AP-1 is required for the maintenance of apico-basal polarity in the C. elegans intestine

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SUMMARY
Epithelial tubes perform functions that are essential for the survival of multicellular organisms. Understanding how their polarised features are maintained is therefore crucial. By analysing the function of the clathrin adaptor AP-1 in the C. elegans intestine, we found that AP-1 is required for epithelial polarity maintenance. Depletion of AP-1 subunits does not affect epithelial polarity establishment or the formation of the intestinal lumen. However, the loss of AP-1 affects the polarised distribution of both apical and basolateral transmembrane proteins. Moreover, it triggers de novo formation of ectopic apical lumens between intestinal cells along the lateral membranes later during embryogenesis. We also found that AP-1 is specifically required for the apical localisation of the small GTPase CDC-42 and the polarity determinant PAR-6. Our results demonstrate that AP-1 controls an apical trafficking pathway required for the maintenance of epithelial polarity in vivo in a tubular epithelium.

KEY WORDS: Epithelial polarity, Membrane traffic, Apical sorting, CDC-42, PAR-6, AP-1, C. elegans

INTRODUCTION
Cell polarity establishment is a complex but well-studied process in various models, including neurons, migrating cells following wound healing or the C. elegans one-cell embryo. In epithelial cells, polarity is established through cell-cell and cell-matrix interactions (Yeaman et al., 1999) followed by the formation of three essential modules. Two of these modules, the Crumbs/PALS1/Patj module and the PAR-3/PAR-6/PKC-3 (aPKC) module, are localised at the apical domain, whereas the Dlg/Scribble/Lgl module is at the basolateral domain (St Johnston and Ahringer, 2010). Targeting these modules to their precise localisation is crucial for proper polarity establishment. For example, membrane traffic is essential during apical lumen formation in MDCK cysts: a transcytosis route based on Rab11a, Rab18 and Rab8a is required for Cdc42 and apical PAR proteins accumulation at the presumptive apical domain during lumen formation, providing a mechanism for polarity establishment in a tubular epithelium in vitro (Bryant et al., 2010). However, several questions remain to be solved. For example, the sorting mechanism required after basolateral endocytosis to separate the bona fide basolateral proteins from the future apical proteins was not investigated. Furthermore, the role of this pathway in vivo or in polarity maintenance has not been established.

Polarity maintenance depends mainly on differential trafficking of essential proteins to the apical or basolateral domains. Although apical PAR proteins play a crucial role in controlling endocytosis (Balklava et al., 2007) membrane traffic has also been implicated in the control of PAR localisation (Lu and Bilder, 2005; Nakayama et al., 2009). In Drosophila, endocytic proteins including shibire (dynamin), Rab5, avalancher (syntaxin 7), tsg101 and vps25 have been defined as tumour suppressors and are essential for epithelial polarity and signalling (Shivas et al., 2010; Vaccari and Bilder, 2009). However, the entanglement of polarity loss and signalling defects prevents a detailed analysis of the direct role of membrane traffic on epithelial polarity. Moreover, the results are consistent with several hypotheses, including transcytosis, lysosomal degradation or removal of mistargeted proteins (Shivas et al., 2010). In the C. elegans intestine, it has been shown recently that glycosphingolipids (GSL) are essential for the maintenance of a single lumen (Zhang et al., 2011): knock-downs of genes controlling GSL biosynthesis induce the formation of ectopic apical lumens and mistargetting of apical proteins to the basolateral membrane. GSL are enriched in lipid rafts (Simons and Ikonen, 1997) and these observations suggest that GSL and lipid rafts are required for apical sorting in vivo. Altogether, these studies show that membrane traffic plays a crucial role in polarity maintenance.

In mammalian epithelial cultured cells, several studies have investigated in details the function of clathrin and its epithelial-specific adaptor complex AP-1B. Both are implicated in basolateral sorting whereas neither AP-1B nor clathrin seem to be required for the sorting of apical proteins and overall epithelial polarity maintenance (Deborde et al., 2008; Folsch et al., 1999; Gan et al., 2002), and no clathrin adaptor has been involved in apical sorting (Weisz and Rodriguez-Boulan, 2009). In C. elegans, the loss of AP-1 induces an embryonic arrest at a late stage during morphogenesis (Shim et al., 2000) (supplementary material Fig. S1A-C) consistent with a role in epithelial polarity maintenance (Chisholm and Hardin, 2005). C. elegans AP-1 is composed of four subunits: βapb-1 (shared with AP-2 and therefore not examined in this study), γapg-1, σaps-1 and the μ subunit encoded by two genes, unc-101 or apm-1 (supplementary material Fig. S1A). RNAi knockdowns of aps-1 or apg-1 subunits or the depletion of both μ subunits induce a threefold stage embryonic arrest during elongation (Shim et al., 2000); apm-1(RNAi) alone induces a larval arrest at the L2 stage (see also Materials and methods; supplementary material Fig. S1B,C). We decided to assess the function of AP-1 in the C. elegans intestine and our results demonstrate a strict requirement for AP-1 in apical sorting and apicobasal polarity maintenance in vivo in a model of a tubular epithelium.
MATERIALS AND METHODS

Genetics

*Caenorhabditis elegans* strains were maintained and crossed as described (Brenner, 1974). The strains used in this study are shown in supplementary material Table S1.

