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CVAK104 is a Novel Regulator of Clathrin-mediated SNARE Sorting

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Clathrin-coated vesicles (CCVs) mediate transport between the plasma membrane, endosomes and the *trans* Golgi network. Using comparative proteomics, we have identified coated-vesicle-associated kinase of 104 kDa (CVAK104) as a candidate accessory protein for CCV-mediated trafficking. Here, we demonstrate that the protein colocalizes with clathrin and adaptor protein-1 (AP-1), and that it is associated with a transferrin-positive endosomal compartment. Consistent with these observations, clathrin as well as the cargo adaptors AP-1 and epsinR can be coimmunoprecipitated with CVAK104. Small interfering RNA (siRNA) knockdown of CVAK104 in HeLa cells results in selective loss of the SNARE proteins syntaxin 8 and vti1b from CCVs. Morpholino-mediated knockdown of CVAK104 in *Xenopus tropicalis* causes severe developmental defects, including a bent body axis and ventral oedema. Thus, CVAK104 is an evolutionarily conserved protein involved in SNARE sorting that is essential for normal embryonic development.

Key words: AP-1, AP-2, CVAK104, epsinR, siRNA, SNARE, *Xenopus*

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Clathrin-coated vesicles (CCVs) are important transport intermediates that facilitate the movement of cargo proteins between the plasma membrane, the endosomal system and the *trans* Golgi network (TGN) (1). The outer shell of the CCV consists of a clathrin lattice, attached to the membrane by interactions with various adaptor proteins (APs), which are also responsible for the sorting of integral membrane cargo proteins into the CCV (2). There are two structurally similar AP complexes associated with CCVs, AP-1 and AP-2, both of which sort a broad range of cargo molecules. Although AP-1 and AP-2 perform analogous functions, they are confined to different CCV pathways. While AP-2 is the principle adaptor for clathrin-mediated endocytosis, AP-1 is involved in intracellular trafficking between the TGN and endosomes. The directionality of the AP-1 pathway, i.e., whether it is TGN-to-endosome, endosome-to-TGN or both, is still unclear (1,3–5).

In addition to the AP complexes, there is a growing number of so-called 'alternative adaptors', which mediate the sorting of specific cargo molecules. An example is the intracellular adaptor epsinR, which sorts the SNARE vti1b (6). There are also numerous regulatory proteins required for the formation, fission, uncoating, movement and targeting of CCVs (3). The importance of these 'accessory proteins' has been increasingly recognized over the past few years, and several proteomic investigations have been carried out to determine the complement of proteins present in CCVs (7–9). One of the novel proteins that was thus discovered is coated-vesicle-associated kinase of 104 kDa [CVAK104 (10)], a protein with a predicted kinase fold that was found to copurify with CCVs from rat brain (7) and HeLa cells (9), as well as with AP preparations from bovine brain (10). CVAK104 is highly conserved among eukaryotes, suggesting that it may be an important regulator of CCV-mediated protein trafficking. A biochemical characterization of CVAK104 showed that it is able to bind to clathrin and to AP-2, and to phosphorylate the β subunit of AP-2 *in vitro*, implying a role in clathrin-mediated endocytosis (10).

In this study, we investigate the function of CVAK104 using a combination of cell and developmental biology. We present evidence that in spite of its ability to interact with AP-2 *in vitro*, CVAK104 is involved in non-endocytic, intracellular CCV trafficking. Our results complement and extend those of an independent study on CVAK104 by Düwel and Ungewickell (11), which was published while this manuscript was in preparation.

Results

Identification of CVAK104 in CCVs

We have recently developed a comparative proteomics approach to identify novel components of CCVs (9). CCV-enriched fractions are prepared from HeLa cells and 'mock CCV' fractions from cells that were depleted of clathrin by small interfering RNA (siRNA) knockdown. Comparison of control and mock CCV fractions allows the identification of *bona fide* CCV proteins as those depleted or absent from the mock CCV fraction. To facilitate the comparison of mock and control CCVs, we have used (among other techniques) fluorescent two-dimensional difference gel electrophoresis (2D-DIGE). Fractions were labelled with

two different fluorescent dyes, pooled and analysed in single 2D gels (Figure 1A). This technique allows us to distinguish proteins that are present in similar quantities in both fractions (red + green = yellow spots), and thus contaminants, from proteins that are depleted or absent from the mock CCVs (red spots) and thus candidate CCV proteins. Using this approach in conjunction with mass spectrometry (MS), we were able to identify both known and unknown CCV components (9). One of the most promising (and at the time uncharacterized) of these proteins was the kinase-like CVAK104, which appears as a series of red spots in Figure 1A.

A diagram of the domain organization of CVAK104 is shown in Figure 1B. CVAK104 is related to a yeast protein, Scy1p, and the homology between the two proteins extends well beyond the N-terminal kinase-like domain. Interestingly, all of the members of the Scy1 family have kinase-like domains that lack key residues for enzymatic activity. The clathrin- and AP-2-binding domain on CVAK104 was mapped by Düwel and Ungewickell (11) to residues 699–929 at the C-terminus of the molecule. Secondary structure predictions indicate that the portion of the protein between the kinase-like domain and the clathrin-binding domain has a high α-helical content, while

