# Clathrin is not required for SNX-BAR-retromer-mediated carrier formation

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## Summary

Clathrin has been implicated in retromer-mediated trafficking, but its precise function remains elusive. Given the importance of retromers for efficient endosomal sorting, we have sought to clarify the relationship between clathrin and the SNX-BAR retromer. We find that the retromer SNX-BARs do not interact directly or indirectly with clathrin. In addition, we observe that SNX-BAR-retromer tubules and carriers are not clathrin coated. Furthermore, perturbing clathrin function, by overexpressing a dominant-negative clathrin or through suppression of clathrin expression, has no detectable effect on the frequency of SNX-BAR-retromer tubulation. We propose that SNX-BAR-retromer-mediated membrane deformation and carrier formation does not require clathrin, and hence the role of clathrin in SNX-BAR-retromer function would appear to lie in pre-SNX-BAR-retromer cargo sorting.

**Key words:** Clathrin, Retromer, SNX-BAR

## Introduction

Recent studies have highlighted the importance of retromer complexes in regulating a wide variety of cellular processes based primarily, but not exclusively, on their ability to regulate retrograde endosome-to-TGN (trans-Golgi network) transport (Cullen and Korswagen, 2012). Retromers are multi-subunit complexes that bear many similarities to classical coat complexes such as COPI, COPII and clathrin coats (Seaman, 2005; Bonifacino and Hurley, 2008; Collins, 2008; Cullen and Korswagen, 2012). Like these quintessential coat complexes, retromers function to couple cargo recognition and sorting to membrane deformation, resulting in the formation of cargo-enriched carriers. The cargo recognition sub-complex of both the SNX-BAR (sorting nexin with a Bin/Amphiphysin/Rvs domain) retromer and the SNX3 retromer consist of a stable trimer of VPS26, VPS29 and VPS35 that recognises a sorting motif located in the cytoplasmic tails of an ever-expanding subset of internalized transmembrane receptors, including the cation-independent mannose 6-phosphate receptor (CI-MPR) and sortilin. (Seaman, 2007). The cargo-recognition sub-complex engages a second sub-complex that mediates membrane deformation. For the SNX-BAR retromer, the membrane-deforming sub-complex comprises a heterodimer of sorting nexin-1 (SNX1) or SNX2 coupled to either SNX5 or SNX6 (Seaman and Williams, 2002; Wassmer et al., 2007; Wassmer et al., 2009). The BAR domain is capable of sensing and driving membrane curvature (Carlton et al., 2004). It is postulated that through the formation of dimer–dimer contacts, SNX-BAR heterodimers form higher order helical arrays that stabilise and drive tubule formation (Frost et al., 2009; Mim et al., 2012) (reviewed by van Weering et al., 2010). The coordinated action of tubule constriction, possibly mediated by the dynamin-related protein EHD1 (Gokool et al., 2007), coupled to longitudinal forces generated through WASH-mediated actin polymerization (Derivery et al., 2009; Gomez and Billadeau, 2009) and the association with the dynein–dynactin minus-end-directed microtubule motor (Hong et al., 2009; Wassmer et al., 2009), have been argued to aid the efficiency of tubule scission.

Clathrin has been implicated in SNX-BAR-retromer-mediated trafficking (Johannes and Popoff, 2008; McGough and Cullen, 2011). Retrograde transport of the β-subunit of Shiga toxin and CI-MPR, both SNX-BAR-retromer cargoes (Arighi et al., 2004; Seaman, 2004; Bujny et al., 2007; Popoff et al., 2007), is dependent on clathrin or clathrin-interacting proteins such as EpsinR (Saint-Pol et al., 2004; Popoff et al., 2007; Popoff et al., 2009). In addition, a putative clathrin-binding box has been identified in SNX1 and SNX2, which in isolation is capable of binding clathrin (Skånland et al., 2009). Two models have been proposed to describe the sequential action of clathrin and the SNX-BAR retromer (Johannes and Popoff, 2008). In one, clathrin organises an endosomal sub-domain of clathrin-binding proteins and cargo adaptors, which initiates membrane deformation prior to SNX-BAR-retromer processing the tubule into a cargo-enriched transport carrier. In an alternative model, clathrin organises and enriches cargo into a sub-domain prior to membrane deformation. Binding of SNX-BAR retromer to the edge of the flat clathrin sub-domain leads to BAR-domain-mediated membrane deformation thereby targeting the enriched cargo into a tubular carrier. In this study we have sought to distinguish between these models and hence clarify the relationship between SNX-BAR retromer and clathrin.

## Results and Discussion
### SNX-BAR retromer does not associate with clathrin

In light of the links between clathrin and the SNX-BAR retromer, we initially sought to address their spatial relationship. Fixed-cell confocal imaging of endogenous clathrin heavy chain (CHC)
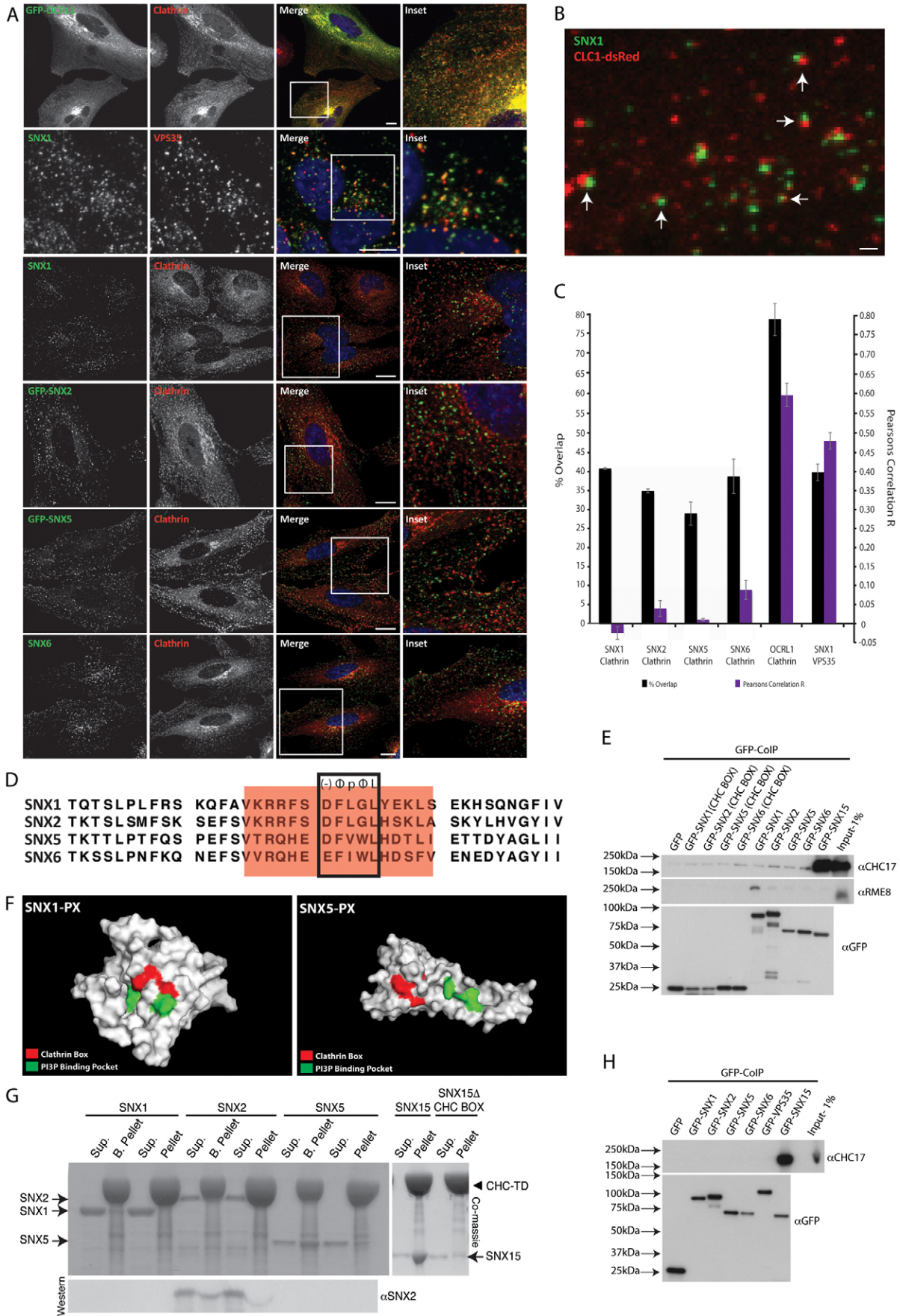
**Fig. 1.** See next page for legend.

