Control of E-cadherin apical localisation and morphogenesis by a SOAP-1/AP-1/clathrin pathway in C. elegans epidermal cells

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**ABSTRACT**

E-cadherin (E-cad) is the main component of epithelial junctions in multicellular organisms, where it is essential for cell-cell adhesion. The localisation of E-cad is often strongly polarised in the apico-basal axis. However, the mechanisms required for its polarised distribution are still largely unknown. We performed a systematic RNAi screen *in vivo* to identify genes required for the strict E-cad apical localisation in *C. elegans* epithelial epidermal cells. We found that the loss of clathrin, its adaptor AP-1 and the AP-1 interactor SOAP-1 induced a basolateral localisation of E-cad without affecting the apico-basal diffusion barrier. We further found that SOAP-1 controls AP-1 localisation, and that AP-1 is required for clathrin recruitment. Finally, we also show that AP-1 controls E-cad apical delivery and actin organisation during embryonic elongation, the final morphogenetic step of embryogenesis. We therefore propose that a molecular pathway, containing SOAP-1, AP-1 and clathrin, controls the apical delivery of E-cad and morphogenesis.

**KEY WORDS:** Epithelial polarity, E-cadherin, Membrane traffic, AP-1, *Caenorhabditis elegans*

**INTRODUCTION**

Adherens junctions and their essential component E-cadherin (E-cad) play a key role in maintaining the integrity of epithelial and preventing epithelium-mesenchyme transitions. Many studies have highlighted the role of membrane traffic in junction maintenance, and intracellular routes have been defined *in vivo* in *Drosophila*. In particular, it was shown that dynamin, Rab5, Rab11 or the apical PAR module control the clustering, endocytosis and recycling of E-cad (Blankenship et al., 2007; Georgiou et al., 2008; Harris and Tepass, 2008; Langevin et al., 2005; Leibfried et al., 2008; Levayer and Lecuit, 2013; Levayer et al., 2011; Shaye et al., 2008). In zebrafish embryos, the loss of the adaptor complex AP-1 induces a decrease in E-cad staining at the plasma membrane (Montpetit et al., 2008). In mammalian cells, many studies have also examined the factors implicated in E-cad endocytosis, recycling or degradation (Fujita et al., 2002; Ling et al., 2007; Palacios et al., 2001; Wang et al., 2007). However, whereas E-cad has a polarised distribution along the apico-basal axis in most cell types and tissues (Lynch and Hardin, 2009; St Johnston and Ahringer, 2010), surprisingly, the sorting mechanisms required for this polarised localisation are largely unknown, except for a potential role of the exocyst subunit Exo70 and of clathrin in mammalian cells (Deborde et al., 2008; Xiong et al., 2012). As a result, the phenotypic consequences of the loss of E-cad polarised distribution are not known.

To identify factors required for E-cad apical localisation *in vivo* in *C. elegans* epidermal cells we performed a systematic RNAi screen. We found that clathrin, its adaptor AP-1 and the AP-1 physical interactor p200/HEATR5B are all required to prevent E-cad basolateral localisation. The analysis of the AP-1 loss of function phenotype further shows that the associated embryonic lethality is caused by junction mislocalisation and disorganisation of the actin cytoskeleton during morphogenesis.

**RESULTS**

Identification of genes required for E-cad apical localisation

To identify genes required for E-cad polarised localisation we chose to examine the junctions of the *C. elegans* epidermis (supplementary material Fig. S1A-C), where E-cad is apical. We observed E-cad *in vivo* with a functional E-cad::gfp (Achilleos et al., 2010) co-expressed with a dlg-1::rfp construct (Diogon et al., 2007) to localise the boundary between the apical and lateral domains. In these cells, E-cad is mostly apical, as we could only detect a very weak lateral localisation: quantification showed that the lateral/cytoplasmic ratio of E-cad in control larvae was $<1.1$ at 48 h after hatching ($n=186$; Fig. 1C) and down to 1 at 72 h ($n=175$; supplementary material Table S2). We performed RNAi on 503 conserved genes which were selected based on their predicted function in membrane traffic or cytoskeleton organisation (Shaye and Greenwald, 2011), or on their identification in genome-wide *C. elegans* membrane traffic screens (Balklava et al., 2007; Winter et al., 2012) (supplementary material Table S1). E-cad localisation was observed after 48 h and 72 h of RNAi. About 10% of these genes affected E-cad expression levels or induced an intracellular accumulation (supplementary material Table S1). We also found that the depletion of ten genes induced a strong and consistent lateral mislocalisation of E-cad; four of these genes encode the clathrin heavy chain (chs-1), the σ and γ subunits of the clathrin adaptor AP-1 (aps-1 and apg-1) and the AP-1-interacting protein HEATR5B/p200/Laa1p [c13f10.4, hereafter called soap-1 for Sorting of apical proteins (Lui et al., 2003)] (Fig. 1Ab-Ah). Each AP-1 µ subunit alone was sufficient to target E-cad to the apical membrane but simultaneous depletion of both µ subunits recapitulated the complete loss of AP-1 (Fig. 1Ad-AF; 3D reconstructions shown in supplementary material Movies 1A,B). After quantification we found an average lateral/cytoplasmic ratio $\geq 1.7$ of E-cad at the basolateral membrane at 48 h and 72 h for all positive genes (Fig. 1C and supplementary material Table S2); the apical/cytoplasmic ratio was not strongly affected (Fig. 1B) except for clathrin, which leads to rapid decrease in E-cad...
These four factors are therefore essential to prevent E-cad lateral accumulation.