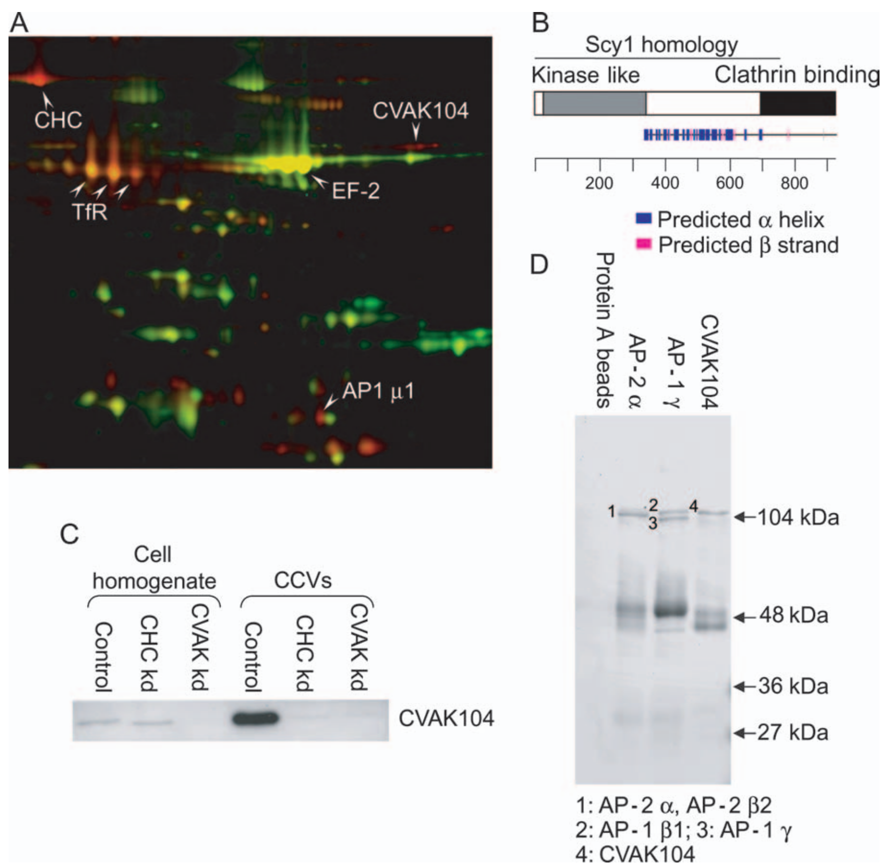

Figure 1: Identification and characterization of CVAK104. A) 2D-DIGE analysis of CCVs. The CCV-enriched fractions were prepared from untreated HeLa cells, and 'mock' CCV fractions from clathrin-depleted HeLa cells. Fractions were labelled with fluorescent dyes and analysed in single 2D gels. The figure shows a false-colour overlay of control CCV (red) and mock CCV (green) fractions. Red spots correspond to proteins that are depleted from the mock CCVs, and thus to candidate CCV components. Yellow spots correspond to contaminants, which are similarly abundant in both fractions. Arrowheads indicate proteins that were identified by MS. B) Schematic diagram of CVAK104 organization, showing the positions of the kinase-like domain and the clathrin-binding domain [mapped by Düwel and Ungewickell (11)]. Homology between the CVAK104 and the *Saccharomyces cerevisiae* protein Scy1p was determined by BLAST (http://www.ncbi.nlm.nih.gov/BLAST/). The predicted secondary structure downstream from the kinase-like domain was determined by JPRED (http://www.compbio.dundee.ac.uk/). C) Western blots of cell homogenates (~7.5 μg protein/lane) and CCV fractions (~2 μg protein/lane) were probed with a new polyclonal antibody against CVAK104. The antibody labels an ~104-kDa band, which disappears when CVAK104 is knocked down with siRNA. The protein is highly enriched in CCVs, and undetectable in mock CCVs. This particular enrichment/depletion profile is characteristic of proteins involved in intracellular clathrin-mediated transport (9). D) HeLa cells were extracted with 0.5% IGEPAL and immunoprecipitated with antibodies against CVAK104, AP-2 α and AP-1 γ in conjunction with protein A, or with protein A beads only (control). Proteins identified by MS are indicated with numbers. The identification of AP-1 and AP-2 subunits other than those directly recognized by the antibodies shows that the experimental conditions were appropriate to co-immunoprecipitate stable protein complexes. CHC, clathrin heavy chain; kd, knockdown; TfR, transferrin receptor; EF, ribosomal elongation factor.

the clathrin-binding domain is likely to be disordered. This type of structure, consisting of one or more folded domains, followed by a disordered segment containing motifs that bind to clathrin and/or adaptors, is found in many of the components of CCVs, including AP complexes, alternative adaptors and accessory proteins (2).

To characterize CVAK104, we raised a polyclonal antibody against its disordered C-terminal domain. This antibody recognizes a band of ~104 kDa on Western blots of HeLa cell homogenates, which disappears almost completely when CVAK104 is knocked down with siRNA (Figure 1C). The Western blot confirms that CVAK104 is highly enriched in control CCV preparations (the homogenate lanes contain four times as much protein as the CCV lanes), and it is almost completely undetectable in mock CCVs, indicating that it is a *bona fide* component of CCVs. We have previously shown that AP-1 and AP-2 have different enrichment and depletion profiles under these conditions, with AP-1 showing much greater enrichment in CCVs and greater depletion from mock CCVs than AP-2 (9). Based on these criteria, the enrichment and depletion profile of CVAK104 is indicative of an association with AP-1 positive, non-endocytic CCVs.

To identify putative interaction partners of CVAK104, the antibody was used to immunoprecipitate detergent (IGEPAL) extracts of HeLa cells (Figure 1D). A single precipitated band was detectable on Coomassie blue-stained gels, which was shown by MS to correspond to CVAK104. No obvious candidate binding partners were observed, suggesting that CVAK104's interactions with other proteins are transient or weak.

Localization, dynamics and membrane association of CVAK104

To determine the localization of CVAK104, we used the antibody for immunofluorescence microscopy, mixing together control and CVAK104-depleted cells (Figure 2A). CVAK104 is localized to the perinuclear region of the cell as well as to more peripheral structures, and this pattern disappears when CVAK104 is knocked down using siRNA, confirming the specificity of the labelling. Double labelling for other coat proteins shows extensive (although not complete) colocalization between CVAK104 and clathrin (Figure 2B), and between CVAK104 and AP-1 (Figure 2C). However, CVAK104 shows no apparent colocalization with AP-2 (Figure 2D), even though it can bind to AP-2 *in vitro* (10).

To further characterize the nature of CVAK104's association with membranes, we used the drug brefeldin A (BFA), which prevents the small guanosine triphosphatase ADP ribosylation factor (ARF) from binding to membranes. The BFA causes AP-1 and other intracellular coat components to redistribute to the cytosol, but it does not affect the association of AP-2 with the plasma membrane (12). The HeLa cells were treated with BFA for 5 minutes, then imaged by immunofluorescence microscopy (Figure 2E). Upon treatment with BFA, CVAK104 became largely cytosolic, indicating that its association with membranes is mediated by ARF. Collectively, these findings suggest that CVAK104 is associated with intracellular rather than endocytic CCVs.

To observe the dynamics of CVAK104 in living cells, we transiently transfected HeLa cells with a CVAK104-green fluorescent protein (GFP) fusion protein. Like native CVAK104, CVAK104-GFP localizes to intracellular vesicular structures with no significant labelling of the plasma membrane (Figure 3A). The video link (Video S1) shows that many of these structures are highly mobile. To ensure that GFP tagging does not alter the targeting of CVAK104, we also imaged CVAK-GFP by immunofluorescence microscopy in fixed HeLa cells. The fusion protein has a very similar distribution to endogenous CVAK104 and colocalizes substantially with AP-1 (Figure S1A). This suggests that the observed live-cell movements of CVAK-GFP are likely to reflect those of untagged CVAK104. To determine how clathrin affects the targeting of CVAK104, we transiently expressed CVAK104-GFP in HeLa cells that had been depleted of clathrin heavy chain by siRNA knockdown (Figure 3B and Video S2). In general, the distribution of CVAK104-GFP appeared to be more cytosolic in clathrin-depleted cells; however, a significant pool of CVAK104-GFP remained associated with vesicular structures, which were largely immobile. In addition, we observed an association of CVAK104-GFP with mobile tubular structures (white arrows in Figure 3B), indicating that CVAK104 is capable of binding to membranes in a clathrin-independent manner.