with the retromer SNX-BARs, revealed little overlap between the two (Fig. 1A). Most retromer SNX-BAR-labelled endosomes were clathrin negative. Another retromer component VPS35 also showed little colocalisation with dsRed-tagged clathrin light chain (CLC-dsRed; supplementary material Fig. S1A). In addition, when clathrin and the SNX-BAR retromer were found on the same endosomes they often appeared to be juxtaposed, as if occupying different sub-domains (Fig. 1B). The resulting data were quantified in terms of pixel overlap, and the correlation of pixel intensity where the two signals overlap [Pearson's correlation $R$, where 0 indicates no relationship (random colocalization), a value between 0 and 1 indicates a positive relationship and a value between $-1$ and 0 shows a negative relationship]. Analysis of pixel overlap revealed a large proportion of retromer SNX-BAR pixels were positive for clathrin (Fig. 1C). This is most probably due to the shear amount of clathrin signal, present not only on membranes but also in a cytoplasmic pool, and the inability to spatially resolve the signals in the clathrin-enriched TGN region. Indeed analysis of the degree of overlap with the clathrin channel rotated through 180° still results in a significant percentage overlap (supplementary material Fig. S1B). However, the more relevant Pearson's correlation analysis showed little correlation between the clathrin and SNX-BAR-retromer signals at points where they overlapped (Fig. 1C). This is in contrast to the high Pearson's correlation between SNX1 and VPS35, and between clathrin and

OCRL1, proteins known to interact with each other (Fig. 1C). Furthermore, while rotation of one channel through 180° has no effect on the Pearson's values for the retromer SNX-BARs and clathrin, it dramatically reduces the Pearson's value between SNX1 and VPS35, and between clathrin and OCRL1, suggesting any degree of overlap for the retromer SNX-BARs with clathrin is random (supplementary material Fig. S1B).

Recently, Skånland and colleagues identified an inverted clathrin-binding box within the PX domains of SNX1 and SNX2 (Skånland et al., 2009). SNX5 and SNX6 contain an analogous clathrin-binding box at a similar location (Fig. 1D). To investigate whether the isolated clathrin-binding boxes interact with clathrin we performed co-immunoprecipitation experiments with GFP-tagged clathrin binding boxes, using full-length GFP–SNX15, which directly associates with CHC (C. M. Danson and P.J.C., unpublished data), as a positive control. Under these conditions, we were unable to observe an association between isolated clathrin-binding boxes and endogenous clathrin (Fig. 1E). To exclude the possibility that the clathrin-binding boxes in the full-length protein are functional, we determined if full-length recombinant SNX1, SNX2 or SNX5 (we were unable to purify sufficient quantities of SNX6) could interact with the purified GST-tagged terminal domain of clathrin. Although the positive control SNX15 interacted strongly, we failed to observe an association between the retromer SNX-BARs and clathrin (Fig. 1G). We did observe both SNX2 and SNX5 binding to the denatured form of clathrin terminal domain but not the correctly folded clathrin terminal domain, which is consistent with previous data establishing that SNX5 does not directly bind this isoform of CHC (Towler et al., 2004). Extending this, the SNX-BAR retromer did not indirectly associate with clathrin, as both the GFP-tagged retromer SNX-BARs and GFP–VPS35 failed to co-immunoprecipitate endogenous CHC (Fig. 1E,H). Finally, we used the NMR structures of the SNX1 and SNX5 PX domains to visualise the location of the predicted clathrin-binding boxes (Zhong et al., 2005; Koharudin et al., 2009) (Fig. 1F). Although the boxes are surface exposed, they are in close proximity to key residues that line the phosphatidylinositol 3-monophosphate (PtdIns3$P$)-binding pocket, which mediates membrane association (Cozier et al., 2002). Thus, when SNX1 and SNX5 are associated with PtdIns3$P$-enriched endosomal membranes the putative clathrin-binding boxes are unlikely to be accessible to clathrin. The inaccessibility of the clathrin-binding boxes, coupled with their inability to bind clathrin, are inconsistent with retromer SNX-BARs directly associating with clathrin.

## SNX-BAR-retromer tubules and carriers are clathrin negative

As described previously, the role of clathrin in SNX-BAR-retromer function may lie in the process of membrane deformation during SNX-BAR-retromer carrier formation (Johannes and Popoff, 2008). In order to visualise the relationship between the SNX-BAR retromer and clathrin during tubulation, we performed live-cell imaging of GFP–SNX1 and CLC–dsRed. In over 70 SNX-BAR-retromer tubulation events, clathrin was not detected on the tubule or subsequent carrier, even when tubulation occurred from an endosome vacuole that was clathrin positive (Fig. 2A–C; supplementary material Fig. S1B). Over 70% of tubulation events occurred from clathrin-negative endosomes (Fig. 2C),

