GCAGUUAUGUUGGAAGAUU-3' were amplified from the respective V5-constructs and cloned into pcDNA3.1 (Invitrogen). The clarified lysate was diluted 1:1 with sucrose buffer and divided into four samples (S13). One sample was supplemented with 1/4 volume gel-loading buffer 1 (4 M LiCl, 1 mM EDTA), 1 M NaCl, 1 mM EDTA supplemented with 10% glycerol, and protease inhibitors). After 30 min on ice, insoluble material was separated by a second spin at 91,000 r.p.m. (16,500 g) for 16 h at 4 °C, and the salt-extracted membrane fraction (S100, 1 M NaCl) or detergent-extracted membrane fraction (S100, 1% Triton X-100) were supplemented with 1/4 volume gel-loading buffer 1. The corresponding pellets were resuspended in equal volumes of gel-loading buffer 2 to generate samples P100 (1 M NaCl) and P100 (1% Triton X-100). Samples were incubated for 5 min at 95 °C, and 50 μl of each sample was resolved on 4–12% SDS–PAGE gels (Invitrogen) and analysed by IB.

Sedimentation velocity ultracentrifugation. Salt-extracted membrane fractions (S100, 1 M NaCl) were prepared as above using HeLa cells from one confluent 10 cm dish as starting material. Five hundred microlitres of S100 fraction were diluted 1:1 with high-salt buffer to a final sucrose concentration of 5%. A 20–50% sucrose gradient was prepared by overlaying 400 μl of a 45% sucrose cushion with high-salt buffer supplemented with 20%, 19.5%, 18%, 16.5%, 15%, 13%, 11%, 9.5%, 8.5%, 6.5% or 5% glycerol (1 ml per step, 11 steps total). Gradients were overlaid with 1 ml of salt-extracted membrane fraction centrifuged at 169,000 g for 16 h at 4 °C in a SW41 rotor (Beckman). Twelve fractions of 1 ml each were collected from the top and precipitated by the addition of 1/10 volume of 6.1 M trichloroacetic acid. Precipitates were recovered by a 30 min spin at 13,000 r.p.m. (16,500g) in a refrigerated tabletop centrifuge and each pellet was washed with 1 ml ice-cold acetone. Pellets were re-suspended in 60 μl gel-loading buffer 2. Samples were incubated for 5 min at 95 °C and 20 μl of each fraction was resolved by SDS–PAGE gels and IB. Sedimentation coefficients (s20,w) were estimated as previously described.34

Gel filtration. One millilitre of salt-extracted membrane fraction was prepared as above using two confluent 10 cm dishes of HeLa cells as starting material. Proteins were separated on a Superose 6 10/300 GL column (GE Bioscience) equilibrated in high-salt buffer supplemented with 10% glycerol. Forty-eight fractions of 500 μl each were obtained and precipitated using trichloroacetic acid as described above. Air-dried pellets were resuspended in 60 μl gel-loading buffer and incubated for 5 min at 95 °C, and 20 μl of each second fraction was analysed by SDS–PAGE gels and IB. Stokes radii (in Å) were determined as previously described. The molecular mass of EARP was calculated assuming a partial specific volume of 0.720.75 cm3 g−1. © 2015 Macmillan Publishers Limited. All rights reserved
supplemented with 30 μl Protein A or Protein G beads (GE Healthcare) and antibody, or 30 μl Strept-Avidin beads. Samples were incubated for 2 h with rotation at 4 °C. Beads were washed three times with 1 ml lysis buffer each and resuspended in 40 μl gel-loading buffer. Samples were incubated for 5 min at 95 °C and analysed by SDS-PAGE on 4–12% SDS gradient gels and 1B.

**METHODS**

**Yeast two-hybrid assays.** Yeast strain AH109 was co-transformed with plasmids using standard methods. Three days after transformation, colonies were picked and overnight liquid culture was set up in SD medium lacking leucine and tryptophan (Clontech). On the following day, cultures were diluted to an optical density at 600 nm of 0.1, and 5 μl of each culture was spotted onto SD agar plates lacking leucine and tryptophan (+His) or SD agar plates lacking leucine, tryptophan and histidine (−His) supplemented with 0.2 mM 3-AT (MP Bio). Cell growth was analysed three days after plating.