The binding site on CVAK104 for clathrin is located in its C-terminal disordered region (11). Because the N-terminal domain of CVAK104 has a predicted kinase fold, we speculated that this part of the protein may also be a functionally important unit. To assess whether the kinase-like domain carries any targeting information, we attached yellow fluorescent protein (YFP) to a truncated version of CVAK104 (residues 1–387) and transiently expressed it in HeLa cells (Figure 3C and Video S3). This construct too was able to localize to membranes, associating both with mobile vesicular structures and with mobile tubules. The vesicular but not the tubular structures showed substantial colocalisation with endogenous CVAK104 and AP-1, as ascertained by immunofluorescence of fixed HeLa cells (Figure S1B). Expression of the truncated YFP construct in HeLa cells that had been depleted of clathrin heavy chain showed increased labelling of the mobile tubular structures (Figure 3D and Video S4). Together, our findings demonstrate that the kinase-like domain of CVAK104 contains targeting information that allows binding to an intracellular compartment, independently of clathrin.

To determine the identity of the CVAK104-positive membranes, we monitored the uptake of transferrin in HeLa

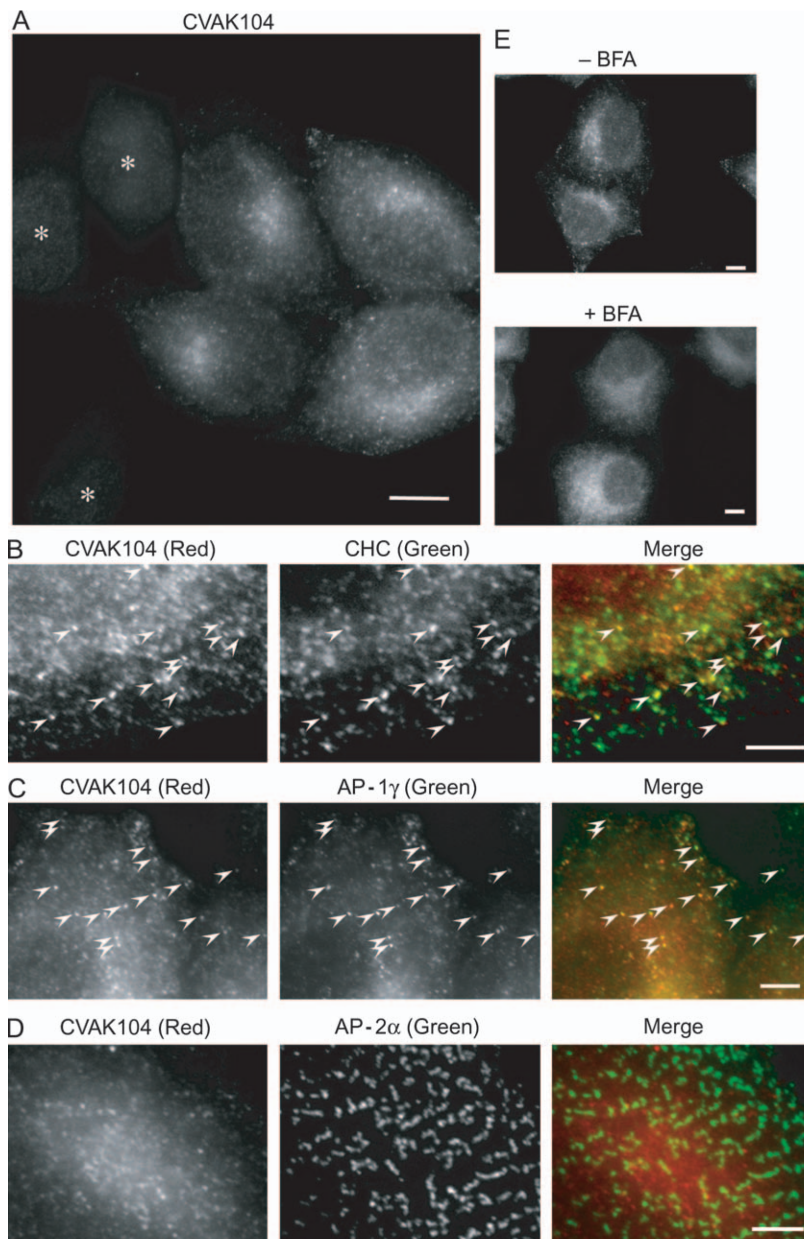

Figure 2: Immunolocalization of CVAK104. A) HeLa cells were either treated with siRNA against CVAK104 or untreated (control), then mixed and labelled with anti-CVAK104 antibody. Asterisks indicate CVAK104-depleted cells. Scale bar: 10 μm. B–D) Double labelling for CVAK104 and coat proteins. B) HeLa cells were double labelled for CVAK104 and CHC. The two patterns show significant colocalization. C) HeLa cells were double labelled for CVAK104 and AP-1 γ. Again the two patterns show significant colocalization. D) HeLa cells were double labelled for CVAK104 and AP-2 α. The two patterns show no significant colocalization. Scale bars: 5 μm. E) HeLa cells were treated with BFA (20 μg/mL) for 5 minutes at 37 °C or treated with medium only (control), and labelled with anti-CVAK104 antibody. Upon BFA treatment, CVAK104 loses its membrane association and becomes largely cytosolic. Scale bars: 10 μm. CHC, clathrin heavy chain. Arrowheads indicate areas of colocalisation.

cells expressing CVAK104-GFP (Figure 3E and Videos S5,S6). Fluorescently labelled transferrin was pre-bound at 4 °C, and then the cells were warmed to 37 °C and imaged immediately. There was substantial colocalization between CVAK104-GFP and transferrin at early time-points (Figure 3E, 1 and 10 minutes; Video S5) but not at later time-points (Figure 3E, 60 minutes; Video S6), indicating that like AP-1, CVAK104 is at least partially associated with early and/or recycling endosomes.

Functional characterization of CVAK104 in HeLa cells

To investigate the function of CVAK104, we used siRNA to deplete the protein in HeLa cells and then examined the phenotype. In most respects, the cells appeared to be normal. CVAK104 depletion had no apparent effect on the localization of either AP-1 or AP-2, or on the steady-state distribution or surface expression of the chimeric proteins CD8-furin and CD8-CIM6PR (unpublished data), both of which are highly sensitive to disturbances in AP-1-mediated trafficking (13). Transferrin recycling, which is also affected by AP-1 knockdown (13), was similarly unaltered by CVAK104 depletion (unpublished data). We also investigated whether CVAK104 influences the protein composition of CCVs by preparing CCV-enriched fractions from control cells and from cells that had been depleted of CVAK104 by siRNA knockdown and then analysing the two fractions by 2D-DIGE. The only protein that changed substantially was CVAK104 itself (unpublished data), indicating that the absence of CVAK104 does not result in any major changes in CCV composition.