**Fig. 1. Retromer SNX-BARs do not associate with CHC. (A)** Retromer SNX-BARs do not colocalise with CHC. HeLa cells were fixed and stained for endogenous SNX1 or SNX6 (green) and endogenous CHC or VPS35 (red) or virally transduced with GFP-SNX2 or GFP-SNX5 (green), or transiently transfected with GFP-OCRL1, prior to being fixed and stained for endogenous CHC (red). Scale bars: 10 μm. **(B)** SNX-BAR-retromer-labelled endosomes lie juxtaposed to clathrin-labelled punctae. HeLa cells transiently expressing CLC–dsRed were fixed and stained for endogenous SNX1. Scale bar: 1 μm. **(C)** Analysis of the colocalisation shown in A: the percentage overlap of the individual SNX–BAR or OCRL1 signals with the clathrin signal, and the degree of correlation when signals overlap (Pearson's correlation coefficient). Error bars show the s.d. ($n$=3 with 20 cells per condition). **(D)** Sequence alignment of the retromer SNX-BARs reveals the conserved putative clathrin-binding box ($-$)ΦpΦL [L, leucine; Φ, bulky hydrophobic; p, polar; ($-$), negative]. The amino acids fused to GFP to create isolated clathrin-binding boxes used in E are shaded pink. **(E)** Isolated clathrin-binding boxes fail to bind clathrin. Extracts from HEK293 cells transiently expressing GFP-tagged clathrin-binding boxes from SNX1, SNX2, SNX5 and SNX6 and full-length SNX1, SNX2, SNX5, SNX6 and SNX15, were subjected to a GFP-nanotrap prior to western blotting for endogenous CHC and RME-8. **(F)** Putative clathrin-binding boxes are predicted not to be accessible when the SNX-BARs are bound to PtdIns3$P$-enriched endosomal membranes. NMR structures of SNX1 and SNX5 are shown. Key residues required for PtdIns3$P$ binding are shown in green (SNX1: Arg186 and Arg238; SNX5: Arg42, Lys44, Lys46, Lys96 and Arg103), whereas the clathrin-binding boxes are shown in red. **(G)** Full-length SNX1, SNX2 or SNX5 do not directly bind the terminal domain of CHC. Glutathione resin containing the correctly folded clathrin or a denatured clathrin (boiled for 20 minutes) were incubated with purified 2 μM SNX1, SNX2 or SNX5. SNX15 or SNX15 without its clathrin-binding box were used as positive and negative controls. **(H)** Clathrin is not detected in SNX-BAR-retromer immunoprecipitates. Cell extracts derived from HEK293 cells transiently transfected with GFP, GFP-SNX1, GFP-SNX2, GFP-SNX5, GFP-SNX6, GFP-VPS35 and GFP-SNX15, were subjected to GFP-nanotrap analysis and blotted for CHC and GFP.
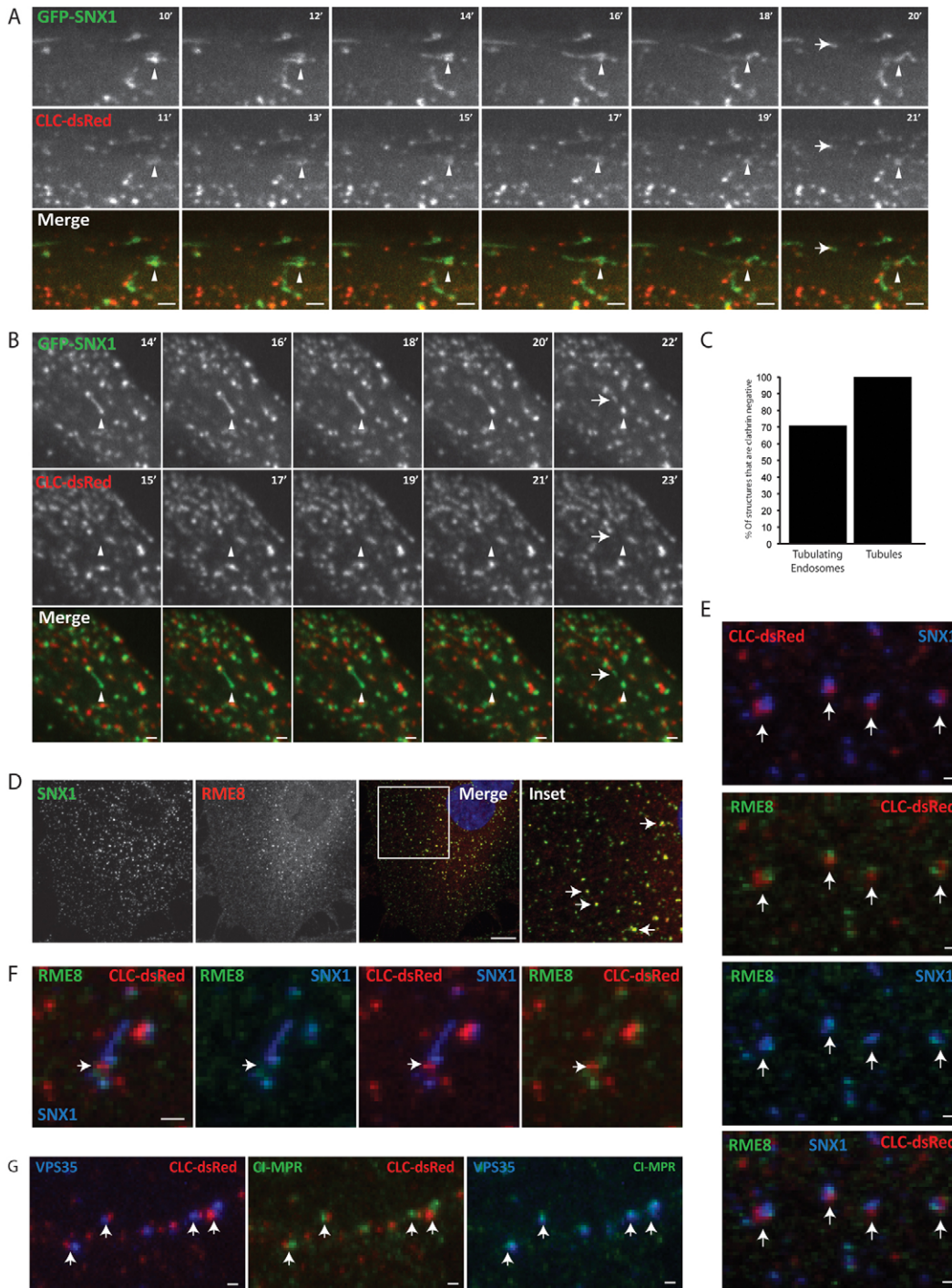
**Fig. 2. Clathrin is absent from SNX1-retromer tubules and carriers.** RPE-1 cells were transiently co-transfected with pEGFP-SNX1 (green) and CLC-dsRed (red) and imaged live after a 16 hour incubation period. (**A,B**) Frames depicting the formation and scission of a GFP–SNX1 tubule from a vesicle positive for both SNX1 and clathrin (A), or positive for SNX1 but negative for clathrin (B; in both cases the arrowheads indicate the dual-expressing vesicle, whereas the arrow indicates the carrier after scission; supplementary material Movies 1, 2). Scale bars: 4 μm. (**C**) Of 77 SNX1-decorated tubules all were clathrin negative, whereas of 77 tubulating endosomes, 22 were clathrin positive. (**D**) SNX1 colocalises with RME-8. HeLa cells were fixed and stained for endogenous SNX1 (green) and endogenous RME-8 (red). Arrows indicate endosomes positive for both. Scale bar: 10 μm. (**E**) RME-8 localises to SNX1-positive, clathrin-negative sub-domains. HeLa cells transiently expressing CLC–dsRed were fixed and stained for endogenous SNX1 (blue) and endogenous RME-8 (green). Scale bars: 1 μm. (**F**) SNX1 tubules emanate from SNX1- and RME-8-positive sub-domains. HeLa cells transiently expressing CLC-dsRed were fixed and stained for endogenous SNX1 (blue) and endogenous RME-8 (green). Arrow indicates a tubulating endosome. Scale bar: 1 μm. (**G**) Similar to SNX1, VPS35 is juxtaposed to clathrin, in a domain positive for CI-MPR. HeLa cells transiently expressing CLC-dsRed were fixed and stained for endogenous VPS35 (blue) and endogenous CI-MPR (green). Scale bars: 1 μm.
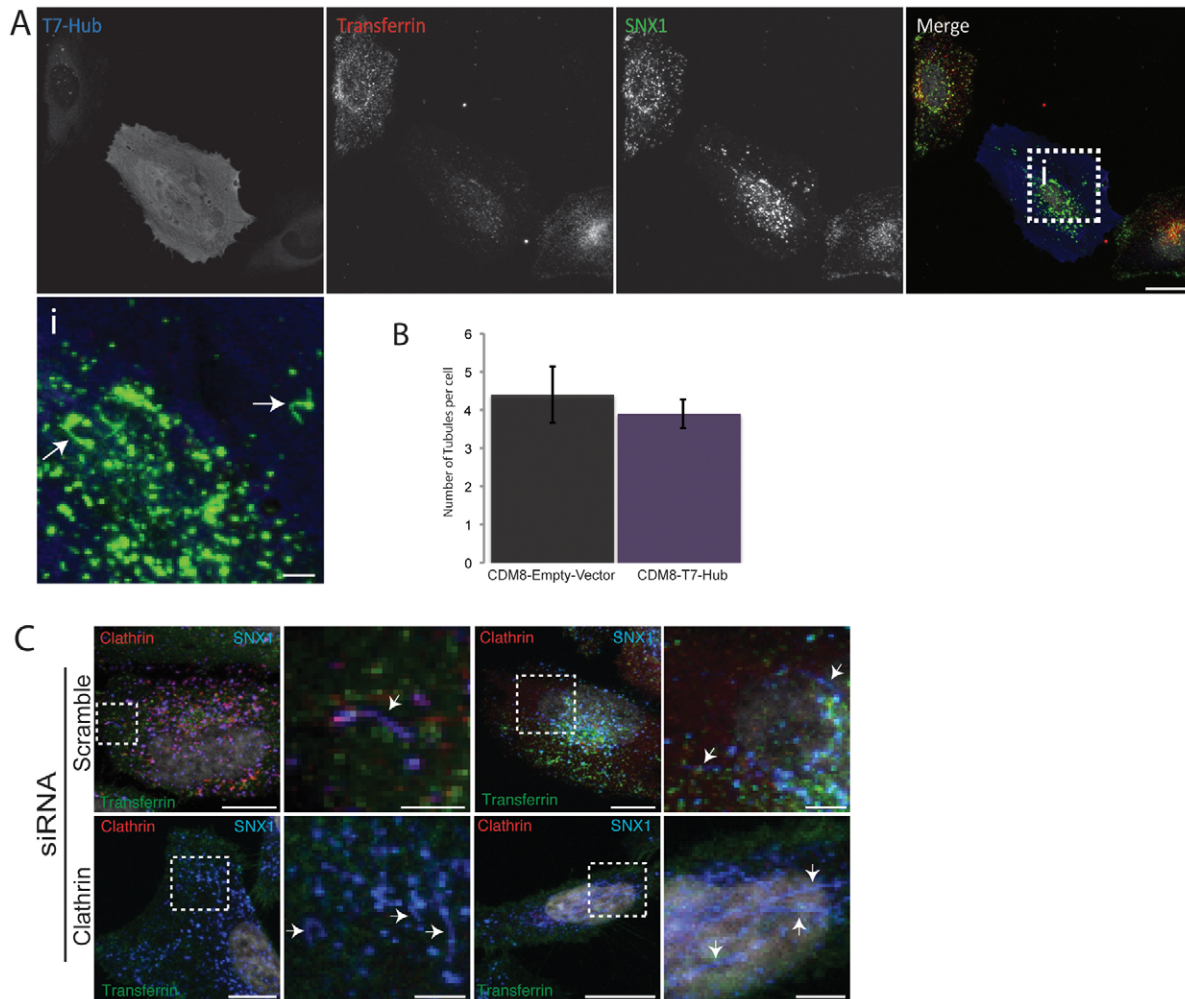
**Fig. 3. Clathrin is not required for SNX-BAR-retromer tubulation.** (**A**) Overexpression of the terminal domain of CHC does not affect SNX-BAR-retromer tubulation. HeLa cells were transiently transfected with a CDM8–T7–CHC-hub domain construct or a empty CDM8 construct. Cells were incubated in serum-free medium for 1 hour prior to addition of serum-free medium containing 25 µg/ml of fluorescently labelled transferrin and then fixed after 30 minutes and stained for endogenous SNX1 (green) and T7 (red) after a 24 hour incubation period. Scale bar: 10 µm. Examples of SNX1 tubules are indicated with arrows in the enlarged image i (scale bar: 3 µm). (**B**) Number of SNX1 tubules per cell ($n=3$ and 10 cells per condition). (**C**) RNAi-mediated suppression of CHC does not affect SNX-BAR-retromer tubulation. CHC was knocked down in HeLa cells. Coverslips were incubated in serum-free medium for 1 hour prior to addition of serum-free medium containing 25 µg/ml of fluorescently labelled transferrin and then fixed after 20 minutes. Cells were subsequently stained for endogenous SNX1 and CHC, to identify efficient knockdown. Arrows indicate SNX1 tubules. The regions within the dashed lines are enlarged in the panels to the right. Scale bars: 10 µm; 1 µm in enlarged images.