**TF uptake and chase.** TF uptake and chase in HeLa cells was carried out using a modification of a previously described protocol. For uptake of Alexa Fluor 568-conjugated TF (Invitrogen), mock- or siRNA-treated cells were placed in uptake medium (MEM, 1% ovalbumin, 25 mM HEPES) supplemented with 40 μg ml⁻¹ Alexa Fluor 568-conjugated TF for 20 min. After washing three times with pre-warmed MEM, cells were chased in chase medium (MEM, 25 mM HEPES, 100 μg ml⁻¹ human holo-Tf) pre-warmed to 37 °C. Live-cell imaging was set to start 10 min after the beginning of the chase. For uptake of biontin-conjugated TF, cells were incubated in uptake medium supplemented with 40 μg ml⁻¹ TF-biotin for 30 min. After washing twice with citrate buffer (pH 4.6) to remove surface-bound TF, cells were incubated in chase medium for 30 min before harvesting for subcellular fractionation. TF-biotin was detected using streptavidin–peroxidase polymer as per the manufacturer’s instructions.

**Fluorescence microscopy.** Immunofluorescence microscopy was carried out as previously described on a Zeiss LSM710 microscope (Carl Zeiss). Live-cell imaging was conducted with a NIKON Eclipse Ti Microscope System equipped with an environmental chamber (temperature controlled at 37 °C and CO₂ at 5%), high-speed EM charge-coupled device cameras (iXon DU897 from Andor and Evolve 512 from Photometrics) and NIS-Elements AR microscope imaging software. TIRF images were acquired with an Apo TIRF 100X objective (numerical aperture 1.49) and iXon DU897. Spinning-disc confocal z-stacks were taken with a Plan Apo VC 60X objective (numerical aperture 1.40) and Evolve 512. Dual-colour imaging was achieved by fast switching excitation lasers so that images from green and red channels were aligned automatically.

**FACS analysis.** Flow cytometry was carried out to quantify TF uptake in the experiment shown in Supplementary Fig. 3c. HeLa cells transfected with mock, Vps54 or syndetin siRNAs were incubated with 40 μg ml⁻¹ TF–Alexa47 at 37 °C for different times, and washed twice with ice-cold citrate buffer (pH 4.6) and three times with PBS to remove surface-bound TF. After detachment cells from the plates by incubation with 2 mM EDTA on ice for 1 h, cells were resuspended in ice-cold PBS and kept on ice until analysis by FACS. Suspended cells were analysed using a FACSCALIBUR (BD Biosciences) equipped with a 635 nm red diode laser and standard filters to detect Alexa Fluor 647 emission. CellQuest acquisition and analysis software was used to quantify at least 1 × 10⁶ cells from each group in each independent experiment.

**Statistical analysis.** All numerical results are reported as the mean ± s.e.m. and represent data from a minimum of three independent experiments. No statistical method was used to predetermine sample size. The experiments were not randomized, and the investigators were not blinded to allocation during experiments and outcome assessment. GraphPad InStat software (GraphPad) was used for analysis of statistical significance. Unless otherwise indicated, a two-tailed Student t-test for unpaired data was used to evaluate single comparisons between different experimental groups. Normal distribution of the data sets in comparisons passed the D’Agostino–Pearson omnibus normality test. Differences were considered statistically significant for a value of P < 0.05. Figure 4: the F-test indicates that the Rab4A group has significantly smaller variance than the Rab11A group. Figure 6: mock, 301 cells; Vps54 KD, 297 cells; syndetin KD, 250 cells; syndetin KD + Vps53, 73 cells; syndetin KD + mSyndetin, 61 cells; quantified in three independent experiments. Figure 7: mock, 162 cells; 5x6 KD, 143 cells; quantified in three independent experiments. Supplementary Fig. 3: mock, 171 cells; Vps54 KD, 167 cells; syndetin KD, 142 cells; quantified in three independent experiments. Immunofluorescence and live-cell imaging results shown in Figs 3–7 and Supplementary Figs 2 and 3 are representative of at least three independent experiments. Results in Figs 2d–h, 6a and 7a are representative of at least three independent experiments. Results in Figs 2b,c, 6e and Supplementary Fig. 4 are representative of two independent experiments. The experiment in Fig. 2a was done once.

