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## The apical protein Apnoia interacts with Crumbs to regulate tracheal growth and inflation

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# PLOS Genetics

## The apical protein Apnoia interacts with Crumbs to regulate tracheal growth and inflation --Manuscript Draft--

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<b>Abstract:</b>	<p>Most organs of multicellular organisms are built from epithelial tubes. To exert their functions, tubes rely on apico-basal polarity, on junctions, which form a barrier to separate the inside from the outside, and on a proper lumen, required for gas or liquid transport. Here we identify apnoia (apn), a novel Drosophila gene required for tracheal tube elongation and lumen stability at larval stages. Larvae lacking Apn show abnormal tracheal inflation and twisted airway tubes, but no obvious defects in early steps of tracheal maturation. apn encodes a transmembrane protein, primarily expressed in the tracheae, which exerts its function by controlling the localization of Crumbs (Crb), an evolutionarily conserved apical determinant. Apn physically interacts with Crb to control its localization and maintenance at the apical membrane of developing airways. In apn mutant tracheal cells, Crb fails to localize apically and is trapped in retromer-positive vesicles. Consistent with the role of Crb in apical membrane growth, RNAi-mediated knockdown of Crb results in decreased apical surface growth of tracheal cells and impaired axial elongation of the dorsal trunk. We conclude that Apn is a novel regulator of tracheal tube expansion in larval tracheae, the function of which is mediated by Crb.</p>
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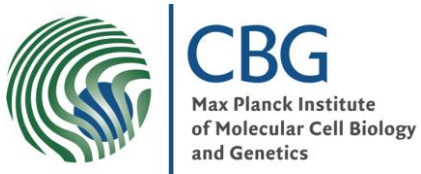
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Dresden, 31.10.2018

Dear Editor,

we would like to submit the revised version of our manuscript entitled **“The apical protein Apnoia interacts with Crumbs to regulate tracheal growth and inflation”** for publication in PLoS Genetics.

We have addressed all points raised by the reviewers and listed them in a separate file.

We hope that you find our work interesting and are looking forward to your response.

Sincerely,

Elisabeth Knust  
Kassiani Skouloudaki

## Responses to reviewer's questions

### Reviewer #1:

In their manuscript entitled "The apical protein Apnoia interacts with Crumbs to regulate tracheal growth and inflation," Skouloudaki, Papadopoulos and colleagues identify a novel Crumbs-interacting protein, Apnoia (Apn), which they name based on the tracheal defect observed in loss of function animals. Apn was initially identified by yeast two-hybrid as a Crumbs interactor and later shown to substantially co-localize with Crumbs in tracheal cells, although most Apn is apical as compared to the subapical localization of Crb. A CRISPR-Cas9 generated deletion of the coding region resulted in a lethal mutation in which animals die during the second larval instar with twisted and uninflated tracheal tubes. Tube maturation was assessed and found to be defective at the stage of liquid clearance and gas-filling. Additionally, authors report that dorsal trunk tubes displayed a reduced length. Based on measurements of cells within the dorsal trunk, the authors conclude that growth along the AP axis is reduced, but not circumferential growth. In addition, the authors report that Crumbs localization is altered such that Crb is mostly within intracellular vesicles. Blocking endocytosis in an *apn* mutant background resulted in apical accumulation of Crbs, interrupted by the authors as indicating a requirement for Apn in Crb retention at the apical membrane, perhaps via Rab11-dependent trafficking from an endosomal compartment. The intracellular Crb in *apn* mutant animals was largely colocalized with the retromer component, VPS-35. The authors further report that knockdown of Crb by RNAi recapitulates the *apn* loss of function phenotype.

The work is interesting and appropriate for the journal. It seems largely well done, and should be considered for publication after addressing the following concerns:

- A major deficit of this manuscript is that the authors test a tracheal requirement for Crumbs and Apn by eliminating protein function in the entire animal. Crumbs, of course, is broadly expressed, and while *apn* expression does seem to be fairly tracheal-specific, there remains a worry that the phenotype is non-autonomous. In principle these deficits could be easily addressed.

Since *apn* is expressed only in tracheae, its elimination in the entire animal is expected to influence only the tracheal tissue. In addition, tracheae-specific depletion of *apn* by RNAi exhibits the same phenotype (Suppl. Fig 3B). Finally, re-introduction of the *apn* cDNA in the tracheae rescues the mutant phenotype (Suppl. Fig 3C, F).