This screen also included other AP-1 interactors (Lui et al., 2003) and genes known to affect E-cad trafficking in other organisms, such as dynamin, rab-5, rab-11, the exocyst complex and the type Iγ phosphatidylinositol phosphate kinase (ppk-1 in C. elegans) (Ivanov and Naydenov, 2013); we also targeted genes implicated in apical sorting in C. elegans, such as glycosphingolipid (GSL) biosynthetic enzymes (Zhang et al., 2011). We found a weaker E-cad signal following the depletion of some GSL biosynthetic enzymes, probably owing to the penetrant larval lethality, but the localisation of E-cad was not affected (supplementary material Table S2); n correspond to the number of larvae; a-k refers to the RNAi conditions shown in the subpanels of A. *†, not quantifiable. To calculate the ratio, pixel intensity was calculated along two identical lines at the membrane and in the neighbouring cytoplasm as shown for aps-1 (see also Material and Methods). Error bars indicate s.e.m. The lateral localisation observed in rab-5(RNAi) and rab-11(RNAi) larvae is statistically significant (***,P<0.001). Ap, apical; Lat, lateral. Scale bar: 10 µm.

**Fig. 1. Identification of genes required for E-cad apical sorting.** (A) Larvae expressing E-cad::GFP and DLG-1::RFP were observed by confocal microscopy 72 h (or 48 h for chc-1(RNAi) due to the strength of the phenotype) after RNAi induction. Left panels (Apical) show an apical view; middle panels (Lateral) show sections taken 3 µm below the apical section shown on the left in the same larva; right panels (Z) show the Z-reconstruction obtained in the same larva. In control larvae, E-cad is only visible apically as two parallel lines separating the seam cells and the dorso-ventral epidermis. In aps-1(RNAi), apg-1(RNAi), apm-1(RNAi); unc-101(sy108), soap-1(RNAi) and chc-1(RNAi) larvae, a clear lateral signal (arrowheads) can be observed in the middle panels; see also 3D reconstructions in supplementary material Movies 1A-B. Note that the chc-1(RNAi) larvae arrest earlier (the picture in Ah shows a 48 h-old larva) as shown by the presence of seam/seam junctions (arrow in Apical column of Ah; see supplementary material Fig. S1B,C). In dyn-1(RNAi), rab-5(RNAi) and rab-11(RNAi) larvae, E-cad accumulates inside seam cells at an apical focal place; note that in these larvae, E-cad tends to be lost from its normal junction localisation. In about 50% of rab-11(RNAi) larvae, E-cad is visible at the lateral membrane (arrowheads) as shown in Ak′. (B,C) Quantification of the apical/cytoplasmic (B) or the lateral/cytoplasmic (C) ratio of E-cad signal at 48 h (quantifications were also performed at 72 h for lateral localisation and gave similar results; see supplementary material Table S2); n corresponds to the number of larvae; a-k refers to the RNAi conditions shown in the subpanels of A. *†, not quantifiable. To calculate the ratio, pixel intensity was calculated along two identical lines at the membrane and in the neighbouring cytoplasm as shown for aps-1 (see also Material and Methods). Error bars indicate s.e.m. The lateral localisation observed in rab-5(RNAi) and rab-11(RNAi) larvae is statistically significant (***,P<0.001). Ap, apical; Lat, lateral. Scale bar: 10 µm.
these genes in E-cad secretion and/or recycling to the apical membrane. In ~50% of rab-5- or rab-11-depleted larvae, we could detect a weak basolateral E-cad signal (Fig. 1A′,C), suggesting that these factors have also a limited but statistically significant role in E-cad polarised sorting. However, there was no E-cad lateral accumulation following dynamin depletion despite a strong cellular and developmental phenotype. Quantification of the apical signal showed that RAB-5 and RAB-11 are required for the maintenance of the E-cad apical pool, contrary to AP-1 and SOAP-1, which do not affect the apical localisation of E-cad (Fig. 1B). Altogether, this suggests that SOAP-1, AP-1 and clathrin, as well as, to a minor degree, RAB-5 and RAB-11, are required for the apical sorting of E-cad, whereas dynamin, RAB-5, RAB-11 and the exocyst complex are required for E-cad transport.

**E-cad localisation in the intestinal epithelium**

The first observations that AP-1 and clathrin were implicated in apical sorting were made in the *C. elegans* intestine (Shafaq-Zadah et al., 2012; Zhang et al., 2012). To test the role of these factors on E-cad localisation in another epithelial tissue we carefully examined the apico-basal distribution of E-cad in the intestine. Using the DLG-1 signal to identify the subapical domain, we found that E-cad is only visible at the lateral membrane in control larvae (n=28; Fig. 2A,B). We next depleted *aps-1* by RNAi in larvae and never observed E-cad at the apical membrane (n=33; Fig. 2C); similar results were observed following *rab-5* and *rab-11* depletion (data not shown). We concluded that the localisation of E-cad and the function of AP-1 are strikingly different between the intestine and the epidermis, and we decided to further investigate the apical targeting of E-cad in the epidermis.