Plasmid construction

The gfp::cdc-42 construct was generated using the Multisite Gateway system (Invitrogen). Briefly, this construct was generated by making three donor vectors containing the vha-6 promoter, the gfp fusion sequence fused in frame to cdc-42 genomic sequence (a fusion PCR was used to generate this fragment), and the unc-54 3′UTR. The three vectors were then recombined to give a unique plasmid that was sequenced. Stable transgenic lines were obtained by co-injecting this plasmid and the pRF4 plasmid carrying the rol-6(sa1006) marker in WT N2 worms (Mello et al., 1991) to generate the FL83 strain. GFP::CDC-42 was only visible from the threefold stage, which prevented observations of its localisation earlier during elongation. The sequence of the primers used to generate the three donor vectors are shown in supplementary material Table S2.

RNAi

We found that RNAi against *aps-1* or *apg-1* by injection or feeding induced an identical 2.5/3-fold arrest phenotype for 90-100% embryos. We decided to use the feeding method with *aps-1(RNAi)*, which gives more than 95% elongation arrest while *apg-1(RNAi)* is slightly less penetrant. Essential penetrance of all phenotypes described following RNAi is well above 80% unless otherwise stated. To examine larval phenotypes, young L1 larvae were fed on a fresh RNAi plate. All phenotypes were recorded 15-18 hours later at a time where embryos were still alive, as assessed by Nomarski microscopy.

To observe embryonic phenotypes, RNAi was performed on L4 larvae and/or embryos. To fix embryos, a freeze-crack methanol protocol (Leung et al., 1999) was used to prepare samples for electron microscopy. Electron microscopy

Control and RNAi embryos were let to develop for 9-11 hours (3- to 4-fold embryos) before fixation by high pressure freezing with an EM-PACT-2 (Leica, Vienna, Austria) followed by freeze substitution with an AFS (Leica, Vienna, Austria) as described (Lieggeois et al., 2006). Ultra-thin sections were obtained with a Leica Ultracut ultramicrotome and observed on a Philips CM12 with a Gatan Orius 1000 or a Jeol 100CX2 with a Gatan Orius 200.

The presence of ectopic lumens and junction length were quantified as follows: each embryo was sectioned every 5-7 μm so that different cells were observed in different 5-7 μm regions. We then used only one section of high quality per region for each embryo to measure electron-dense structures (junctions) and assess the presence or absence of ectopic lumens. For junction length, we observed 12 cells from four different control embryos and 30 cells from six different *aps-1(RNAi)* embryos. Ectopic lumens were scored in 17 sections from six different *aps-1(RNAi)* embryos, whereas no ectopic lumen was observed in four different control embryos.

RESULTS

AP-1 is required for sorting basolateral and apical cargos

The *C. elegans* intestine is a simple model of tubular epithelium composed of two rows of cells with a lumina forming in the middle and easily identifiable apical and basolateral domains (Fig. 1A). We first tested the efficiency of *aps-1(RNAi)* and *apg-1(RNAi)* to remove the AP-1 complex by performing RNAi experiments against *aps-1* or *apg-1*, which encode two different subunits of AP-1 in a strain expressing an AP-1::GFP fusion protein (see Materials and methods). We observed a complete disappearance of the AP-1::GFP signal following *aps-1(RNAi)* and a diffuse localisation following *apg-1(RNAi)* (supplementary material Fig. S1D), a result similar to what has been observed in mammalian cells following RNAi depletion of one AP-1 subunit (Lui-Roberts et al., 2005). The penetrance of the embryonic arrest following *aps-1(RNAi)* or *apg-1(RNAi)* was very high, and all the phenotypes described below displayed a very strong penetrance (>80%) unless otherwise stated. Deletion alleles *aps-1(tm935)*, *apg-1(tm1249)* and *apg-1(ok2578)* induced an L1/L2 larval lethality (supplementary material Fig. S1E) presumably owing to maternal contribution and we decided to analyse the embryonic arrest induced by the RNAi depletion of *aps-1*.