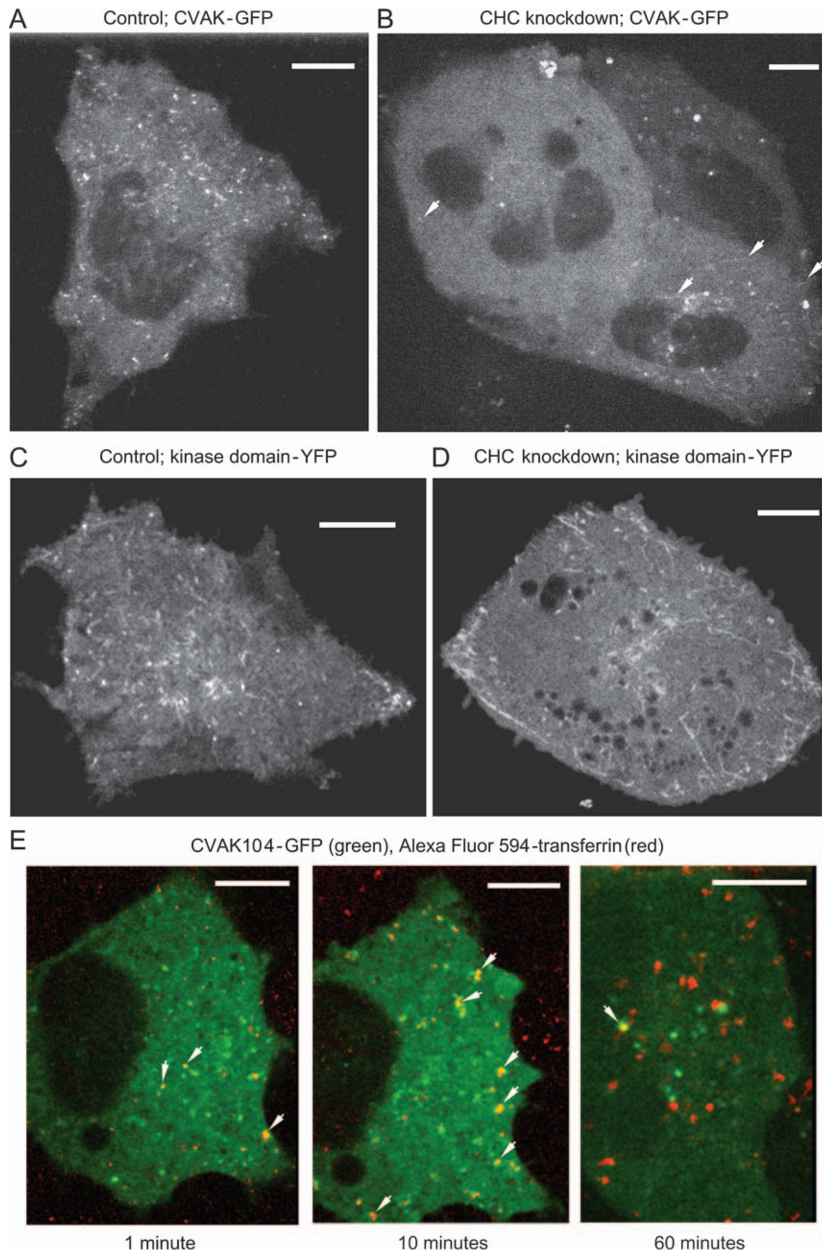

Figure 3: Live-cell imaging of tagged CVAK104 constructs. A and B) CVAK104 was tagged with GFP at the C-terminus and transiently expressed in HeLa cells. A) In control cells, CVAK104-GFP is present in small and highly mobile compartments. B) In clathrin-depleted cells, CVAK104-GFP is more cytosolic but also labels largely immobile vesicular compartments as well as mobile tubular structures (white arrows). C and D) The N-terminal kinase-like domain of CVAK104 was tagged with YFP and transiently expressed in HeLa cells. C) In control cells, the construct localizes to mobile tubular and vesicular structures. D) In clathrin-depleted cells, the construct localizes mainly to tubular mobile structures. E) CVAK104-GFP was transiently expressed in HeLa cells. Cells were allowed to endocytose pre-bound Alexa Fluor-labelled transferrin, and imaged either ∼1, ∼10 or ∼60 minutes after beginning of uptake by spinning-disk confocal microscopy. Frequent colocalization (white arrows) can be observed at early, but not at late stages of the experiment. Time-points 1 and 10 minutes show images of the same cell, taken from a continuous movie (Video S5). Representative still images are shown here; the corresponding movies are available as Videos S1–S6 (online supplemental material). Scale bars: 10 μm.

Next, we considered the possibility that CVAK104 might contribute to the sorting of a subset of CCV cargo molecules. In our previous proteomic analysis of CCVs, we established that at least 10 TGN and endosomal SNARES are associated with CCVs (9). This strongly suggests that many post-Golgi SNARES are trafficked in a clathrin-dependent manner; however, it is not generally known how they are selectively incorporated into CCVs (14).

To investigate a potential function for CVAK104 in post-Golgi SNARE trafficking, CCVs (or mock CCVs) were prepared from control, clathrin-depleted and CVAK104-depleted cells. Western blots of the fractions were then probed for 10 different SNAREs, including syntaxin 6, 7, 8 and 16; vti1a and vti1b; VAMP 3, 4 and 7 and SNAP29, all of which are constituents of CCVs (9). While the majority of SNAREs were unaffected or only marginally affected by CVAK104 depletion, there was a significant and highly reproducible loss of syntaxin 8 and vti1b (Figure 4 and unpublished data), indicating that CVAK104 helps to package these SNAREs into CCVs. We suspect that syntaxin 8 and vti1b are sorted as a dimer because they are known to interact with each other and to depend on each other for stability, to the extent that vti1b knockout mice also lose syntaxin 8 (15).