suggesting that clathrin is not required for formation of SNX-BAR-retromer tubules. As overexpression of BAR proteins is known to lead to the formation of excessively long and persistent endosomal tubules (Carlton et al., 2004), therefore to exclude the possibility that the need for clathrin in SNX-BAR-retromer tubulation was being overridden as a result of increasing SNX1 levels, we analysed SNX1 tubulation in fixed cells using an antibody against endogenous SNX1. SNX1 tubules observed under fixed conditions also appeared negative for clathrin (supplementary material Fig. S1C), and emanated most frequently from clathrin-negative endosomes (supplementary material Fig. S1F). Similar results were observed for SNX2 and SNX5 tubules (supplementary material Fig. S1D,E).

Overall, these data correlate with an ultrastructural analysis that revealed the absence of clathrin on SNX-BAR-retromer-labelled tubular carriers (Mari et al., 2008). In addition,

purification of clathrin-coated vesicles followed by multi-variant proteomic profiling has shown little enrichment of SNX-BAR-retromer components (Borner et al., 2012), again consistent with SNX-BAR-retromer carriers being devoid of clathrin.

### RME-8 colocalises with SNX1 on clathrin-negative sub-domains

RME-8 is involved in SNX-BAR-retromer-mediated trafficking (Shi et al., 2009), possibly through the regulation of endosomal clathrin dynamics. RME-8 interacts with Hsc70, stimulating its ATPase activity, which disrupts CHC–CHC interactions (Chang et al., 2004). Loss of RME-8 led to both an increase in the amount of clathrin on endosomes and a slower turnover of endosomal clathrin (Shi et al., 2009). This suggests that SNX-BAR retromer may regulate endosomal clathrin dynamics,
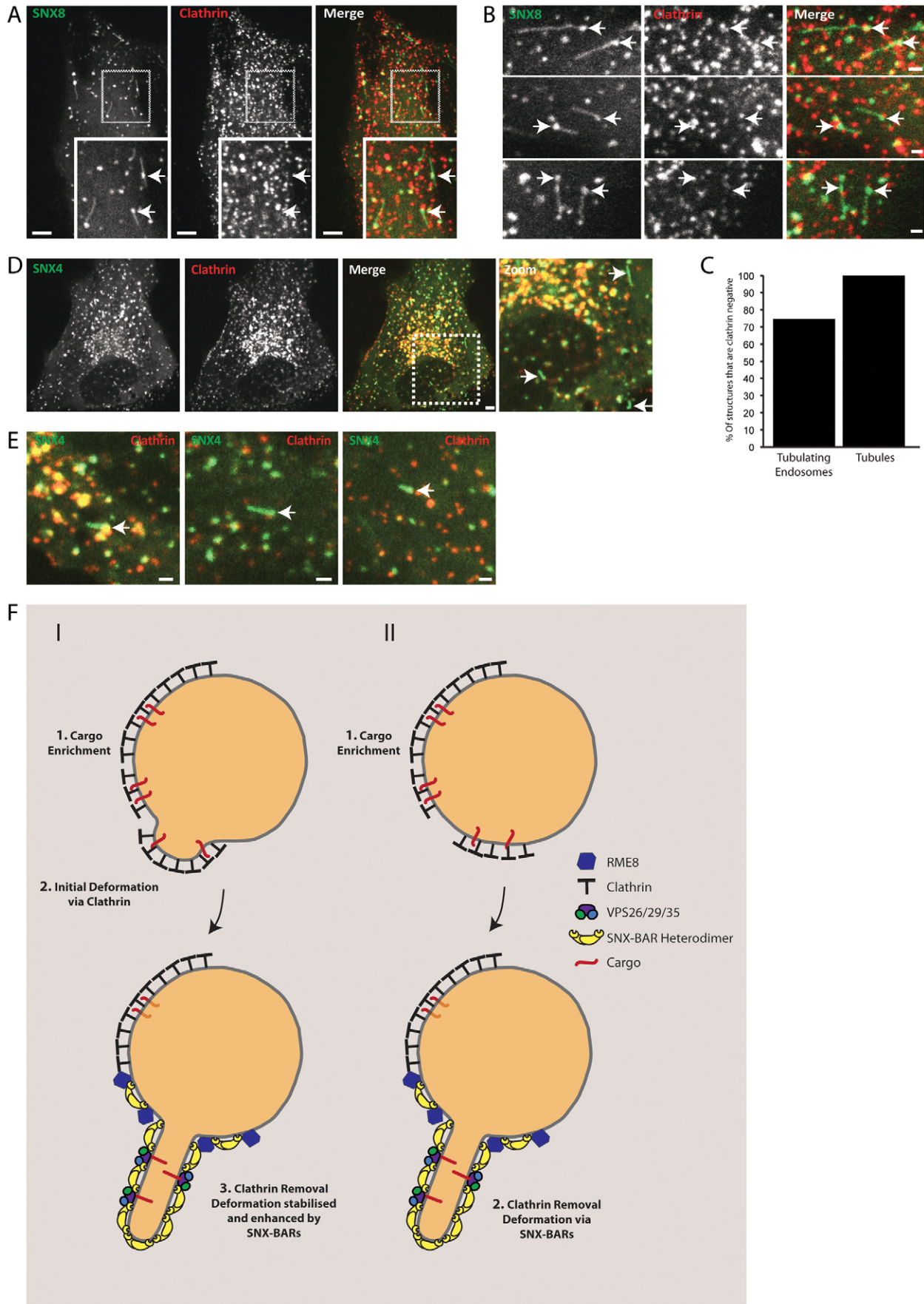
**Fig. 4.** See next page for legend.

allowing for the formation of SNX-BAR-retromer-positive but clathrin-negative sub-domains on endosomes. We confirmed the previously reported interaction between RME-8 and SNX1 (Fig. 1E). Indeed, fixed-cell confocal imaging revealed extensive colocalisation between endogenous RME-8 and SNX1 (Fig. 2D), including on SNX1 tubulating endosomes (Fig. 2F; supplementary material Fig. S2A). Furthermore, triple labelling of endogenous RME-8 and endogenous SNX1, with CLC–dsRed revealed that on endosomes where SNX1 and clathrin were juxtaposed on discreet sub-domains, RME-8 was found on the SNX1-positive but clathrin-negative domains (Fig. 2E). Interestingly, tubules were observed to emanate from these SNX1-, RME-8-positive sub-domains (Fig. 2F). However, RME-8 was not present on the tubules themselves. Intriguingly triple labelling of endogenous VPS35, endogenous CI-MPR with CLC–dsRed showed that CI-MPR also localised to these SNX-BAR-retromer-positive, clathrin-negative areas (Fig. 2G). However, knockdown of RME-8 did not lead to an increase in the colocalisation between SNX1 and clathrin and SNX1 tubules were still clathrin negative (supplementary material Fig. S2; Fig. 2B–D). We conclude that although RME-8 may help maintain the segregation of the SNX-BAR retromer from clathrin and prevent clathrin accumulation on SNX-BAR-retromer tubules, it is not solely responsible for segregation of the SNX-BAR retromer from clathrin.

## Clathrin is not required for SNX-BAR-mediated retromer carrier formation

The observation that SNX-BAR-retromer tubules most frequently form on clathrin-negative endosomes led us to hypothesise that clathrin is not required for the formation of SNX-BAR-retromer tubules. In order to address this hypothesis we overexpressed a T7-tagged hub fragment of clathrin, comprising the C-terminal third of CHC, and counted the number of endogenous SNX1 tubules in fixed cells. The clathrin hub fragment competes with endogenous CHC for binding to CLC and thereby perturbs clathrin function (Liu et al., 1998). We observed the same number of SNX-BAR-retromer tubulation events in cells expressing the clathrin hub fragment or alternatively when endogenous CHC was knocked down through RNAi suppression (Fig. 3A–C). In both instances clathrin function was perturbed sufficiently to inhibit internalisation of fluorescently labelled transferrin, which 20, 30 and 40 minutes after addition was found

to primarily label the plasma membrane, while in control cells it was found on intracellular punctae (supplementary material Fig. S3). These data are consistent with clathrin functioning independently of membrane deformation by the SNX-BAR retromer during the formation of cargo-enriched carriers. Finally, to determine whether clathrin may play a role in membrane deformation events in other SNX-BAR-mediated endosomal sorting pathways, we performed live-cell imaging of CLC–dsRed with GFP–SNX8, implicated in retrograde transport (Dyve et al., 2009), and GFP–SNX4, involved in transferrin receptor recycling (Traer et al., 2007). This revealed that membrane tubules and carriers formed by these SNX-BARs were also devoid of clathrin (Fig. 4A–E), suggesting that the absence of clathrin from SNX-BAR tubules and carriers might be conserved across the SNX-BAR family.

In summary, our data are in agreement with a model in which clathrin enriches cargo on a subset of SNX-BAR-retromer-decorated endosomes, possible through the recruitment of cargo adaptors and/or by spatially confining cargo within an endosomal sub-domain (Fig. 4F). The retromer SNX-BARs, which are capable of deforming membrane *in vitro* independently of other accessory proteins, then removes clathrin, possibly through an interaction with RME-8, prior to the generation and processing of tubular carriers.

## Materials and Methods

### Cell culture and transfections
HeLa cells were maintained in DMEM (Gibco-Invitrogen) plus 10% fetal calf serum (Sigma-Aldrich) and penicillin/streptomycin (PAA). Plasmid containing the stated construct was transfected using Lipofectamine LTX reagent (Invitrogen) 24 hours before analyses. cDNA of human SNX-BAR proteins was cloned into the pEGFP-C1 vector (Clontech). CLC–dsRed was a gift from Prof. George Banting, University of Bristol, UK. GFP–OCRL1 was a gift from Dr Martin Lowe, University of Manchester, UK. CDM8-T7 and CDM8-T7-CHC-hub were kind gifts from Prof. Frances Brodsky, UCSF, CA, USA. pGEX-CHC N-terminal domain construct was a kind gift from Dr Stephen Royle, University of Liverpool, UK. HEK293 cells were grown to 85% confluence in 15 cm dishes prior to transfection with 5 μg of plasmid DNA using PEI, and incubated for 24 hours.