Because AP-1B has been implicated in basolateral targeting in mammals (Folsch et al., 1999; Gan et al., 2002), we first examined the localisation of the transmembrane basolateral marker SLCF-1, CoolSNAP HQ2 camera and Metamorph software). All images were examined and assembled using ImageJ 1.43 and Adobe Photoshop CS3 10.0.
which is a putative monocarboxylate transporter (Mouchiroud et al., 2010). We found that SLCF-1 was only localised at the basolateral cortex of control embryos whereas *aps-1*(RNAi) induced an homogenous cortical distribution of SLCF-1 (Fig. 1B,C). We next examined the distribution of two transmembrane apical markers. In wild-type embryos both proteins are strictly apical (Fig. 1B). In *aps-1*(RNAi) embryos, the Na⁺/H⁺ exchanger NHX-2 (Nehrke, 2003) remained apical but was also observed in lateral patches (Fig. 1C). By contrast, the oligopeptide transporter PEPT-1 (Nehrke, 2003) displayed a homogenous cortical localisation at apical and basolateral membranes, and was also present in lateral patches (Fig. 1C). Fluorescence intensity was measured along a line crossing the whole intestine (Fig. 1B,C, dotted line) and showed that SLCF-1 and PEPT-1 are visible both at the apical and basolateral membrane in *aps-1*(RNAi) embryos (Fig. 1D). The loss of asymmetric distribution of SLCF-1 and PEPT-1 was observed in more than 90% of embryos (n>100). The absence of NHX-2 from the basolateral membrane in *aps-1*(RNAi) embryos indicates that the junctional diffusion barrier is intact, an hypothesis that was confirmed by several observations (see below). We therefore concluded that AP-1 could be required for sorting both basolateral and apical transmembrane proteins without affecting their transport to the plasma membrane.

To confirm this hypothesis, we depleted several components of the membrane traffic machinery. Because of the early embryonic lethal phenotype associated with the loss of function of several of these genes, the knockdown experiments were performed in larvae. We first recapitulated the sorting phenotype induced by *aps-1*(RNAi) (Fig. 2B). We then asked whether both µ subunits or only one was responsible for these sorting defects. We found that, in *apm-1*(RNAi) larvae, both SLCF-1 and PEPT-1 were mis-sorted, while no phenotype was observed in *unc-101(sy108)-null* mutants (Fig. 2C,D). We next tested the function of essential trafficking components such as dynamin (DYN-1) and the small GTPases RAB-5 and RAB-11. The asymmetric distributions of PEPT-1 and SLCF-1 were not affected in *dyn-1*(RNAi) or *rab-5*(RNAi), whereas both proteins were accumulating in vesicular structures still attached or closely apposed to their respective apical or basolateral plasma membrane (Fig. 2E,F). To examine the function of recycling endosomes and polarised transport, we next targeted RAB-10 and RAB-11, which can be used to target basolateral (Chen et al., 2006) or apical (Prekeris et al., 2000) routes, respectively. We found that PEPT-1 was never affected in *rab-10*(RNAi) worms, whereas SLCF-1 was seen at the basolateral membrane and on large intracellular vacuoles that accumulate in RAB-10-depleted adults (Chen et al., 2006) (Fig. 2G). Conversely, RAB-11 depletion had no effect on SLCF-1 localisation, whereas PEPT-1 systematically accumulated intracellularly close to the apical membrane (Fig. 2H). At least 50 larvae were observed for each condition and fluorescence quantification was used to validate the results (Fig. 2A-H). Only *aps-1*(RNAi) and *apm-1*(RNAi) larvae showed a symmetrical distribution of SLCF-1 and PEPT-1 observed in more than 90% of the cases and never seen in other RNAi conditions. These results demonstrate that endocytosis, as tested with DYN-1 and RAB-5, and recycling, as tested with RAB-11 and RAB-10, are not required for the polarised sorting of SLCF-1 and PEPT-1 but only for their transport. We concluded that AP-1/APM-1 can sort both apical and basolateral cargos that are then transported by different routes.

A surprising observation is that there is little intracellular accumulation of PEPT-1 and SLCF-1 in *aps-1*(RNAi) embryos, suggesting that AP-1 is mostly required for sorting rather than transport. To examine the effect of *aps-1* depletion on intracellular organelles, we quantified the number of intracellular punctae formed by several markers in intestinal cells of threefold stage embryos. We found that the number of RAB-5, RAB-7 and RAB-10 punctae was not significantly affected (P>0.1) in *aps-1*(RNAi) embryos (supplementary material Fig. S2A,B) whereas Golgi/TGN
displayed a normal morphology (supplementary material Fig. S3B). However RAB-11-positive structures were absent from the subapical cortex in aps-1-depleted embryos (>90%, n>100) (supplementary material Fig. S2A), suggesting that AP-1 is specifically required for the maintenance of RAB-11-positive endosomes. However, rab-11 depletion induces an intracellular accumulation of PEPT-1, whereas aps-1 depletion leads to mis-sorting of this cargo to the apical and basolateral membrane. We concluded that in absence of AP-1, apical cargos probably use default intracellular pathways bypassing the requirement for RAB-11 endosomes.