**Identification of a SOAP-1/AP-1/clathrin interaction in the epidermis**

We next examined the interactions between SOAP-1, AP-1 and clathrin. AP-1 is a clathrin adaptor capable of recruiting clathrin to membranes (Robinson, 2004). We therefore observed the localisation of clathrin using an integrated *chl-1::gfp* construct expressed under the control of its own promoter. We found that the loss of AP-1 induces a strong decrease in the number and intensity of clathrin puncta in the epidermis (Fig. 3A-C), consistent with an AP-1-dependent recruitment of clathrin on membranes. We next explored a potential interaction between SOAP-1 and AP-1: it has been shown that the depletion of SOAP-1 in *y*east or in *Drosophila* leads to a loss of AP-1 punctate accumulation, suggesting that SOAP-1 could contribute to AP-1 recruitment to membranes (Fernández and Payne, 2006; Le Bras et al., 2012). We identified a similar effect on several AP-1 reporters (*aps-1::gfp, apm-1::gfp* and *unc-101::gfp* all expressed under the control of their own promoters) following soap-1 depletion (Fig. 3D-K). Quantifications were performed using a plugin written to also quantify cytoplasmic accumulation (see Materials and Methods); unexpectedly, we found that *chl-1::gfp*, following *aps-1* depletion, and *APS-1::GFP* and *APM-1::GFP*, following soap-1 depletion, did not accumulate in the cytoplasm (supplementary material Fig. S3), suggesting a global decrease in the level of expression of clathrin and AP-1. We propose that AP-1 controls clathrin membrane recruitment and could itself be recruited by SOAP-1.

![Fig. 2. AP-1 independent E-cad lateral sorting in the intestine of larvae. Single confocal sections in the intestine of larvae expressing E-cad::GFP and DLG-1::RFP. P0 larvae were observed 48 h after the induction of *aps-1(RNAi)* at the L1 stage. (A) E-cad and DLG-1 localisation at the apical membrane focal plane (arrows): E-cad is only visible on the lateral membrane (arrowheads). The second row (A′) of pictures shows enlargements of the respective white boxes above. The graph shows a line scan performed along the blue box in A′: the DLG-1 signal peaks before the E-cad signal. Note the weak DLG-1 signal (*) at the level of the subapical membrane in this focal plane. (B) E-cad and DLG-1 localisation at the lateral membrane focal plane (arrowheads). The second row (B′) of pictures shows enlargements of the respective white boxes above. The graph shows a line scan performed along the blue box in B′: the DLG-1 signal peaks before the E-cad signal. (C) E-cad and DLG-1 localisation at the apical membrane focal plane (arrows) in control (n=28) and *aps-1(RNAi)* (n=33) larvae. E-cad remains lateral (arrowheads) and is not visible at the apical membrane (arrows). The graphs show line scans performed along the blue box shown in C and C′: the DLG-1 signal peaks before the E-cad signal in control and *aps-1(RNAi)* larvae. Most *aps-1(RNAi)* larvae displayed a significant E-cad lateral signal in epidermal cells, demonstrating that the APS-1 depletion was efficient. Scale bar: 10 μm.](image-url)
AP-1 interaction with RAB-11+ endosomes

We found that the depletion of dynamin, RAB-5 or RAB-11 induced an intracellular accumulation of E-cad, indicating that these factors are required for E-cad transport; we also observed a weak but significant E-cad lateral localisation following RAB-5 or RAB-11 depletion. We therefore sought to identify where AP-1 could control E-cad apical sorting. To this aim we examined the integrity of essential membrane traffic organelles following AP-1 depletion. Electron microscopy (EM) analysis revealed that ER, Golgi cisternae, multivesicular bodies (MVBs) and lysosomes were not affected (Fig. 4A,B). By contrast, intestinal cells in the same animals revealed a strong accumulation of MVBs and lysosomes (supplementary material Table S3). To examine the two most important classes of endosomes, which are not easily identified by EM, we investigated the localisation of the early endosome marker RAB-5 and of the recycling endosome marker RAB-11. We observed a significant decrease in GFP::RAB-11 signal (puncta number and intensity), whereas GFP::RAB-5 was not significantly affected (Fig. 4C-H; supplementary material Fig. S3). We concluded that AP-1 is required for the maintenance of RAB-11+ endosomes. Because epistatic relationships between AP-1 and RAB-11 could not be established (double RNAi with AP-1 does not work), we next tested whether RAB-5, RAB-11 or the exocyst complex were required for the maintenance of the APS-1::mCherry signal. We found that it was not affected by the depletion of rab-5 (Fig. 4I-K) or the exocyst subunit sec-15 (data not shown), whereas it required rab-11 expression (Fig. 4L-N). To further investigate the link between AP-1, RAB-5 and RAB-11 we performed colocalisation studies; we found that AP-1 and RAB-11, but not RAB-5, were closely apposed and often colocalised, as validated by quantification (Fig. 4O-Q). We therefore propose that the SOAP-1/AP-1/clathrin module is sorting E-cad to the apical membrane at the level of RAB-11+ endosomes.