### Antibodies
Mouse monoclonal antibody against SNX1 (clone 5) and CHC were purchased from BD Biosciences, Oxford, UK. Mouse monoclonal antibody against SNX6 was purchased from Sigma-Aldrich, Poole, UK. Rabbit CHC and VPS35 antibodies were purchased from Abcam 330, Cambridge, UK. Mouse monoclonal GFP antibody (mix of clones 7.1 and 13.1) was purchased from Roche, Burgess Hill, UK. Rabbit polyclonal RME-8 antibody was a kind gift from Dr Peter McPherson, McGill University, Canada.

### RNA interference
HeLa cells were grown to 60% confluence and transfected with 10 pmol CHC SMARTpool siRNA or 10 pmol scrambled control siRNA (siRNAs by Dharmacon RNAi Technologies, Thermo-Fisher) using HiPerFect transfection reagent (Qiagen) according to product instructions. 48 hours later cells were split and transfected with a second round of siRNA. Cells were processed for imaging after a further 48 hours.

### Fixed-cell imaging
Coverslips were fixed and stained as described previously (Harterink et al., 2011), prior to imaging on a confocal laser-scanning microscope (SP5-AOBS, Leica Microsystems). Colocalization was quantified using VOLOCITY image analysis software (PerkinElmer Inc.). Volume integration of voxel intensity was calculated using the Pearson's coefficient, measuring the protein of interest relative to a marker. Thresholds were set independently for each channel.

### Live-cell imaging
Cells were transferred to $CO_2$-independent medium (Gibco-Invitrogen) supplemented with 10% fetal calf serum and imaged at 37℃ on a spinning disc confocal system (Perkin-Elmer UltraVIEW ERS 6FE confocal microscope with Yokogawa CSU22 spinning disk) using a 63× lens. Time-lapse imaging was executed at a frame rate of 0.5 Hz for dual colour combinations.

**Fig. 4. Clathrin is absent from SNX4 and SNX8 tubules.** RPE-1 cells were transiently co-transfected with pEGFP-SNX8 or pEGFP-SNX4 (green) and CLC-dsRed (red) prior to live cell imaging after a 16 hour incubation period. (**A**) Images of GFP–SNX8 tubules lacking clathrin. Scale bars: 10 μm. (**B**) Further examples of SNX8 tubules forming from both clathrin-positive and -negative endosomes are indicated by arrows. Scale bars: 1 μm. (**C**) Of 70 SNX8-decorated tubules all were clathrin negative, whereas of 70 tubulating endosomes, 17 were positive for clathrin. (**D**) Image of GFP–SNX4 tubules (arrows) lacking clathrin. Scale bar: 1 μm. (**E**) Further examples of SNX4 tubules devoid of clathrin (arrows). Scale bar: 1 μm. (**F**) Models for the role of clathrin in SNX-BAR-retromer function. Scale bars: 1 μm. (I) Clathrin organises an endosomal sub-domain of clathrin-binding proteins and cargo adaptors, which initiates membrane deformation prior to SNX-BAR-retromer processing of the tubule into a cargo-enriched transport carrier. (II) Clathrin is required for cargo enrichment but not SNX-BAR-retromer-mediated membrane deformation. Binding of SNX-BAR retromer to the edge of the flat clathrin sub-domain leads to BAR-domain-mediated membrane deformation thereby targeting the enriched cargo into a tubular carrier.

### GFP-nanotrap immunoprecipitations

GFP-nanotrap immunoprecipitations were performed as previously described (Harterink et al., 2011), using HEK-293T cells transiently expressing the stated constructs.

### Recombinant protein expression and production

Recombinant protein expression and production was described by Harterink et al. (Harterink et al., 2011).

Supplementary material available online at
http://jcs.biologists.org/lookup/suppl/doi:10.1242/jcs.112904/-/DC1