**AP-1 depletion induces the formation of ectopic apical lumens**

In almost all aps-1(RNAi) depleted threefold stage embryos we observed the formation of lateral patches stained by the apical markers NHX-2 and PEPT-1, which can be distinguished from intracellular vesicles by their localisation between two cells as seen for PEPT-1 (Fig. 1C, right panel, empty arrowhead). To characterise these lateral patches, we used electron microscopy (EM) to examine 9- to 11-hour-old embryos, which corresponds to three- and fourfold stages (supplementary material Fig. S1F). This time window was chosen to identify primary rather than secondary defects. In wild-type embryos, we could identify a single lumen with normally shaped microvilli, well defined junctions and closely apposed lateral membranes (Fig. 3A). In aps-1-depleted embryos, one main lumen was visible with elongated junctions (Fig. 3B, supplementary material Fig. S3A-D,F) and partially disorganised microvilli (supplementary material Fig. S3B). But we also frequently (70%, n=17) observed ectopic lumens defined by short microvilli and additional junctions (Fig. 3B, supplementary material Fig. S3A,C,D). These ectopic lumens were located either between adjacent cells (Fig. 3B, supplementary material Fig. S3A) or within cells following lateral membrane invagination (supplementary material Fig. S3C) or in one instance without obvious connection to the plasma membrane (supplementary material Fig. S3D). Disorganised microvilli, ectopic lumens and ectopic junctions were also observed in apg-1(RNAi) embryos (supplementary material Fig. S3E). We concluded that AP-1 depletion can induce the conversion of lateral membrane into apical membrane (supplementary material Fig. S3G).

**AP-1 is required for the asymmetric apical localisation of PAR-6**

The loss of AP-1 induces the formation of ectopic lumens and affects the polarised distribution of PEPT-1 and SLCF-1. Although the former is implicated in growth, amino acid homeostasis and fat storage (Spanier et al., 2009), the latter is involved in longevity
(Mouchiroud et al., 2010); their mislocalisation is therefore unlikely to be responsible for the formation of ectopic lumens. We reasoned that other apical proteins could be affected and induce the formation of ectopic lumens; for example, PAR-6 depletion triggers the formation of apical patches reminiscent of ectopic lumens (Totong et al., 2007). We therefore decided to examine the localisation of polarity determinants at the 1.5-fold stage, which corresponds to the end of polarity establishment, and in threefold stage embryos for the polarity maintenance phase. We first examined the apical PAR module. During epithelial polarisation at the beginning of embryonic elongation, PAR-3, PAR-6 and PKC-3 accumulate at the presumptive apical domain (Achilleos et al., 2010; Totong et al., 2007). This step of polarity establishment was not affected, as shown by staining of these endogenous proteins in 1.5-fold stage embryos, where they were restricted to their normal apical localisation (Fig. 4A, supplementary material Fig. S4A). In the more elongated threefold stage aps-1(RNAi) embryos, we found that PAR-6 was both apical and basolateral in more than 80% of embryos (Fig. 4B-D, supplementary material Fig. S4A) and could be observed in lateral patches (Fig. 4B, supplementary material Movies 1 and 2). To quantify the basolateral enrichment, we calculated the apical/cyttoplasmic and basal/cyttoplasmic fluorescence ratios in control (n=13) and aps-1(RNAi) (n=15) threefold stage embryos; we found a 60% increase of the basal/cyttoplasmic ratio of PAR-6, which was not present in control embryos (P<3×10−4; Fig. 4C) whereas the apical/cyttoplasmic ratio was stable. We also quantified lateral staining along a line parallel to the apical domain and crossing two lateral membranes (Fig. 4B,D). In contrast to PAR-6, PAR-3 and PKC-3 were not found at the basolateral membrane (Fig. 4B,D, supplementary material Fig. S4A) except for PAR-3 in about 15% of the embryos. However, both PAR-3 and PKC-3 were also present in lateral patches (Fig. 4B). Based on EM observations, ectopic junctions form around ectopic lumens. To establish whether the lateral patches observed by fluorescence correspond to ectopic lumens, we co-stained embryos for PKC-3 and AJM-1, a junctional protein (Kuppen et al., 2001). AJM-1 was found at its normal belt-like localisation on either side of the apical PKC-3 signal but we also observed rings of AJM-1 surrounding doughnut-shaped PKC-3-positive structures (Fig. 4E). This observation strongly suggests that lateral patches correspond to ectopic lumens identified by EM. Finally, in order to confirm PAR-6 mislocalisation we used a PAR-6::GFP fusion. We found that it became mislocalised in the cytoplasm and on ectopic lumens in intestinal cells of threefold stage aps-1(RNAi) embryos (supplementary material Fig. S4B). A cytoplasmic localisation was also observed in apg-1(RNAi) embryos (supplementary material Fig. S4B) and in aps-1(tm935) mutant larvae (supplementary material Fig. S4C). We concluded that AP-1 is specifically required for the apical localisation of PAR-6.