Vti1b requires both AP-1 and epsinR for correct packaging into CCVs; in fact, the observed loss of vti1b from CCVs in

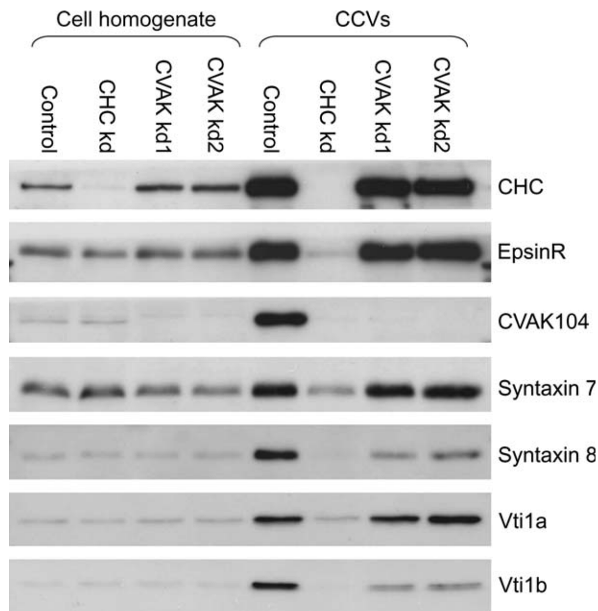

Figure 4: CVAK104 kd results in specific depletion of syntaxin 8 and vti1b from CCVs. Cell homogenates and CCV fractions were prepared from control and siRNA-treated cells and analysed by Western blot. CVAK kd1 and CVAK kd2 denote samples from two independent kd experiments. Approximately 10 μg protein of whole cell lysates or 2.5 μg of CCV fractions were loaded in each lane. Although the four SNAREs, syntaxin 7, syntaxin 8, vti1a, and vti1b, are all sorted by clathrin, only syntaxin 8 and vti1b are depleted from CCVs in a CVAK104 kd. CHC, clathrin heavy chain; kd, knockdown.

a CVAK104 knockdown is comparable with that reported for AP-1 or epsinR knockdowns (6). We therefore investigated a possible link between CVAK104 and AP-1/epsinR. Because our initial biochemical characterization indicated that CVAK104's interactions with other proteins are labile (Figure 1D), we performed CVAK104 immunoprecipitations from digitonin extracts of HeLa cells. These gentle conditions leave many of the more delicate cargo protein interactions intact (unpublished observation). Analysis by Western blot revealed that CVAK104 co-immunoprecipitates clathrin, AP-1 γ and epsinR (Figure 5), suggesting that CVAK104 may directly regulate the function of these proteins. Under the same conditions, no co-immunoprecipitation of the cation-independent mannose 6-phosphate receptor (CIMPR) or the AP-3 μ subunit was observed.

CVAK104 and Xenopus *development*

Mice with a deletion in the vti1b gene and concomitant destabilization of syntaxin 8 have been reported to be viable and healthy (15). Thus, if the only function of CVAK104 is to assist the sorting of syntaxin 8 and vti1b, CVAK104 depletion in an animal should also produce a very mild phenotype. To test this hypothesis, one-celled *Xenopus tropicalis* embryos were injected with a morpholino (MO) antisense oligonucleotide to knockdown CVAK104. Figure 6A (panels 1, 2, 5 and 6) shows that the phenotype

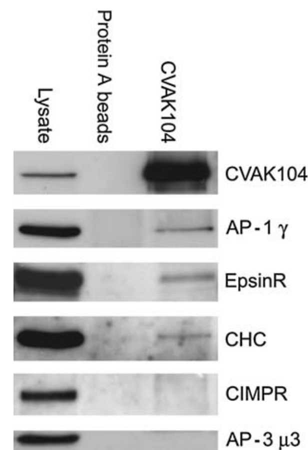

Figure 5: CVAK104 interacts with clathrin, AP-1 and epsinR. HeLa cells were extracted with 1% digitonin and immunoprecipitated with an antibody against CVAK104 in conjunction with protein A linked to Sepharose beads, or with protein A beads only (control). Immunoprecipitates were analysed by Western blot. CHC, clathrin heavy chain.

of CVAK104-depleted embryos is in fact severe. Abnormalities include reduced axial extension, bent anterior–posterior axis and ventral oedema.

To ensure that the CVAK104 knockdown phenotype was specifically caused by the depletion of the protein and not by off-target effects, we carried out a 'rescue' experiment, in which we co-injected the embryos with the MO as well as with messenger RNA (mRNA) encoding myc-tagged human CVAK104. The human and frog proteins are 77% identical and because of sequence variation at the mRNA level, human CVAK104 is not targeted by the MO. The tagged protein was detectable by Western blotting in both control and MO-treated embryos (Figure 6B). Expressing human CVAK104 in control embryos had no apparent effect (Figure 6A, panels 4 and 8), indicating that elevated levels of the protein are not toxic. The embryos that were co-injected with human CVAK104 mRNA and MO showed a high level of rescue at both tailbud and tadpole stages (Figure 6A, panels 3 and 7), indicating that the effects of MO knockdown are indeed specific to depletion of CVAK104.

To obtain reference phenotypes for defects in clathrin-mediated trafficking, we also performed MO knockdowns of AP-1 (μ1A) and AP-2 (μ2) (Figure 6C). The AP-2-depleted embryos arrested very early during development, and none survived beyond the tailbud stage. In contrast, AP-1 knockdown resulted in a phenotype that shares some similarities with that of CVAK104 knockdown, including a shortened and bent anterior–posterior axis and ventral oedema. Together, these observations indicate that, like AP-1 and AP-2, CVAK104 is needed for normal embryonic development, suggesting that it has other functions in addition to its role in sorting syntaxin 8 and vti1b.