**Accession number.** The secondary accession for the protein sequence of syndetin (CCDC132 isoform a) is NP_060137.2 (NCBI protein database).

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**Supplementary Figure 1** Phylogenetic analysis of Syndetin orthologs. An initial BLASTP search revealed that the first 492 amino acids of human Syndetin comprised the largest conserved domain (DUF 2450). This sequence was used in two consecutive rounds of PSI-BLAST on genomes from different organisms. Orthologs were defined as (i) being large proteins (>500 amino acids in all super-groups, with the exception of some smaller proteins in the SAR/CCTH super-group), (ii) having a high α-helical content, (iii) having either DUF 2450 or DUF 2451 domains, and (iv) yielding human Syndetin and not Vps54 as one of three top hits in reverse PSI-BLAST searches. Listed are representative species, NCBI reference sequences and length of the Syndetin orthologs.

| Kingdom           | Species                          | NCBI Reference | Length |
|-------------------|----------------------------------|----------------|--------|
| **Opisthokonta**  | Homo sapiens                      | NP_060137.2    | 964    |
|                   | Xenopus tropicalis               | NP_001107716.1| 691    |
|                   | Danio rerio                      | XP_001923725.4| 835    |
|                   | Strongylocentrus purpuratus      | NP_001182788.2| 1062   |
|                   | Aplysia californica              | XP_005099172.1|        |
|                   | Nematostraela vectensis          | XP_001634514.1| 522    |
|                   | Trichoplax adherens              | XP_002110770.1| 864    |
|                   | Apis mellifera                   | XP_001121588.2| 935    |
|                   | Daphnia pulex                    | EFX99003.1    | 916    |
|                   | Caenorhabditis elegans           | NP_499394.2   | 954    |
|                   | Sauingoea rossetta               | XP_004922385.1| 1077   |
|                   | Physcomitrella patens            | XP_00178351.1 | 1003   |
|                   | Orzya sativa                     | NP_001065215.1| 1074   |
| **Archaeplastida**| Arabidopsis thaliana             | BAH19449.1    | 1124   |
|                   | Solanum lycopersicum             | XP_004250992.1| 1092   |
|                   | Volvox carteri                   | XP_002952743.1| 1184   |
|                   | Chlamydomonas reinhardtii        | XP_001690508.1| 912    |
|                   | Dictyostelium discoideum         | XP_0038199.1  | 1133   |
|                   | Acanthamoeba castellanii         | XP_004336688.1|        |
|                   | Naegleria gruberi                | XP_002683118.1|        |
|                   | Leishmania major                 | XP_001683735.1|        |
|                   | Trypanosoma cruzi                | EKF39648.1    |        |
|                   | Trichomonas vaginalis            | XP_001317963.1|        |
|                   | Emiliana huxleyi                 | XP_005774142.1|        |
|                   | Phytophthora sojae               | EGZ28453.1    |        |
|                   | Albugo laibachii                 | CCA20924.1    |        |
|                   | Saprolegnia diclina              | EQC32289.1    |        |
| **Excavata**      | Thalassiosira pseudonana         | XP_002289837.1| 1551   |
|                   | Blastocystis hominis             | CBK22207.2    |        |
|                   | Guillardia theta                 | XP_005827472.1|        |
|                   | Ectocarpus siliculosus           | CB33644.1     |        |
|                   | Tetrahymena thermophila          | XP_0001019529.2|       |
| **SAR/CCTH**      | Phymarelium tetraurelia          | XP_001453912.1|        |