AP-1 depletion does not affect the apico-basal diffusion barrier in the epidermis

In epithelial cells, the separation between the apical and basolateral domains is made by an apico-basal diffusion barrier. An E-cad lateral mislocalisation could therefore be explained by a defect in the integrity of this barrier. The barrier function is achieved by the tight junctions in mammalian cells but it has not yet been defined in Drosophila or C. elegans. We first tested the potential role of the two known C. elegans junctional complexes (supplementary material Fig. S1A) by observing the localisation of CHE-14, a transmembrane protein which displays a diffuse localisation throughout the epidermal apical membrane (Michaux et al., 2000). These experiments were performed in embryos to observe the known developmental phenotype associated with the loss of junctional complexes (Chisholm and Hardin, 2005). We found that CHE-14 remains apical when α-catenin (HMP-1 in C. elegans) is targeted (Fig. 5B,B′; n=11/11), whereas it becomes homogenously distributed at the plasma membrane upon dlg-1 depletion (Fig. 5C,C′; n=9/10). Similarly, E-cad was also observed at the lateral membrane in dlg-1(RNAi) embryos (supplementary material Fig. S4A,B). These results strongly suggest that the diffusion barrier is formed by the DLG-1/AJM-1 complex (DAC) and not by the E-cad complex. Upon aps-1 depletion, CHE-14 remained apical to DLG-1 (Fig. 5D,D′; n=14/14) and the DLG-1 belt-like appearance was not affected, demonstrating that the loss of AP-1 does not affect the function of the apico-basal diffusion barrier. Interestingly, we observed that CHE-14 was visible at the lateral membrane above the DLG-1 signal (Fig. 5D′), as in hmp-1(RNAi) embryos (Fig. 5B′). We concluded that the E-cad lateral mislocalisation is not due to a simple diffusion from an apical position along the plasma membrane but to an intracellular missorting defect.

AP-1 has a specific function in E-cad apical localisation

We next tested whether the sorting function of AP-1 was restricted to E-cad, and we therefore examined the localisation of a variety of polarised markers in AP-1-depleted animals (Fig. 6). First, we observed that a cuticle was visible in threefold stage control and aps-1(RNAi) embryos, a hallmark of apical secretion by the epidermis (Fig. 4A,B). Then, we examined the localisation of the apical PAR proteins, which we found to be mislocalised to the lateral membrane in the intestine (Shafaq-Zadah et al., 2012). We did not observe any mislocalisation in the epidermis, but it should be noted that the expression of these markers becomes too weak to be examined later during morphogenesis at the time of the developmental arrest (Fig. 6A-F). We further confirmed normal apical secretion by observing that the apical transmembrane proteins LRP-1 (Yochem...
et al., 1999) and VHA-5 (Liégeois et al., 2006) were not affected by AP-1 depletion in larvae (Fig. 6G-J). We next carefully examined the localisation of DLG-1 and AJM-1, which are the main components of the DAC junction, and we found that the belt-like shape of these markers was not altered at the timing of elongation arrest (Fig. 6K-L‴), although AJM-1 and DLG-1 tend to form more intracellular puncta in aps-1(RNAi) embryos. Z-projections also suggest that the DAC junction might be elongated along the apico-basal axis (Fig. 6K‴-L‴). We then observed the localisation of the basolateral polarity determinant LET-413, the C. elegans Scribble orthologue (Legouis et al., 2000); this protein is lateral in control embryos and its localisation was unaltered in aps-1(RNAi) embryos.
Fig. 5. AP-1 depletion does not affect the apico-basal diffusion barrier. (A-D′) P0 adults expressing CHE-14::GFP and DLG-1::RFP were treated by RNAi for 48 h and F1 embryos examined following hmp-1, dig-1 or aps-1 depletion. CHE-14 is an apical protein which can also be detected in the cytoplasm (blue arrowheads). Each RNAi condition induces a developmental arrest at a different morphogenetic step: hmp-1(RNAi) embryos arrest after the onset of elongation, whereas dig-1(RNAi) embryos arrest at twofold and aps-1(RNAi) arrest at threefold stages. They all express CHE-14 at a similar level. Each picture shows a maximal projection throughout the epidermis. Z-reconstructions at the level of the dashed lines are shown in the bottom panels (A′–D′): CHE-14 is not seen below (white arrowheads) the DLG-1 staining except in dig-1(RNAi) embryos, in which DLG-1 is depleted. Note that the DAC junctions (red arrowheads) seem elongated and slightly detached from the apical pole and that CHE-14 is visible above the DLG-1 signal (white arrows) in hmp-1 (B′) and aps-1 (D′)-depleted embryos. For all conditions, n≥10. Scale bars: 10 μm.