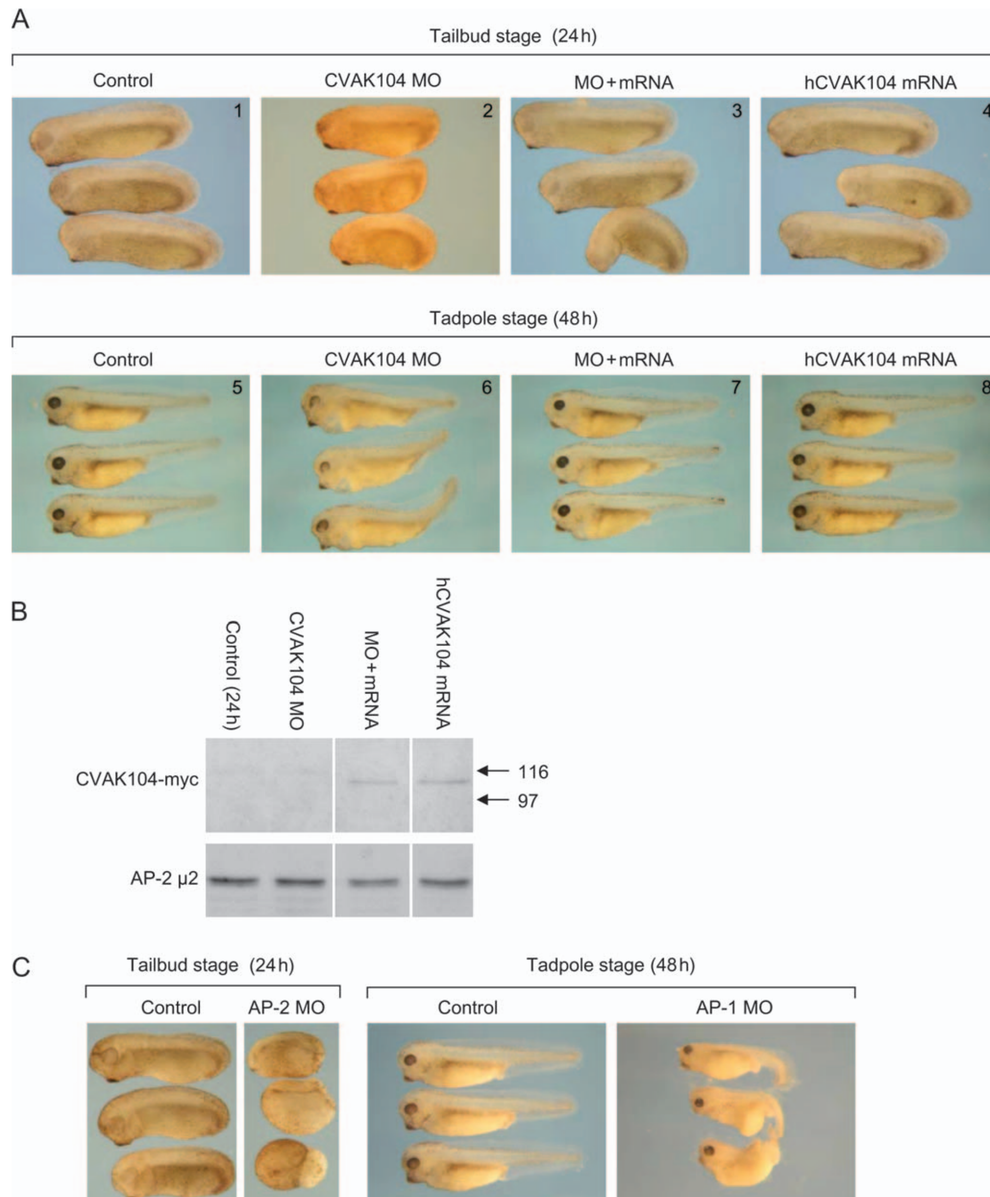

Figure 6: CVAK104 knockdown and rescue in *Xenopus*. A) Fertilized *X. tropicalis* eggs were injected with a control MO (panels 1 and 5) or a MO against CVAK104 (panels 2 and 6) at the one-cell stage and imaged either 24 h or 48 h post-fertilization. To rescue the knockdown phenotype, fertilized eggs were injected with anti-CVAK104 MO + mRNA encoding human CVAK104-myc (panels 3 and 7), then imaged either 24 h or 48 h post-fertilization. Expression of human CVAK104-myc resulted in high levels of rescue both at tailbud and tadpole stages. Expression of human CVAK104-myc alone had no apparent detrimental effects (panels 4 and 8). B) Western blots of extracts from 24 h embryos were probed with anti-myc antibody. AP-2 μ2 was included as a loading control. Human CVAK104-myc was detectable in embryos that had been injected with mRNA. C) Fertilized eggs were injected with either a control MO, a MO against AP-2 μ2, or a MO against AP-1 μ1A, then imaged either 24 h or 48 h post-fertilization. Both knockdowns cause severe phenotypes, but the AP-1 knockdown phenotype is more similar to the CVAK104 knockdown phenotype.

Discussion

In this study, we have identified and characterized CVAK104 as a novel accessory protein for intracellular CCV-mediated trafficking. Our findings are in good agreement with those of an independent study by Düwel and Ungewickell (11), and they significantly advance our understanding of CVAK104 function by demonstrating its involvement in SNARE sorting and in embryonic development.

CVAK104 is unlikely to be involved in endocytosis

Conner and Schmid (10) and Düwel and Ungewickell (11) showed that CVAK104 can bind to AP-2 *in vitro*, suggesting that it functions in clathrin-mediated endocytosis.

However, our findings argue against a role for CVAK104 at the plasma membrane and instead indicate that it functions in intracellular trafficking. First, by Western blotting, CVAK104 shows enrichment in CCVs and depletion from mock CCVs similar to that of AP-1, not AP-2 (Figure 1C) (9). Second, CVAK104 colocalizes with AP-1 but not with AP-2 (Figure 2). Third, endocytosed transferrin colocalizes with CVAK104-GFP in an early endosomal compartment but not at the plasma membrane (Figure 3E). Similar colocalization data were reported by Düwel and Ungewickell (11). Fourth, CVAK104 is important for the sorting of syntaxin 8 and vti1b, which reside in intracellular membranes (Figure 4). Fifth, AP-1 and epsinR can be coimmunoprecipitated with CVAK104 (Figure 5). Sixth, knockdown of CVAK104 in *Xenopus* embryos results in defects that are similar to those caused by knockdown of AP-1 but are dissimilar to those caused by knockdown of AP-2 (Figure 6). It is also worth noting that the yeast homologue of CVAK104, Scy1p, colocalizes with clathrin on intracellular membranes (16).

CVAK104 targeting is only partially dependent on clathrin

CVAK104 binds to clathrin heavy chain *in vitro* (10), and Düwel and Ungewickell (11) have characterized this interaction extensively. They showed that CVAK104's C-terminal domain (residues 699–929) mediates the interaction with clathrin, and it is also capable of targeting a GFP fusion protein to clathrin- and AP-1-positive membranes. In addition, they reported that CVAK104 dissociates from membranes in clathrin-depleted cells (although by Western blotting, they saw only a 30% reduction in the amount of CVAK104 cofractionating with a membrane-containing pellet). In contrast, we find that clathrin knockdown reduces but does not abolish CVAK104's membrane association. Moreover, when we expressed a YFP fusion of the N-terminal kinase-like domain of CVAK104, which lacks any clathrin-binding activity (11), we found that this construct was able to localize to intracellular membranes, both in the presence and in the absence of clathrin (Figures 3C,D and S1B). These membranes show partial colocalization with internalized transferrin (unpublished data) and are likely to correspond to the donor compartment from which CVAK104-positive vesicles bud. Presumably, the C-terminal domain with the clathrin-binding sites is needed for CVAK104 to be packaged efficiently into CCVs. The ability of the N-terminal domain to be targeted to intracellular membranes on its own may also help to explain why CVAK104 is associated with intracellular rather than plasma membrane-derived CCVs.