The other apical polarity module is the Crumbs/PALS1(Stardust) module, which is implicated in epithelial polarity in Drosophila and mammals (St Johnston and Ahringer, 2010). However, it is not required for viability in C. elegans (Seghert et al., 2004). To confirm this result, we obtained deletion mutants for crb-1 (Crumbs), eat-20 (Crumbs-like) and tag-117 (PALS1/Stardust); homozygous mutants for each of these genes were viable and did not display any obvious phenotype. To assess a possible redundancy between crb-1 and eat-20, we performed crb-1(RNAi) in eat-20(gk218) and eat-20(RNAi) in crb-1(ok931) mutants without identifying any gross phenotype. We also assessed the localisation of EAF-20::GFP (Shibata et al., 2000) in wild-type and aps-1(RNAi) embryos without identifying any change in its localisation in intestinal cells (not shown). We next examined the localisation of LET-413/Scribble, which represents the Scribble/Dlg complex in C. elegans but its weak staining in the intestinal cells of threefold stage embryos prevented a detailed analysis of its localisation; however, it was not affected in the epidermis (not shown). Finally, we also examined the E-cadherin/catenin complex (CCT) and the DLG-1/AJM-1 complex (DAC; supplementary material Fig. S5A) and found that the belt-like localisation of these proteins was not affected in aps-1(RNAi) embryos (Fig. 4E, supplementary material Fig. S5B), while they were also found surrounding ectopic lumens (Fig. 4E). Altogether, these results suggest that PAR-6 is the only essential polarity determinant to require AP-1 for its correct localisation.

Ectopic lumens could result from a failure to establish polarity correctly and a single lumen or form later. To determine the timing of ectopic lumen formation, we examined PKC-3 localisation in correlation with elongation (supplementary material Fig. S1F). We never observed ectopic lumens before the 2.5-fold stage in aps-1(RNAi) embryos (n=43; Fig. 4A) whereas they were observed in 74% of arrested threefold stage embryos (n=42; Fig. 4B). A similar result was obtained with PEPT-1 and no ectopic lumen was seen in twofold stages embryos by electron microscopy (not shown). We therefore concluded that AP-1 prevents de novo formation of ectopic apical lumens along the lateral membrane after normal polarity establishment and single lumen formation.

**AP-1 controls CDC-42 apical localisation**

We next wanted to identify the potential link between AP-1 and PAR-6, which is a cytoplasmic protein not known to directly interact with membranes. We had already ruled out a role for several PAR-6 interactors, including PAR-3, PKC-3 and Crumbs, which do not become basolateral in aps-1(RNAi) embryos. Because CDC-42 can interact with PAR-6 and control its cortical recruitment in C. elegans one-cell embryos (Gotta et al., 2001), we decided to investigate the role of AP-1 and other membrane traffic components on CDC-42 localisation. These experiments were performed in larvae to bypass the early requirement of dynamin, RAB-5 and RAB-11 during embryogenesis. In control larvae, GFP::CDC-42 was found mostly at the apical domain but was also visible at the lateral membrane and was absent from the basal membrane (Fig. 5A,B); a similar localisation has been observed with a rescuing GFP::CDC-42 under the control of its own promoter (Lukas Neukomm and Michael Hengartner, personal communication). In aps-1(RNAi) larvae, we found a specific
accumulation of CDC-42 at the basal cortex as shown after fluorescence quantification (Fig. 5A,B). We next examined the role of dynamin and RAB-5; their depletion reduced the relative amount of CDC-42 at the apical membrane, but we did not observe any quantitatively significant basal accumulation (Fig. 5A,B). However, CDC-42 could occasionally be detected at the basal cortex following dynamin depletion, suggesting that dynamin may have a minor role in removing basal CDC-42. Finally, we found that RAB-11 depletion strongly affected CDC-42, which became very faint and essentially cytoplasmic (Fig. 5A,B). We concluded that dynamin, RAB-5 and RAB-11 are implicated in CDC-42 transport and localisation, whereas only AP-1 is required to prevent CDC-42 basal accumulation. We also characterised GFP::CDC-42 localisation in threefold stage embryos. In contrast to the larval localisation, which was predominantly apical, CDC-42 had a symmetric distribution; it was found both at the basolateral and apical domains in control embryos (Fig. 5C, supplementary material Movie 3). In aps-1(RNAi) embryos the CDC-42 apical enrichment was strongly affected whereas the basolateral localisation was maintained. The apical staining was not visible in 30% of embryos ($n=46$; Fig. 5C, supplementary material Movie 4) and was significantly reduced compared with the cytoplasmic signal in the remaining embryos (Fig. 5D), while there was a small but significant increase in basolateral/cytoplasmic ratio. We concluded that AP-1 contributes to CDC-42 apical localisation both in embryos and larvae.