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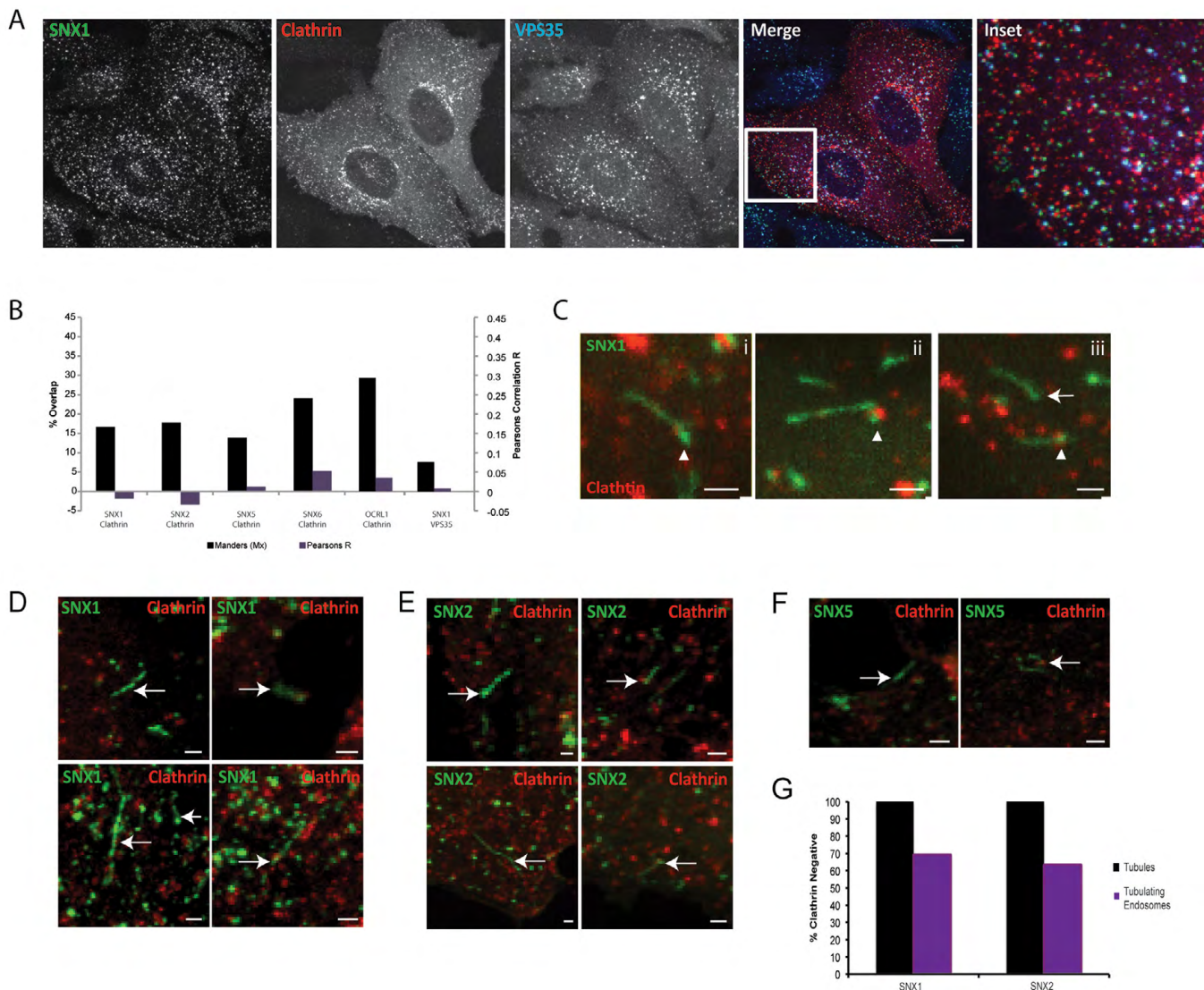
**Fig. S1. (A)** The retromer component VPS35 does not colocalise with CHC. HeLa cells transiently expressing CLC-dsRed were fixed and stained for endogenous SNX1 (green) and endogenous VPS35 (far-red). Scale bar represents 10 μm. **(B)** 10 sample images for each condition in Fig. 1A were re-analyzed with one channel rotated through 180 degrees to generate an image with randomized pixel overlap and correlation. **(C)** Further examples of clathrin-negative GFP-SNX1 tubules emanating from clathrin-positive and clathrin-negative endosomes. RPE-1 cells were transiently co-transfected with pEGFP-SNX1 (green) and CLC-dsRed (red) and cells were subsequently imaged live after a 16 hour incubation period. Scale bar represents 1 μm. **(D)** Clathrin is absent from endogenous SNX1-retromer tubules. HeLa cells were fixed and stained for endogenous SNX1 (green) and endogenous clathrin heavy chain (red). Examples of clathrin-negative SNX1 tubules emanating from clathrin-positive and clathrin-negative endosomes are shown. Of the 52 SNX1-decorated tubules all were clathrin negative, while of the 52 tubulating endosomes that were analysed, 16 were clathrin positive. Shown as percentages in bar graph format in Fig. S1F. Scale bar represents 1 μm. **(E)** Clathrin is absent from GFP-SNX2 retromer tubules. HeLa cells transiently expressing GFP-SNX2 were fixed and stained for endogenous CHC (red). Examples of clathrin-negative GFP–SNX2 tubules emanating from clathrin-positive and clathrin-negative endosomes are shown. Of the 33 SNX2-decorated tubules all were clathrin-negative, while of the 33 tubulating endosomes that were analysed, 12 were clathrin-positive. Shown as percentages in bar graph format in Fig. S1F. Scale bar represents 1 μm. **(F)** Clathrin is absent from GFP-SNX5 retromer tubules. HeLa cells transiently expressing GFP-SNX5 were fixed and stained for endogenous CHC (red). Examples of clathrin-negative GFP-SNX5 tubules emanating from clathrin-positive and clathrin-negative endosomes are shown. Scale bar represents 1 μm. **(G)** Percentage of SNX1 and GFP-SNX2 tubules and tubulating endosomes which were clathrin-negative.