CVAK104 is involved in trafficking of post-Golgi SNAREs

The SNAREs are particularly important components of transport vesicles because they enable the vesicles to fuse with the appropriate acceptor compartment; however, little is known about how they are trafficked. In a previous study, we established that numerous post-Golgi SNAREs are present in CCVs (9). Here, we show that knocking down CVAK104 significantly reduces the levels of syntaxin 8 and vti1b in CCVs, indicating that CVAK104 may be part of the sorting machinery for these particular SNAREs. Intriguingly, there was no concomitant change in the level of syntaxin 7, even though it is part of the same Q-SNARE complex and all three proteins are strongly depleted from mock CCVs prepared from clathrin-depleted cells. This has important implications for SNARE trafficking. To be fusogenic, the three Q-SNAREs must form a heterotrimer, so the observation that syntaxin 7's enrichment in CCVs is unaffected by CVAK104 knockdown strongly suggests that although all three SNAREs rely on CCVs for transport, they do not traffic as a fusion-competent complex. Whether they are sorted into different CCVs, or are present in the same CCV but are kept apart (e.g., by syntaxin 7 adopting its closed conformation), remains to be determined. Either way, this presents a possible explanation for how SNAREs can be trafficked by CCVs without causing non-specific fusion events before reaching their final destination.

What could be CVAK104's mechanism of action? CVAK104 knockdown does not lead to any global changes in CCV composition, as judged by 2D-DIGE, and we have not observed a direct interaction between CVAK104 and syntaxin 8 or vti1b (unpublished data). This suggests that CVAK104 is unlikely to function as a cargo adaptor, such as epsinR, which binds and sorts vti1b (6). However, we have shown that clathrin, AP-1 and epsinR can be coimmunoprecipitated with CVAK104 (Figure 5), and all three of these proteins are required for efficient sorting of vti1b into CCVs (6). Because CVAK104 knockdown does not affect the amount of epsinR (or clathrin) present in CCVs (Figure 4), it seems likely that CVAK104 may indirectly affect protein sorting by regulating the activity of adaptors (e.g., epsinR, AP-1) and/or accessory proteins. Although CVAK104 has a predicted kinase fold, it lacks key catalytic residues, and there is some discrepancy in the literature over whether or not CVAK104 has any enzymatic activity (10,11). We show in the present study that this domain is capable of targeting to intracellular membranes, and it is possible that it may have a different function, such as to act as a binding domain for other proteins and/or lipids.

CVAK104 knockdown has been reported to cause missorting of the lysosomal enzyme cathepsin D (11), suggesting that CVAK104 may also be required for the incorporation of mannose 6-phosphate receptors into CCVs. In the present study, we did not detect any effect of CVAK104 knockdown on the distribution of the chimeric CD8-CIM6PR; similarly, under conditions where CVAK104 clearly interacts with epsinR and AP-1, we could not detect any interaction with CIMPR (Figure 5). We did, however, observe a very slight decrease in the levels of endogenous CIMPR in CCVs prepared from CVAK104-depleted HeLa cells (unpublished data). In any case, both our own findings

and those of Düwel and Ungewickell (11) are in agreement in that they support a function for CVAK104 in protein sorting at the TGN and/or endosomes.

AP-1, AP-2 and CVAK104 are important for Xenopus *development*

Using *X. tropicalis* embryos as a model system, we have investigated the consequences of interfering with CVAK104 and other CCV components during development. By knocking down CVAK104, AP-1 and AP-2, we were able to show that all three components are required for normal morphogenesis. Deprivation of AP-2 had the most severe consequences; most embryos arrested at very early stages, and none of them survived past the tailbud stage. The AP-1 knockdown also caused serious defects, including ventral oedema, bent anterior–posterior axis and reduced axial extension. This highlights the tremendous importance of clathrin-mediated trafficking during embryogenesis, and it provides reference phenotypes for the knockdown of other components of the CCV machinery. Knocking down CVAK104 also caused ventral oedema, bent anterior–posterior axis and reduced axial extension, although not to the same extent as the AP-1 knockdown. The similarities between the two phenotypes support the idea that AP-1 and CVAK104 function in the same pathway. Interestingly, similar phenotypes have also been observed in embryos injected with MOs that target signalling molecules, such as growth factors and their receptors (17). Our current working hypothesis is that AP-1 and CVAK104 may help to sort these molecules, something that can be tested by analysing CCV fractions from control and siRNA-treated HeLa cells. Although we have so far only examined the consequences of CVAK104 depletion in *Xenopus*, the evolutionary conservation of CVAK104 between frogs and humans, as shown by the rescue experiment, suggests that this protein plays an important role in all vertebrates. Thus, the developing *Xenopus* embryo may provide a powerful tool to investigate CVAK104 function in more detail. Knockdown and attempted rescue with deletion mutants or truncated versions of human CVAK104 may give insights into which domains of the protein are required for full or partial function.

Materials and Methods

RNA interference and CCV preparations

The siRNA-mediated knockdown of clathrin heavy chain was performed with a custom-made duplex (Dharmacon, Lafayette, CO, USA) described by Motley et al. (18). For CVAK104 knockdowns, Dharmacon's SMART-pool siRNA was used (FLJ10074). Knockdowns were performed as described by Borner et al. (9). The CCV-enriched fractions were prepared from control and knockdown HeLa cells as described (9).

Gel electrophoresis and MS

The SDS–PAGE and Western blots were performed using standard methods. Antibodies against the following proteins were used: AP-1 γ (mAb100/3) (Sigma-Aldrich, St Louis, MO, USA); AP-3 μ3 (19); CIMPR (gift from Paul Luzio, Cambridge Institute for Medical Research, University of Cambridge, UK); CVAK104 (this study); clathrin heavy chain (19); c-myc (A-14) (Santa Cruz Biotechnology, Santa Cruz, CA, USA); epsinR (20); SNAP29 (Synaptic Systems, Goettingen, Germany); AP-2 μ2 (AP50), syntaxin 8, vti1a, vti1b, (BD Transduction Laboratories, San Diego, CA, USA); syntaxin 6, syntaxin 7, syntaxin 16 (gifts from Andrew Peden, Cambridge Institute for Medical Research, University of Cambridge, UK); VAMP 3 (Abcam, Cambridge, UK); VAMP4 (21); VAMP7 (22). The 2D-DIGE analysis was performed as described by Borner et al. (23). Gel pieces containing proteins of interest were excised, and analysed by MALDI-TOF or TOF-TOF, or by liquid chromatography (LC) tandem MS (LC-MS/MS), as described in Borner et al. (9).

Immunoprecipitations

For immunoprecipitations, HeLa cells were extracted with 0.5% IGEPAL CA-630 (Sigma-Aldrich) in PBS (150 mM NaCl, 25 mM phosphate buffer, pH 7.5), or with 1% digitonin (Fluka, Neu-Ulm, Germany) in 2-(N-morpholino) ethanesulfonic acid buffer (0.1 M MES, 1 mM $MgC_2H_5O_2$, 0.5 mM EGTA, pH 6.5), supplemented with 0.2 mM protease inhibitor AEBSF (Calbiochem, San Diego, CA, USA). Insoluble material was removed by centrifugation and filtration (pore size of 0.2 μm). Extracts were pre-cleared by incubation with Sepharose-coupled protein A. Antibodies were added to extracts at 1/100 dilutions (no antibody was added to control samples). Sepharose-coupled protein A was added to recover immunoprecipiates. Sepharose beads were washed five times in ice-cold PBS–IGEPAL (0.1%) and once in PBS, or five times in ice-cold MES buffer (1% digitonin). Proteins were eluted with 2.5% SDS/50 mM Tris, pH 8.0 at 90 °C for 2 $\times$ 2 minutes. The following antibodies were used: anti-CVAK104 (this study); anti-AP-2 α (24) and anti-AP-1 γ (25).