**PAR-6 apical localisation is dependent on its interaction with CDC-42**

CDC-42 can interact with PAR-6 and recruit it to the anterior cortex of the one-cell *C. elegans* embryo (Gotta et al., 2001). Owing to the early lethal phenotype induced by *cdc-42(RNAi)*, we performed CDC-42 depletion by RNAi in larvae and found that the apical/cytoplasmic ratio of PAR-6::GFP showed a twofold decrease ($P<5\times10^{-4}$) in *cdc-42(RNAi)* larvae (Fig. 6A). By contrast, the PAR-3::GFP fusion protein was not affected (Fig. 6B). We also quantified GFP::CDC-42 apical accumulation in *par-6(RNAi)* larvae without finding any role for PAR-6 in CDC-42 apical localisation (Fig. 6C). The interaction between PAR-6 and CDC-42 is mediated by the semi-CRIB domain of PAR-6 (Aceto et al., 2006; Gotta et al., 2001). To confirm a requirement for a direct interaction between CDC-42 and PAR-6 in vivo, we used a strain expressing a GFP::PAR-6 where the semi-CRIB domain is deleted and which can bind PKC-3 and PAR-3 but not CDC-42
DISCUSSION

By analysing the phenotype induced by the depletion of the clathrin adaptor complex AP-1 we have shown that it is required for the maintenance of a single lumen in the *C. elegans* intestine. At the cellular level, we found that AP-1 is necessary for the polarised apical localisation of the oligopeptide transporter PEPT-1 and of the essential polarity proteins PAR-6 and CDC-42, as well as for the basolateral distribution of the monocarboxylate transporter SLCF-1. Apical and basolateral sorting in epithelial cells is a complex process and basolateral targeting has been extensively studied (Carmosino et al., 2009; Duffield et al., 2008; Folsch, 2008; Folsch et al., 2009; Gonzalez and Rodriguez-Boulan, 2009) whereas mechanisms underlying apical delivery have proven difficult to characterise (Golachowska et al., 2010; Weisz and Rodriguez-Boulan, 2009). Several hypotheses can be proposed for AP-1 function in apical trafficking. First, AP-1 could be implicated solely in basolateral targeting followed by transcytosis of apical proteins; we do not favour this hypothesis because preventing endocytosis by targeting dynamin should be necessary to understand how AP-1/APM-1 can sort both apical and basolateral cargos and to identify the apical sorting signals potentially recognised by AP-1.

[Fig. 5. AP-1 is required to sort CDC-42 to the apical membrane. (A) RNAi against *aps-1*, *dyn-1*, *rab-5* and *rab-11* was performed in L1 larvae and the localisation of GFP::CDC-42 recorded 48 hours later. The apical membrane is indicated by a white arrowhead and the basolateral membrane by a white arrow. Fluorescence quantification along the dotted line across the intestine for each picture is shown on the right. GFP::CDC-42 accumulates at the apical membrane (white arrowhead) and is also visible at the lateral membrane (white arrow) in control (L4440) larvae. GFP::CDC-42 is visible at the basal membrane only in *aps-1(RNAi)* larvae. It accumulates on large vesicles (empty arrowhead) in *dyn-1(RNAi)* and *rab-5(RNAi)* larvae, whereas the signal is very weak in *rab-11(RNAi)* larvae. (B) Quantification of the cortical/cytoplasmic signal ratio at the apical (left) and basal (right) cortex (*n=15* for each RNAi condition). Fluorescence intensity was measured along a 5 μm line at the apical and basal cortex and in the cytoplasm. ***(*P<1×10−5*) significantly different from control. Data are mean±s.e.m. (C) GFP::CDC-42 localisation in L4440 and *aps-1(RNAi)* embryos. The main panels show a middle focal plane and the insets a top view were the basal staining is visible (see also supplementary material Movies 3 and 4). Contrary to its localisation in larvae, GFP::CDC-42 is present at both the apical (arrowhead) and basolateral (arrows) membranes in control embryos (see also supplementary material Movie 3). GFP::CDC-42 apical localisation is lost in 30% of *aps-1(RNAi)* embryos (*n=46*) with no staining at the level of the arrowhead (see also supplementary material Movie 4). (D) Quantification of the cortical/cytoplasmic signal ratio at the apical (left) and basal (right) cortex. Fluorescence intensity was measured along a 5 μm line at the apical and basal cortex and in the cytoplasm. There is a significant decrease in apical staining in the 70% of embryos (*n=46*) that display an apical localisation (***(*P<1×10−5*) while basolateral localisation is slightly increased (***(*P<1×10−3*)). Data are mean±s.e.m. Scale bars: 10 μm.]