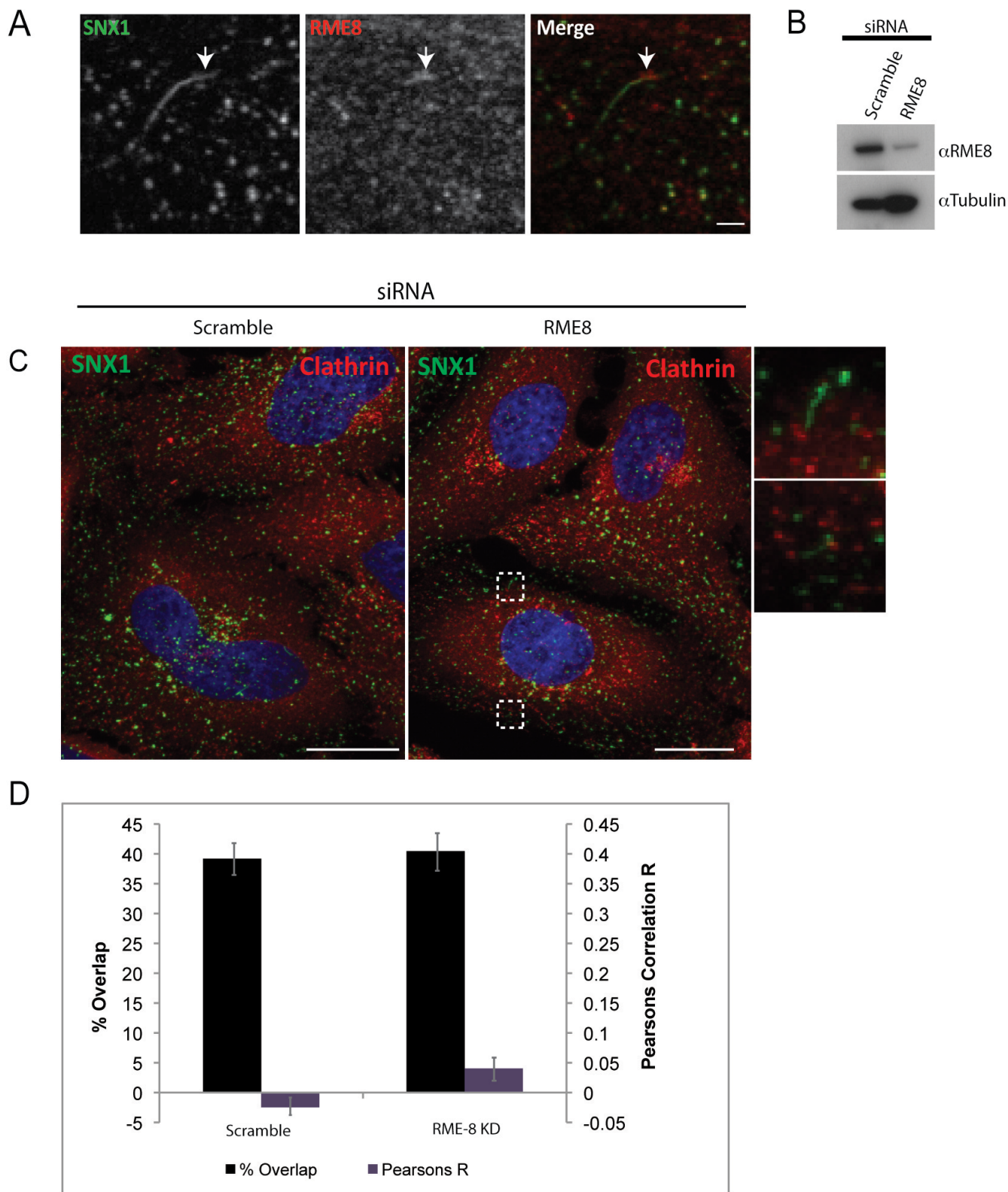
**Fig. S2. (A)** SNX1 colocalises with RME-8 on tubulating endosomes. HeLa cells were fixed and stained for endogenous SNX1 (green) and endogenous RME-8 (red). Scale bar represents 1 μm. **(B)** Western blot confirming knockdown of RME-8 in HeLa cells. **(C)** RME-8 was knocked down in HeLa cells and cells were subsequently fixed and stained for SNX1 and clathrin. Boxed areas are magnified and show SNX1 tubules are still negative for clathrin in RME-8 knockdown cells. **(D)** Colocalisation analysis of SNX1 versus clathrin in RME-8 knockdown cells versus scramble. Shown are the percentage overlap of the individual SNX1 signal with the clathrin signal and the degree of correlation between the two signals when they overlap (Pearson's correlation coefficient). Error bars, SD (*n*=3 with 10 cells per condition).
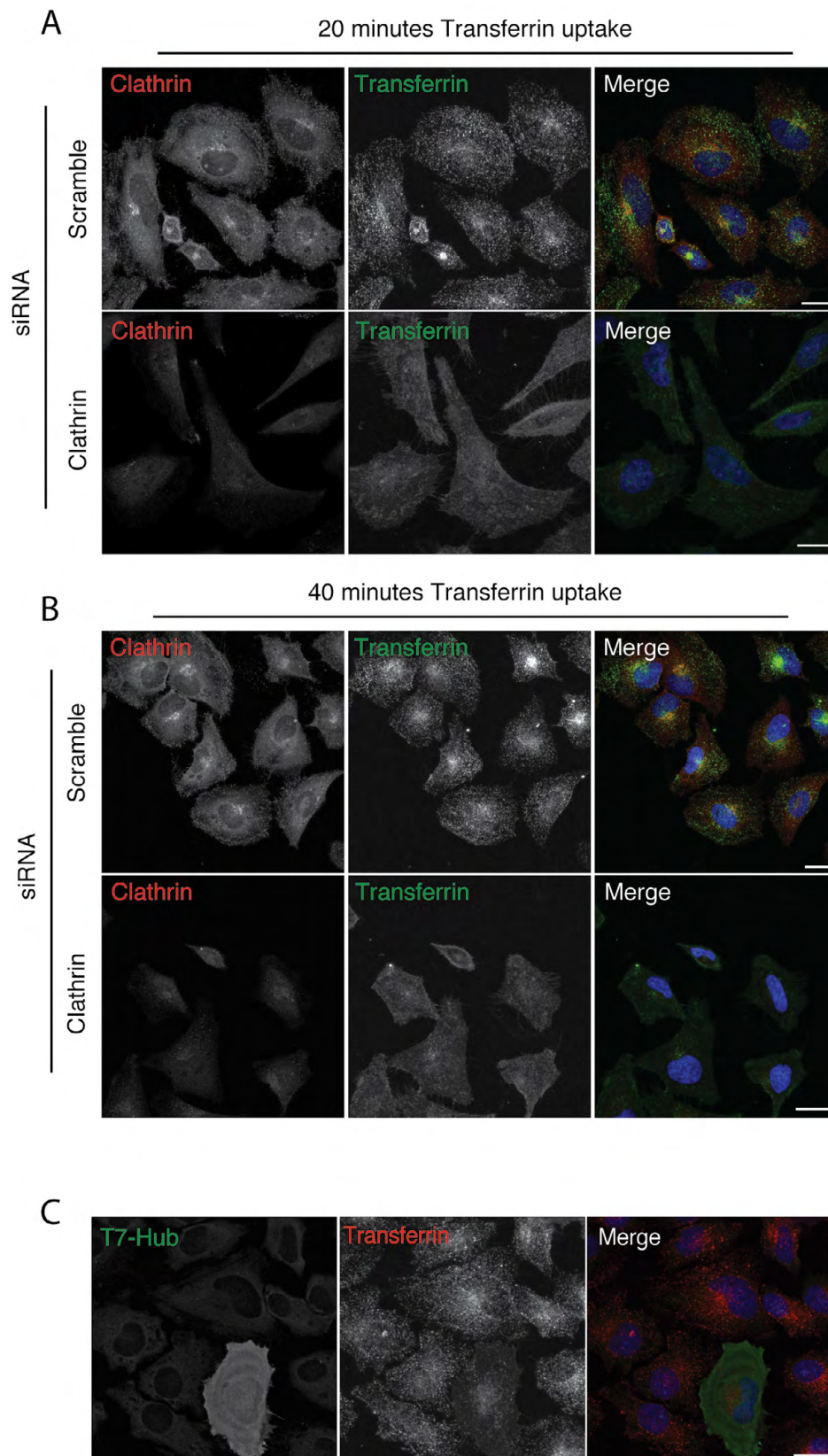
**Fig. S3.** Clathrin knockdown or expression of a T7-tagged clathrin terminal domain inhibits transferrin internalization. **(A,B)** Clathrin was knocked down in HeLa cells. Coverslips were subsequently incubated in serum free medium for one hour prior to addition of serum free medium containing 25 µg/ml of fluorescently labelled transferrin and then fixed after 20 and 40 minutes and stained for clathrin heavy chain to identify cells with efficient knockdown. Scale bar, 10 µm. **(C)** HeLa cells were transiently transfected with a CDM8-T7-CHC-hub domain construct. Cells were incubated in serum free medium for one hour prior to addition of serum free medium containing 25 µg/ml of fluorescently labelled transferrin and then fixed after 30 minutes and stained for T7 (green) after a 24 hour incubation period.