Complicating matters for Crumbs, mosaic loss of function studies in the trachea have been performed (*crb11A22*), and no defect reported (Schottenfeld-Roames et al., 2014). Likewise, pan-tracheal crumbs RNAi has been shown to modify the phenotype of mutants that accumulate ectopically high levels of Crumbs, but has not been shown to cause tracheal tube defects in otherwise wild type backgrounds (Song et al., 2013), at least under the RNAi conditions described. Authors should test cell autonomy or at least tracheal-specific requirements for Crb and Apn, either by eliminating them there specifically, and/or by rescuing specifically in the trachea (in the case of *apn* mutant larvae).

We thank the reviewer for this important question. We have actually used different reagents from Song et al, 2013, indicated in Materials and Methods, in which we describe the *crb* RNAi line used by us. Indeed, previous data performed with *crb*[11A22] as well as with *crb* RNAi #



39177 did not show significant tracheal defects. In line with this, *crb*[11A22] and *crb*RNAi 39177 showed no abnormal trachea phenotype in our experiments (data not shown). Therefore we tested the *crb* RNAi #38373 (Hochapfel F et al., 2017, Cellular and Molecular Life Sciences), which targets the 3'UTR of the *crb* mRNA. We observed a trachea-specific phenotype upon knocked down, using either ubiquitously or tracheal-specific Gal4 drivers.

The same phenotypes were observed upon *Apn* knock-down using the aforementioned drivers. Personal communication with Michael Krahn (University of Muenster) confirmed that these authors also experienced problems with the Song et al *crb* RNAi line #39177, but not with the *crb* RNAi line #38373. Therefore we decided to perform our knockdown experiments with the line #38373 line provided by the Bloomington Stock Center.

Additional points for the authors to address include:

- Can *apn* mutants be rescued with transgene expression during larval stages?

New data added in Suppl. Fig 3C, F show that in approximately 20% of *apn*<sup>1</sup> mutant larvae gas filling (Suppl. Fig 3C) and tube length defects (Suppl. Fig 3F) are rescued upon tracheal-specific transgene expression.

- Is *Crb* localization in other epithelia normal in *apn* mutants?

New data added in Suppl. Fig 4G, H, show that *Crb* localization is not altered in salivary glands of stage L2 mutant in *apn*<sup>1</sup> larvae.

- Are tracheal defects, including *Crb* mislocalization, limited to multicellular tracheal tubes? or are autocellular and seamless tubes also compromised?

New data added in (Suppl. Fig 4A-B'') show that tracheal defects are not limited to multicellular tubes, but also affect autocellular and seamless tubes.

For the PLA shown in figure 1, can authors show images from more closely matched dorsal trunk segments (same metamere and same tube diameter) and provide quantitation?

We provide new data according to reviewer request in Fig. 2A-B'' and Fig. 2C.

## **Reviewer #2:**

Through a modified yeast two hybrid screen, Skouloudaki et al identify an insect-specific transmembrane protein, which they name Apnoia (*Apn*), as an interaction partner of the apical determinant *Crumbs*. Further evidence for a complex comes from co-IPs from

cultured cells and from in vivo localization data. Apn is shown to have high expression in the *Drosophila* tracheal system, localizing to the apical domain of tracheal cells. Generated Apn mutants display tracheal defects specifically at larval stages when tracheal remodeling events are associated with larval molting and growth. The tracheal tubes of apn mutants are abnormally short and twisted, defects associated with abnormal apical domain size and shape. These defects are also associated with a loss of Crumbs from the apical domain and abnormal accumulation of Crb in Vps35-positive vesicles. Blocking endocytosis reverses the loss of cortical Crumbs in apn mutants, and the loss of Crumbs seems to underlie the apn mutant defects since similar defects are observed following crb RNAi. This study should be of interest to cell and developmental biologists, particularly those studying insects. However, a number of points should be addressed.

1. For Figure 1H, I and J, higher magnification X-Y images of the apical surface and circumference should be shown to clarify co-localization patterns. Also, is there any degree of Apn-Crb co-localization over the apical surface to corroborate the PLA data?