The loss of AP-1 induces the lateral localisation of the E-cad complex and actin disorganisation during morphogenesis

Elongation is a morphogenetic process driven first by polarity establishment and junction formation, then by junction reorganisation in seam cells upon actin contraction and, finally, by muscle activity; failure in any of these processes prevents elongation beyond the twofold stage (Chisholm and Hardin, 2005). From the onset of elongation to the twofold stage, these factors were not affected by the loss of AP-1, as assessed by the observation of markers and normal embryo movements inside the eggshell (supplementary material Fig. S4C-H and data not shown). In control embryos, careful observation from the comma stage to the threefold stage showed that E-cad is initially visible at the lateral membrane; however, this signal was almost lost from the twofold stage and very rarely detected in threefold-stage embryos (Fig. 8A,C,E; n=30). In aps-1(RNAi) embryos, the E-cad lateral signal became stronger from the twofold stage (Fig. 8B,D,F; n=29). Three-dimensional (3D) reconstructions showed that E-cad was mislocalised to the lateral membrane rather than intracellularly (Fig. 8E′,F′ and supplementary material Movies 2A-C). Other members of the E-cad complex, such as the transmembrane claudin VAB-9 (Simske et al., 2003) and the p120-catenin (JAC-1) (Pettitt et al., 2003), which are strictly apical in threefold-stage control embryos (Fig. 8G,I), were observed at the lateral membrane in aps-1(RNAi) embryos (Fig. 8H′,J′ and supplementary material Movies 3A-B). This suggests that the lateral E-cad pool can recruit other members of its complex.

The lateral mislocalisation of E-cad could by itself induce elongation arrest. However, actin is required for embryonic elongation, and E-cad interacts with actin via caterinins. We therefore hypothesised that actin itself could also be affected, and examined its localisation with an actin-binding domain fused to GFP (Gally et al., 2009). In control embryos, actin becomes well organised as parallel bundles perpendicular to the antero-posterior axis in the dorso-ventral epidermis from the twofold stage (Fig. 8K,M,O). In aps-1(RNAi) embryos, the initial actin localisation was not perturbed (Fig. 8L,N). However, in threefold-stage embryos, the very precise and regular organisation of actin was lost (Fig. 8O-Q), showing that the AP-1 depletion had a strong impact not only on E-cad-polarised localisation but also on the actin network, which is essential until the end of elongation (Priess and Hirsh, 1986). The late elongation arrest at threefold stage could therefore be explained by the relatively late impact of AP-1 depletion on E-cad-polarised localisation and/or actin disorganisation.

AP-1 depletion induces a mislocalisation of the DAC junction and an expansion of the apical pole

As reported previously (Shafaq-Zadah et al., 2012; Shim et al., 2000), RNAi against the σ, γ or both µ subunits of AP-1 induces a highly reproducible embryonic arrest during elongation at the threefold stage. We used EM to further characterise the phenotype of threefold-stage-arrested embryos. The most striking phenotype was that the electron-dense structures corresponding to the DAC junction (supplementary material Fig. S1A′) (Köppen et al., 2001) were systematically positioned more laterally and were more elongated (n=16 in 6 embryos) than in control embryos (n=12 in 4 embryos) (Fig. 7A-C). The lateral position was not observed in the intestine but the elongation phenotype was similar (Shafaq-Zadah et al., 2012). The expansion of the apical domain and the junction elongation correspond to what we observed with CHE-14 and the AJM-1/DLG-1 localisation. We also observed a rarer phenotype in which the apical pole was sometimes bulging apically from the rest of the body, suggesting that some seam cells undergo at least partial apical extrusion (supplementary material Fig. S5A-B′; apical bulging was observed for 4/36 seam cells in six aps-1(RNAi) embryos and 0/24 seam cells in four control embryos). Finally, we also systematically noticed cellular debris between the eggshell and the embryo itself, as well as inside the intestine lumen (supplementary material Fig. SSB-B′), which were not observed in control embryos (supplementary material Fig. S5A). We hypothesised that these debris could be formed by parts of seam cells detaching from the body and swallowed by the embryo. We found that the external and intestinal lumen debris were indeed positive for CHE-14::GFP, which is strongly expressed in seam cells but not in the intestine (supplementary material Fig. SSC-C′). These data show that AP-1 is required to maintain the DAC junctions at their normal position and/or to control the size of the apical pole while the diffusion barrier function remains intact.
DISCUSSION

We have shown that AP-1 controls E-cad apical localisation both in embryos and larvae and is required for the normal localisation of the E-cadherin-catenin complex and actin organisation in C. elegans epidermal cells. This cellular function of AP-1 is strictly required to prevent late but severe morphogenetic defects leading to lethality during embryonic elongation. Our results also demonstrate that this AP-1 function requires SOAP-1 and clathrin: SOAP-1 probably controls AP-1 membrane recruitment in a manner similar to the recruitment of clathrin by AP-1 itself. This SOAP-1 function is conserved from yeast to Drosophila and could act in parallel to the more traditionally recognised role of the Arf1 pathway, which was shown to recruit AP-1 in mammalian cells (Robinson, 2004). In the epidermis, the depletion of Arf1 isoforms (arf-1.1 or arf-1.2; the latter induces a systematic larval lethality) did not affect E-cad localisation. We therefore propose that a SOAP-1/AP-1/clathrin module controls E-cad apical sorting in the epidermis. In the intestine, AP-1 is also required for apical sorting but triggers a very different phenotype in which the lateral membrane is converted into an apical membrane (Shafaq-Zadah et al., 2012; Zhang et al., 2012). Interestingly, we observed that E-cad is lateral in the intestine of larvae and not missorted upon AP-1 depletion, whereas the loss of soap-1 had no effect on the localisation of PAR-6 (Shafaq-Zadah et al., 2012) or CDC-42 (G.G. and G.M., unpublished results) even in larvae displaying a strong developmental arrest phenotype. However, we cannot exclude that other cargos, perhaps less dynamically trafficked and/or less essential than E-cad, might also rely on AP-1 for their polarised localisation in the epidermis. Altogether, these data demonstrate that AP-1 can control essential cargos differently in different tissues, and further work will be required to identify the specificity of these AP-1 functions.