DUF 2450 | DUF 2451 | Vps54-like domain
**Supplementary Figure 2** Vps54 and Syndetin exhibit distinct organelle targeting and retrograde transport activities. (a) Co-localization of Vps54-EGFP and Syndetin-EGFP with Ang2-13myc in rat hippocampal neurons analyzed with antibodies to GFP, the myc epitope and MAP2 as described in the legend to Figure 3. Bars, 10 μm. (b) Syndetin KD does not affect retrograde transport from endosomes to the TGN. Control (mock), Ang2-, Vps54- and Syndetin-KD HeLa cells were treated with 0.5 μg/ml Cy3-conjugated B-subunit of Shiga toxin (STxB) at 37°C for 15 min and chased for 1 h before fixation and immunostaining. Nocodazole treatment was applied at 10 μM for 2 h following the chase where indicated. Giantin (medial Golgi marker) and TGN46 (TGN marker) were visualized by immunostaining with rabbit antibody to Giantin and sheep antibody to human TGN46 followed by Alexa647 donkey anti-rabbit and Alexa488 donkey anti-sheep antibodies. Bars, 10 μm.
Supplementary Figure 3 Syndetin KD does not affect initial Tf uptake but increases Tf accumulation over time. (a,b) HeLa cells were treated with the indicated siRNAs and incubated with Tf-Alexa568 at 37°C for 3 min. After three washes with ice-cold PBS, cells were fixed. Images were acquired by confocal microscopy and quantified using ImageJ. Values are the mean ± SEM (n=3 independent experiments). The numbers of cells quantified were: mock, 171; Vps54 KD, 167; Syndetin KD, 142. n.s., P = 0.86 (Mock vs. Vps54 KD) and P = 0.69 (Mock vs. Syndetin KD). (c) HeLa cells treated with the indicated siRNAs were incubated with Tf-Alexa647 at 37°C for different times and then washed twice with ice-cold citrate buffer (pH 4.6) and three times with PBS to remove surface-bound Tf-Alexa647. Intracellular Tf-Alexa647 was quantified by FACS after detaching cells from the plates using 2 mM EDTA. At least 1x10^5 cells were analyzed for each group in each independent experiment. Values are the mean ± SEM (n=3 independent experiments).
Supplementary Figure 4 Effects of Syndetin KD on the distribution of internalized Tf-biotin in subcellular fractions and on LC3-II levels. (a) Equal numbers of HeLa cells treated with control (mock) or Syndetin siRNAs were allowed to internalize Tf-biotin for 30 min. After stripping surface-bound Tf-biotin with citrate buffer (pH 4.6), cells were chased for 30 min. Cells were lysed and fractionated by ultracentrifugation on 5-20% iodixanol gradients. Fractions were collected and analyzed by SDS-PAGE gels and immunoblotting for the indicated proteins. Tf-biotin was detected with Streptavidin-HRP polymer according to the manufacturer’s instructions. Molecular mass markers (in kDa) are indicated at left. (b) HeLa cells were treated with siRNAs targeting the proteins indicated on top and analyzed by SDS-PAGE and blotting for the proteins indicated at right. Bafilomycin A1 was applied at 250 nM for 4 h. Molecular mass markers (in kDa) are indicated at left. Uncropped images of the blots are shown in Supplementary Fig. 5c.
**Supplementary Figure 5** Uncropped images of key panels in main figures. Red boxes indicate the cropped portion of each immunoblot presented in the corresponding main figures. Ladder of molecular markers is shown on the left of each panel.
Supplementary Figure 5 continued
Supplementary Figure 5 continued
Supplementary Video Legends

**Supplementary Video 1** Differential localization of Vps54 and Syndetin in live H4 cells. H4 cells stably expressing Vps54-EGFP were transfected with a plasmid encoding Syndetin-mCherry and imaged by live-cell confocal microscopy after 48 h.

**Supplementary Video 2** Co-localization of Syndetin with Rab4A observed by TIRF microscopy of live rat hippocampal neurons. Rat hippocampal neurons were co-transfected with plasmids encoding Syndetin-EGFP and TagRFP-Rab4A on DIV-3 and examined by TIRF microscopy on DIV-7. Inset shows magnifications of the areas boxed with solid lines.

**Supplementary Video 3** Co-localization of Syndetin with Rab4A observed by confocal microscopy of live HeLa cells. Syndetin-KD HeLa cells were transiently transfected with plasmids encoding mouse Syndetin-EGFP and TagRFP-Rab4A and imaged at 37°C after 48 h.

**Supplementary Video 4** Low co-localization of Syndetin with Rab5A observed by confocal microscopy of live HeLa cells. Syndetin-KD HeLa cells were transiently transfected with plasmids encoding mouse Syndetin-EGFP and TagRFP-Rab5A and imaged at 37°C after 48 h.

**Supplementary Video 5** Rapid access of internalized Tf into the Syndetin compartment. Syndetin-KD HeLa cells were transiently transfected with plasmids encoding Syndetin-4xEGFP and, after 48 h, imaged at after addition of Tf-Alexa568 to the medium.