**Movie 1. Formation and scission of a GFP-SNX1 tubule from a vesicle positive for both SNX1 and clathrin.**
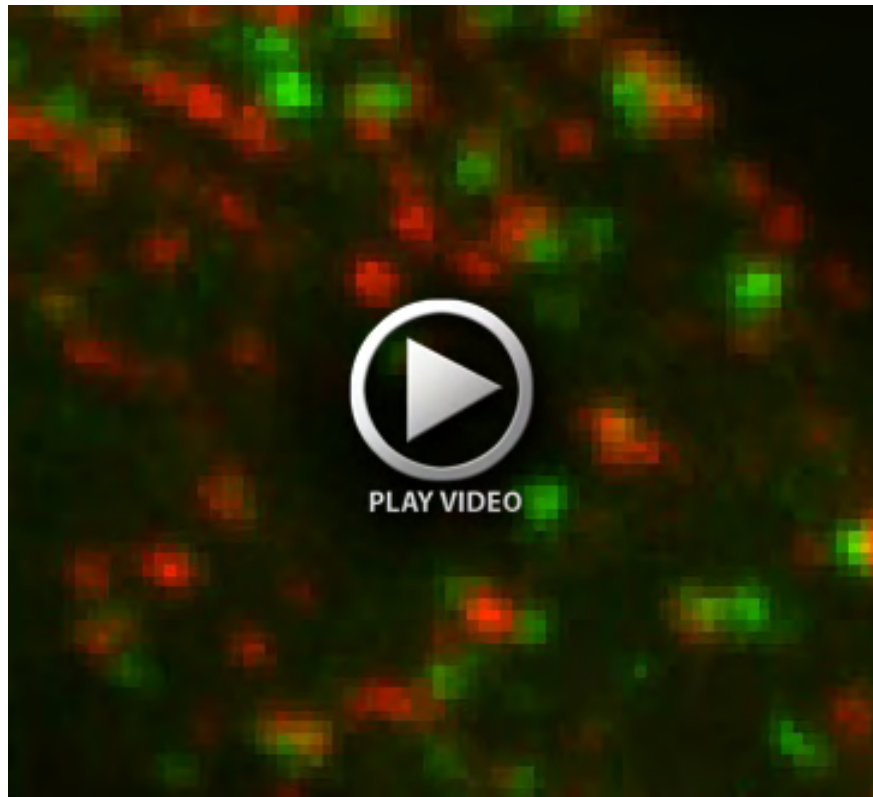


**Movie 2. Formation and scission of a GFP-SNX1 tubule from a vesicle positive for SNX1 but negative for clathrin.**