CVAK104 constructs

An expressed sequence tag encoding full-length human CVAK104 (929 aa; IMAGE Clone ID 6473586; gi39645499) was obtained from the Sanger Centre (UK). A rabbit polyclonal antibody was raised against a glutathione S-transferase fusion of the C-terminal domain of CVAK104 (aa 650–920). For live-cell imaging, DNA encoding full-length CVAK104 (aa 1–929) was amplified by polymerase chain reaction (PCR) and cloned into the *Eco*R1/*Bam*H1 sites of pEGFP-N (Clontech, Mountain View, CA, USA). To obtain a CVAK104-kinase domain fusion, the PCR product encoding full-length CVAK104 was digested with *Eco*R1/*Kpn*1. The resulting fragment (base pair 1–1161) encoded CVAK104-kinase domain (and some flanking sequence; aa 1–387) and was cloned into the *Eco*R1/*Kpn*1 sites of pEYFP-N (Clontech). For expression in *Xenopus*, a fragment encoding full-length human CVAK104-myc (aa 1–929 + c-myc) was amplified by PCR and cloned into the *Eco*R1/*Not*1 sites of vector pCS107.

Immunolocalization, live-cell imaging and transferrin uptake

For immunofluorescence microscopy, HeLa cells were either fixed with 3% paraformaldehyde and permeabilized with 0.1% TX-100 or fixed with methanol at −20 °C. Antibodies against the following proteins were used: AP-1 γ (mAb100/3) (Sigma-Aldrich); AP-2 α (AP.6) (a gift from Frances Brodsky, Department of Pharmacy, School of Pharmacy, University of California San Francisco); clathrin heavy chain (X22) (a gift from Frances Brodsky); CVAK104 (this study); GFP (rabbit polyclonal, Abcam). For some experiments, cells were treated with 20 μg/mL BFA for 5 minutes at 37 °C before fixation. Cells were imaged with a Zeiss Axiovert 200 inverted microscope using a Zeiss Plan Achromat 63$\times$ oil immersion objective, 1.40 NA, a Hamamatsu Orca ER2 camera and Improvision Openlab software. For live-cell work, HeLa cells were transfected with CVAK104-GFP or CVAK104-kinase-domain-YFP for 16–18 h using FuGENE 6 (Roche Diagnostics, Indianapolis, IN, USA). For transferrin uptake, cells were preincubated at 4 °C for 15 minutes in serum-free medium containing 1% BSA and 25 mM HEPES. Cells were then incubated at 4 °C with Alexa Fluor 594-labelled human transferrin (Molecular Probes, Eugene, OR, USA) for 30 minutes. Cells were washed with ice-cold PBS and kept on ice until imaged. All live-cell imaging was performed at 37 °C. Images were acquired using

a Perkin Elmer UltraView Spinning Disk Confocal Microscope, fitted with a Hamamatsu Orca ER2 camera and a Peltier-driven heated stage, using a Zeiss Plan Achromat 63× oil immersion objective, 1.40 NA. Image sequences were processed using ImageJ (http://rsb.info.nih.gov/ij).

Morpholinos

Translation-blocking antisense morpholino oligonucleotides against AP-1 μ1A, AP-2 μ2 and CVAK104 were designed with the following sequences: GCCGAAGATAGTCCTGCAAAGGCAA (AP-1 μ1A) GATCTTCACTCTCTCGC GGCTCCTC (AP-2 μ2) GTTTGTTTAGCATCGACTCCATTTG (CVAK104)

The MOs were modified by addition of lissamine fluorochromes. One-celled *X. tropicalis* embryos were injected with 20 ng of antisense or control (Gene Tools standard control; Gene Tools, Philomath, OR, USA) MO in 2 nL H_2O. In rescue experiments, the 20 ng of antisense MO or control MO was co-injected with 1.2 ng of rescue mRNA. To synthesize rescue mRNA, the rescue construct CVAK104-myc-pCS107 was linearized by digestion with *Asc*I, and transcribed using the Sp6 pol mMESSAGEmMACHINE Kit (Ambion, Austin, TX, USA) according to the manufacturer's instructions. Embryos were observed, photographed and fixed in 4% formaldehyde or frozen for protein analysis at gastrula (stages 10–12), tailbud (stages 24–28) and tadpole (stages 37–41) stages. For protein analysis, tadpoles were homogenized in PBS-AEBSF in a Dounce homogenizer at 4 °C. Debris was pelleted by centrifugation at 5000 × ***g*** for 7 minutes. Supernatants were analysed by Western blot.

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Supplementary Materials

Figure S1: Localisation of CVAK104-GFP and CVAK104-kinase-domain-YFP in fixed HeLa cells. A) CVAK104-GFP was transiently expressed in HeLa cells. Cells were fixed, and AP-1 γ and CVAK104-GFP were detected by immunofluorescence microscopy. CVAK104-GFP shows a distribution that is highly similar to endogenous CVAK104, as well as substantial colocalisation with AP-1 γ (white arrowheads). B) CVAK104-kinase-domain-YFP was transiently expressed in HeLa cells. Cells were fixed, and endogenous CVAK104 and CVAK104-kinase-domain-YFP were detected by immunofluorescence microscopy. The two proteins show substantial colocalisation in vesicular structures (white arrowheads) but not in the kinase-domain-positive tubular structures. Note that the CVAK104 antibody was raised against the C-terminal part of the protein, and therefore does not cross-react with the kinase-domain-YFP fusion protein. Scale bars: 5 μm.

Video S1: CVAK104-GFP expressed in HeLa cells (12×).

Video S2: CVAK104-GFP expressed in HeLa cells depleted of clathrin heavy chain (12×).

Video S3: CVAK104-kinase-domain-YFP expressed in HeLa cells (12×).

Video S4: CVAK104-kinase-domain-YFP expressed in HeLa cells depleted of clathrin heavy chain (12×).

Video S5: CVAK104-GFP and Alexa Fluor 594-labelled transferrin in HeLa cells starting ~1 minute after beginning of transferrin uptake (250×).

Video S6: CVAK104-GFP and Alexa Fluor 594-labelled transferrin in HeLa cells starting ~60 minutes after beginning of transferrin uptake (250×). Note: Scale bars in all movies: 10 μm.

Supplemental materials are available as part of the online article at http://www.blackwell-synergy.com

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