[GFP::PAR-6(CM2) (Aceto et al., 2006)]. We found that this fusion protein was mostly cytoplasmic in the intestine of elongating embryos (Fig. 6D) whereas the wild-type version of GFP::PAR-6 [described in Pacquelet et al. (Pacquelet et al., 2008)] was found to be apical at the same stage (Fig. 6D). We concluded that PAR-6 binding to CDC-42 is required for PAR-6 accumulation at the apical cortex of intestinal cells in vivo.
The de novo formation of ectopic lumens in a tubular epithelium demonstrates that AP-1 is essential for epithelial polarity maintenance. Previous studies have focused on the role of AP-1B in basolateral targeting in mammalian epithelial cells, but did not identify defects in overall cell polarity (Folsch et al., 1999; Gan et al., 2002). In Drosophila epithelial sensory organs, AP-1 acts in recycling endosomes as a negative regulator of targeting to the apical E-cadherin junction for the two basolateral transmembrane proteins Notch and Sanpodo, again without affecting overall polarity (Benchra et al., 2011). It is therefore surprising to identify such an essential function for AP-1 in C. elegans. This difference could be due to the specific shape of tubular epithelia when compared with monolayered models used previously to study AP-1 function; however, the pharynx, which is also a tubular epithelium, is not affected. Alternatively, it could reflect the divergent mechanisms used to establish and maintain polarity in specific epithelial tissues. For example, basolateral targeting motifs are different in MDCK and Caco-2 cells (Monlauzeur et al., 2000), and the PAR-3 function is not identical in epidermal and intestinal C. elegans cells (Achilleos et al., 2010). Interestingly, this novel function could explain the embryonic lethality associated with AP-1γ knockout in mice (Meyer et al., 2000; Zizioli et al., 1999). Moreover, the PAR-3/PAR-6/PKC-3 module is required in many different polarised cell types, including asymmetrically dividing cells, migrating cells, neurons or at the immunological synapse (Suzuki and Ohno, 2006). Because AP-1 is ubiquitously expressed, a role for this complex in CDC-42 and PAR-6 localisation could be essential in other cell types and organisms.

The formation of pathological ectopic lumens in tubular epithelia has been observed in various contexts. Individuals affected by the orphan Microvillus Inclusion Disease (MVID) have ectopic lumens forming inside intestinal cells (Ruemmele et al., 2006) caused by mutations affecting type Vb myosin (Myo5B) (Muller et al., 2008). Ectopic lumens have also been observed in Rab8-knockout mice (Sato et al., 2007). Both proteins are implicated in apical transport (Lapierre et al., 2001; Sato et al., 2007). Ectopic lumens are also induced by the loss of GSL biosynthesis (Zhang et al., 2011). GSL are essential components of lipids rafts (Simons and Ikonen, 1997), which have a role in apical sorting of GPI anchored proteins (Brown and Rose, 1992). Depletion of AP-1, clathrin [see accompanying paper (Zhang et al., 2012)] or of enzymes required for GSL biosynthesis (Zhang et al., 2011) all induce a lateral to apical conversion. Is the formation of ectopic lumens the result of apical depletion or basolateral mis-sorting of polarity determinants? Previous observations suggest that PAR-6 depletion could induce the formation of ectopic lumens (Totong et al., 2007) and we cannot exclude this hypothesis. However, only CDC-42 was found to be depleted from the apical domain in aps-1(RNAi) embryos, whereas endogenous apical PAR-6 does not seem to be affected and both CDC-42 and PAR-6 remain apical in aps-1(RNAi) larvae. We therefore propose that the basolateral relocalisation of PAR-6 could also account for lateral to apical conversion by acting as a recruiting platform for other apical essential factors to initiate the formation of ectopic lumens. Such a model where CDC-42 and PAR-6 localisation is dependent on membrane traffic could explain ectopic lumen formation in various contexts. Whereas Rab8 and Myo5B are required for apical transport, AP-1 and GSL control apical sorting; ectopic accumulation of polarity determinants and lumen formation would therefore be either intracellular (Rab8 and Myo5B) or lateral (AP-1 and GSL).