In our screen, we identified 50 genes affecting normal E-cad expression or localisation. Among these 50 positive genes, 17 encode subunits or accessory proteins of the COPI, COPII and

Fig. 6. AP-1 has a specific function in E-cad apical localisation. In all experiments shown in this figure, we observed a systematic lethality (A-F,K-P: embryonic lethality; G-J: larval lethality) associated with the depletion of AP-1. (A-F) P0 adults were treated by RNAi for 48 h and the F1 embryos examined following aps-1 depletion. Embryos were fixed and stained for PAR-3 (A,B), PAR-6 (C,D) and PKC-3 (E,F). Each picture shows a maximal projection throughout the intestine region and arrowheads point to the epidermal staining. None of these markers was affected at this stage of development. (G,H) Larvae were treated with RNAi for 72 h, then fixed and stained for the apical marker LRP-1. Each picture shows a transverse section in the apico-basal axis. The punctate apical localisation of LRP-1 was not affected. (I,J) Larvae expressing VHA-5::GFP were treated by RNAi for 72 h. Each picture shows a transverse section in the apico-basal axis. The punctate apical localisation of VHA-5 was not affected. (K-L) P0 adults expressing AJM-1::GFP and DLG-1::RFP were treated by RNAi for 48 h and the F1 embryos examined following aps-1 depletion. (K,L) Maximal projections throughout the epidermis. The subapical belt formed by AJM-1 and DLG-1 is not affected; the intracellular puncta co-stained by these two markers are frequent at that stage of development in control embryos but tend to accumulate upon AP-1 depletion. (K’-L’) Z-reconstructions obtained by reslicing and projections of 4-5 planes, showing a slight thickening of the AJM-1/DLG-1 signal. (M-N’) P0 adults expressing LET-413::GFP were treated by RNAi for 48 h and the F1 embryos examined following aps-1 depletion. Each picture shows a maximal projection throughout the epidermis. The LET-413 localisation is not affected at the subapical (arrows) or lateral (arrowheads) levels. M’ and N’ are enlarged pictures of the dashed boxes in M and N, respectively. (O,P) P0 adults were treated by RNAi for 48 h and the F1 embryos were fixed and stained for UNC-52 following aps-1 depletion. The basal secretion of this extracellular marker (arrowheads) was not affected. For all conditions, n≥15. Ap, apical; Bl, basolateral. Scale bars: 10 μm.
V-ATPase complexes, which control secretion and acidification of organelles. GSL biosynthetic enzymes (fasn-1, acs-1, pod-2, splt-1 and let-767) were tested separately and displayed the same phenotype; GSLs are an essential component of lipid rafts and control apical sorting in the C. elegans intestine (Zhang et al., 2011). Targeting these genes by RNAi induces a fast and fully penetrant lethality but we did not identify any E-cad localisation defects, in contrast to what was found in the intestine, where GSLs are essential for apical sorting (Zhang et al., 2011). These observations suggest that GSLs do not control E-cad apical sorting in the epidermis.

Where could the SOAP-1/AP-1/clathrin module be implicated in E-cad trafficking? AP-1 could act to prevent E-cad lateral accumulation, for instance by controlling a transcytotic route; this hypothesis could explain easily the lateral localisation of E-cad at the beginning of embryonic elongation. However, we do not favour this possibility because the loss of dynamin does not induce an E-cad lateral accumulation. In addition, E-cad intracellular localisation upon dynamin, RAB-5, RAB-11 or exocyst complex depletion is mostly restricted to the apical region (Fig. 1; supplementary material Fig. S2). We found a very tight link between AP-1 and RAB-11 and a partially overlapping phenotype: an E-cad lateral localisation was observed in about 50% of rab-11(RNAi) larvae. In epithelial cells, Rab11 plays a central role in the secretory and the recycling pathways (Apodaca et al., 2012), and we therefore propose that AP-1 could control E-cad apical sorting in these two pathways. In the absence of AP-1, E-cad could be targeted randomly to the apical and lateral membranes by a default mechanism not dependent on RAB-11. RAB-11 depletion would lead to a stronger phenotype due to the many functions associated with RAB-11; rab-11(RNAi) induced in P0 hermaphrodites triggers various defects from the one-cell embryo (Bembenek et al., 2010; Sato et al., 2008; Zhang et al., 2008), whereas AP-1-depleted embryos arrest much later at the threefold stage. While examining AP-1-depleted embryos we found that the DAC domain was elongated. Several groups have reported such a phenotype, in particular upon depletion of the MAGUK protein MAGI-1 (Lynch et al., 2012; Stetak and Hajnal, 2011). This protein probably contributes to junction organisation but is not an essential factor: it was identified as an enhancer of the weak loss-of-function hmp-1(fe4) allele (Lynch et al., 2012), and a likely null allele is viable (Stetak and Hajnal, 2011). Other enhancers of hmp-1(fe4) identified include AP-2 subunits (Lynch and Hardin, 2009). Although we did not identify a role for AP-2 in our screen, this observation strongly suggests an AP-2 contribution to junction maintenance. Altogether, these works highlight the need for various screening strategies to identify non-essential factors.

The loss of AP-1 induces a threefold-stage arrest during development. The AP-1 functions identified in neurons (Dwyer et al., 2001) or in the intestine (Shafaq-Zadah et al., 2012; Zhang et al., 2012) cannot account for this lethality. At the developmental level, embryonic elongation requires seam cell elongation driven by actin tension in epidermal cells and functional muscles attached to the epidermis from twofold stage (Chisholm and Hardin, 2005). Because AP-1-depleted embryos display normal movements we believe that the observed embryonic arrest is most likely due to defects linked to epidermal cells.

In AP-1-depleted embryos, the lateral accumulation of E-cad (from twofold stage) was observed first, shortly before the timing of the morphogenetic arrest (threefold stage) when actin disorganisation was observed. This strongly suggests that E-cad mis-targeting and/or actin disorganisation are responsible for the developmental arrest. A potential direct link between E-cad mislocalisation and actin organisation is suggested by the observation that the Hmp-1 and Aps-1 cellular phenotypes are partially similar: both induce a mislocalisation of the DAC junction in the apico-basal axis. This could be due to an arrest of elongation not directly translated into an arrest of membrane addition at the apical pole. If this was the case, the apical membrane could still increase its surface, leading to a more lateral localisation of the DAC junction, and it would be unlikely to affect its diffusion barrier function. However, an important difference is that in AP-1-depleted embryos we did not detect a detachment of actin bundles from the junctions as reported when the E-cad complex is lost (Costa et al., 1998). Instead, we observed a persistence of the E-cad complex at the apical membrane and an irregular organisation and abnormal thickness of actin bundles. Because the E-cad complex can actively recruit and control actin at the level of junctions, E-cad mislocalisation at the lateral membrane could indirectly destabilise actin organisation and affect forces driving elongation. Alternatively, AP-1 could also directly contribute to actin organisation: a proteomic analysis showed that several actin...
nucleators were found on AP-1-enriched liposomes (Baust et al., 2006). We therefore cannot exclude that the loss of AP-1 could also have a direct impact on actin organisation through these actin cofactors, independently of E-cad. Although the Gex (gut on the exterior) phenotype associated with the loss of function of these cofactors (Patel et al., 2008; Soto et al., 2002) occurs earlier than the threefold arrest induced by AP-1 depletion, we cannot rule out a later role of these factors hidden by the earlier phenotype, and therefore propose that AP-1 controls E-cad apical localisation and the embryonic elongation arrest associated with the loss of AP-1 can be due to E-cad missorting and, directly or indirectly, defective actin contractile forces.

**MATERIALS AND METHODS**

**Genetics**

*C. elegans* strains were maintained and crossed as described (Brenner, 1974). The strains used in this study are shown in supplementary material Table S4.

**Plasmid construction**

The *aps-1::gfp* construct under the control of its own promoter was generated as described (Shafaq-Zadah et al., 2012). The epidermal *aps-1::mCherry* construct was generated using the Multisite Gateway system (Invitrogen). The epidermal *aps-1::mCherry* construct was generated by creating three donor vectors: *aps-1* genomic sequence and promoter (*aps-1*) promoter fused to GFP (by PCR fusion), *aps-1* genomic sequence and promoter fused to mCherry::unc-54 (*aps-1*:mCherry::unc-54) promoter, and *aps-1* genomic sequence and promoter fused to GFP::rab-11 (*aps-1*:GFP::rab-11) promoter. Both *aps-1*:mCherry::unc-54 and *aps-1*:GFP::rab-11 were expressed under the control of its own promoter. The epidermal *aps-1* construct was generated using the Multisite Gateway system (Invitrogen). The epidermal *aps-1* construct was generated by creating three donor vectors: *aps-1* genomic sequence and promoter (*aps-1*) promoter fused to GFP (by PCR fusion), *aps-1* genomic sequence and promoter fused to mCherry::unc-54 (*aps-1*:mCherry::unc-54) promoter, and *aps-1* genomic sequence and promoter fused to GFP::rab-11 (*aps-1*:GFP::rab-11) promoter. Both *aps-1*:mCherry::unc-54 and *aps-1*:GFP::rab-11 were expressed under the control of its own promoter.