Genetic interactions and colocalisation studies suggest that AP-1, clathrin and GSL could all interact together to control apical sorting [see accompanying paper (Zhang et al., 2012)]. Interestingly, CDC-42 has been found on AP-1A coated liposomes (Baust et al., 2006), which raises the possibility of a physical interaction between CDC-42 and AP-1, which could be required to prevent or limit CDC-42 basolateral targeting and promote its apical sorting. We propose that by controlling the sorting of both apical and basolateral proteins, including CDC-42 and PAR-6 AP-1 is a crucial factor in apicobasal polarity maintenance, whereas the interactions between AP-1, clathrin and GSL remain to be elucidated.
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Competing interests statement

The authors declare no competing financial interests.

Supplementary material

Supplementary material available online at http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.076711/-/DC1

References

Table S1. Strains used in this study

<table>
<thead>
<tr>
<th>Strain name</th>
<th>Genotype</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>FL33</td>
<td><em>apg-1(tm1249)/+ (outcrossed 5 times)</em></td>
<td>Mouchiroud et al., 2010</td>
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<tr>
<td>FL35</td>
<td>dfEx1[aps-1::gfp + rol-6(su1006)]</td>
<td>Aceto et al., 2006</td>
</tr>
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<td><em>aps-1(tm935)/+ dfEx1[aps-1::gfp + rol-6(su1006)]</em></td>
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<td><em>aps-1(tm935)/+ (outcrossed 6 times)</em></td>
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<tr>
<td>FL83</td>
<td>dfEx3[vha-6p::gfp::cdc-42 + rol-6(su1006)]</td>
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<td>FS254</td>
<td>slcf-1(tm2258); Exfs254[slcf-1::gfp]</td>
<td>A gift from J. Nance (Skirball Institute, New York, USA)</td>
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<td>KK944*</td>
<td><em>itls164[Ppie-1::GFP::PAR-6(CM2) unc-119(+)]; unc-119(ed4) III.</em></td>
<td>Mouchiroud et al., 2010</td>
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<td>KWN246</td>
<td><em>pha-1(e2123) III, myEx133[opt-2(aa1-412)::GFP] + pha-1(+)</em></td>
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<td>JJ1555</td>
<td><em>zuls73[par-3p::PAR-3::GFP; unc-119(+)]; unc-119(ed3)</em></td>
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<td>A gift from M. Labouesse (IGBMC, Illkirch, France)</td>
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<tr>
<td>ZU13*</td>
<td><em>[pie-1::GFP::PAR-6; unc-119(+)]; unc-119(ed3) III</em></td>
<td>Pacquelet et al., 2008</td>
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</tbody>
</table>

*Both KK944 and ZU13, which express *gfp::par-6* under the control of a *pie-1* promoter, can be used to localise PAR-6 in 1.5-fold stage embryos.
Table S2. List of primers used in this study

<table>
<thead>
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<td>vha-6 promotor sens</td>
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<tr>
<td>vha-6 promotor rev</td>
<td>(attB1r)GGGGACTGGCTTTTGTACAAACCTGCTAGGTTTTTAGTGCCTGCGCCCTGAA</td>
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<td>Primer A</td>
<td>AGCTTTGCAATGCCTGCAAGGTCACT</td>
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<tr>
<td>Primer A' sens</td>
<td>(attB4)GGGGACAAATTTGTACAAATAGCAGGCTACGCAGGTCACTCTAGAGATCCCC</td>
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<td>Primer B</td>
<td>CTCCAACGACGACGACGTCTACGTCGCTCGATCTTTTTGTATAGTGTCCTCCCATATGCC</td>
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<td>Primer C</td>
<td>ATGCAGACGATCAAGTGCGTCGTCGTTGGAG</td>
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<tr>
<td>Primer D</td>
<td>GGAGACAAGGAAGACGTCC</td>
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<tr>
<td>Primer D' rev</td>
<td>(attB1r)GGGGACACCTTTGTACAAAGAAAGCTGGTACTAGAGAATATTGCACTTCTTCTTCTCCTCC</td>
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<tr>
<td>3'UTR unc-54 sens</td>
<td>(attB4)GGGGACAGCTTTTGTACAAAGTGACCATCTCGTAGAATTCCAAACTGAGCG</td>
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<tr>
<td>3'UTR unc-54 rev</td>
<td>(attB1r)GGGGACAAACCTTTGTATAAAGTTGCAAGGCGCCTACGCGCAGTCTAGTAGGG</td>
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The primers shown were used to generate the *gfp::cdc-42* plasmid.