**RNAi**

Embryonic and larval RNAi were performed by feeding as described using the Ahringer-Source BioScience library (Fire et al., 1998; Kamath and Ahringer, 2003; Shafaq-Zadah et al., 2012); L4440 corresponds to the standard control RNAi feeding strain. To bypass the early embryonic requirement of most candidate genes we performed the screen by adding bleach-treated adults to RNAi plates, and we observed E-cad localisation 48 h and 72 h later in the same generation (P0); the embryonic development is therefore normal and the RNAi knockdown only starts after hatching. For other experiments, RNAi was induced in young adults and the phenotypes were observed in the next generation (F1).

**Development**

Immunostaining

Fixation of embryos or larvae was performed as described using the freeze-crack methanol protocol (Leung et al., 1999). We used anti-AJM-1 MH27 (1/50) (Köppen et al., 2001), anti-UNC-52 MH2 (1/50) (Francis and Waterston, 1991) and anti-PAR-3 P4A1 (1/50) (Nance et al., 2003) monoclonal antibodies from DSHB (University of Iowa, USA). The anti-PAR-6 (1/50) (Labbe et al., 2006), anti-PKC-3 (1/1000) (Sugiyama et al., 2008) and anti-LRP-1 (1/200) (Yochem et al., 1999) rabbit antibodies were generous gifts from Monica Gotta, Shigee Ohno and Simon Tuck, respectively. Alexa Fluor 488- or 532-conjugated antibodies (Invitrogen) were used as secondary antibodies.

Electron microscopy

Electron microscopy experiments were performed as described (Shafaq-Zadah et al., 2012). Junction length and detachment from the apical pole (Fig. 7A–C) were quantified as follows: each embryo was sectioned every 5–7 µm to ensure that different cells were observed in different 5–7 µm sections. Only one segment of high quality per section was used to measure electron-dense structures (junctons). Measurements were performed on 12 junctions from four different control embryos and on 16 junctions from six different aps-1(RNAi) embryos. An identical approach was used to quantify the number of MVBs and lysosomes in epidermal seam cells and in the intestine: we counted all single-membrane organelles containing internal vesicles or membranes, excluding mitochondria, which have a double membrane. Immuno-electron microscopy was performed as described (Liégeois et al., 2006). The anti-AJM-1 antibody MH27 (1/10, DSHB, University of Iowa) was detected using 5-µm gold particles coupled to a goat anti-mouse antibody (GMHR5L, Oxford Instruments).

Confocal microscopy and signal quantifications

Confocal observations were performed using a Leica SPE (for screening) or SP5 confocal microscope equipped with a 63× objective (LAS AF software). The SP5 confocal microscope is equipped with a sensitive hybrid detector. All images were examined using ImageJ 1.43 and assembled using Adobe Photoshop and Illustrator CS3.

The percentage of embryos or larvae displaying a phenotype was obtained either by direct observation or after quantification. Quantifications (Fig. 1) were performed using ImageJ 1.43 along straight lines (length 10 µm, width 0.3 µm) over the apical, lateral and cytoplasmic parts of one cell for each larva. Quantifications were normalised to the cytoplasmic background; a ratio of 1 therefore indicates no specific membrane staining. Quantifications of colocalisation (Fig. 4Q) were performed using the ImageJ JaCoP plugin (Bolte and Cordelieres, 2006) to determine the Pearson’s correlation coefficient (PCC), which measures the linear relationship between the two signals) as well as the Manders’ coefficients tM1 (proportion of RAB-11 signal coincident with a signal in the APS-1 channel over its total intensity) and tM2 (proportion of APS-1 signal coincident with a signal in the RAB-11 channel over its total intensity). Coefficients were calculated in several regions of interest (ROI) in one plane of each larva examined (n = 23 larvae for each condition). Manders’ coefficients were calculated for pixels above a manually determined threshold for each channel. The coefficients obtained in our experiments enabled us to argue that there is a partial colocalisation between APS-1 and RAB-11.

To quantify the total amount of APS-1, APM-1, RAB-5 and RAB-11 in puncta and cytoplasm (Figs 3 and 4) we used a home-written plugin and Fiji software (Schindelin et al., 2012). We computed the sum of intensity over each plane of a z-stack containing the entire epidermal signal either masking out the cytoplasm or the puncta to quantify the total amount of labeled protein in puncta or in the cytoplasm, respectively. A mask for each plane was computed: to keep only objects of relevant size, we bandpass-filtered planes (0.16–3 µm−1), removed background (rolling-ball diameter: 1.6 µm) (Gonzalez and Woods, 2002) and thresholded each plane separately with the Kapur–Sahoo–Wong method modified to use Renyi entropy (Kapur et al., 1985). Because vesicles can spread over several z-planes, prior to counting particles, we computed a maximum projection and thresholded it as described above, applied the watershed algorithm (Vincent and Soille, 1991) and counted particles with a radius >0.1 µm.

Statistical analysis

Parametric t-tests were used when samples had a Gaussian distribution and similar variances. Other cases were treated using Wilcoxon tests. Significance is indicated as follow: *P<0.05, **P<0.01, ***P<0.001.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

G.G., M.S.-Z. and G.M. designed the experiments, G.G., M.S.-Z., O.N. and R.D. performed experiments and data analysis. J.P. wrote the quantification plug-in. G.G. and G.M. wrote the manuscript.

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Supplementary material

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