In Fig.1G-G" and H-H"(X-Y) as well as Fig.1G"" and H"" (circumference Y-Z) we provide higher magnification images that clearly show a high degree of colocalization of Apn with the other apical membrane proteins. In Fig.1I-I" (X-Y) and Fig.1I"" (circumference Y-Z) we provide strong evidence that Crb and Apn partially colocalize at the apical cell surface.

2. Further controls are needed for the PLA experiments. The same antibodies should be used in the experiment and control (e.g. anti-Crb plus anti-GFP or anti-Apn plus anti-GFP).

We apologize for not making this point clear in our manuscript. We have used a *DECad*-GFP knock-in line as well as a fosmid expressing a GFP-tagged Apn. We stained both lines with GFP and Crb antibodies. This means that the same antibodies have been used in experiment (apn-sfGFP) and control (*DECad*-GFP).

We have included an additional experiment in Fig.2D-D" and Fig.2E-E", in which the same line (apn-sfGFP) was used for PLA, but different antibodies (e.g. anti-Crb plus anti-GFP (Fig.2D-D") and anti-Ecad plus anti-GFP, as control (Fig.2E-E")).

A different apical protein should be tested for a PLA signal with Crb (a different GFP-tagged protein for example) since the signal between Crb and Apn-GFP could be due to random interactions in the apical domain rather than being due to specific complexes.

We followed the reviewer's request and we used the apical protein SAS (SAS-Venus: Stranded-at-Second fused with Venus under the tubulin promoter). We stained both lines with anti-GFP and anti-Crb. This means that the same antibodies have been used in experiment (apn-sfGFP) (Fig.2F-F") and control (SAS-Venus) (Fig.2G-G").

3. To assess whether or not Apn has Crb-independent effects on retromer function, as considered in the Discussion, or if Apn affects the trafficking of other proteins through the retromer, it would be worth testing if the Vps35 compartment enlargement in apn mutants is due to Crb or not (by examining apn mutants expressing crb RNAi).

We thank the reviewer for his/her comment that allows us to clarify this point. We have performed additional experiments, in which *crb* was down regulated by RNAi in *apn*<sup>1</sup> mutants. The results show that indeed the increase in size of Vps35 positive vesicles is to some extent dependent on Crb (new Fig. 10 A-D).

4. The authors say that the *apn* mutant phenotypes are also seen “in larvae upon knock-down of *apn* by RNAi in the tracheae (Fig. 2C)”. However, the label for Fig 2C indicates that the *apn* RNAi is driven by the ubiquitous daughterless-Gal4. RNAi of *apn* specifically in the tracheal system is important for demonstrating that the tracheal defects are tissue-autonomous, and that the body size and viability defects are due the tracheal defects (also the body size and viability defects should be clearly described for the tracheal RNAi of *apn*). An alternate approach would be to attempt rescue of the mutant phenotypes with expression of *Apn* specifically in the tracheal system.

Following to the reviewer’s comment, we performed tracheae-specific depletion of *apn* by RNAi and observed the same gas filling and tube length defects (Suppl. Fig 3B), defects in Crb localization (Fig 5R) as well as body size reduction (Suppl. Fig 2K) as in *apn*<sup>1</sup> mutant animals.

New data added in Suppl. Fig 3C, F show that in approximately 20% of *apn*<sup>1</sup> mutant larvae gas filling (Suppl. Fig 3C) and tube length defects (Suppl. Fig 3F) are rescued upon tracheal-specific transgene expression.

5. Similarly, the authors say that they “knocked-down *crb* in tracheal tubes by expressing *crb* RNAi” but again the panels in Figure 8 indicate the use of *da*-Gal4. RNAi of *crb* specifically in the tracheal system is also important for the author’s conclusions.

We performed tissue specific depletion of *crb* by RNAi, and observed gas filling and tube length defects (Fig 3G), similar as in *apn*<sup>1</sup> mutant animals.

A typo on page 10, line 242: Suppl. Fig. S3 D-E’ should be Suppl. Fig. S3 E-F.’

We have corrected the manuscript according to reviewer’s request.

1       **The apical protein Apnoia interacts with Crumbs to**  
2                   **regulate tracheal growth and inflation**

3  
4           **Kassiani Skouloudaki<sup>1,\*,#</sup>, Dimitrios K. Papadopoulos<sup>1,2,#</sup>, Pavel**  
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17

18 Running title:

19 Apnoia interacts with Crumbs to regulate tracheal growth and inflation

20

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23

24 Author contributions: KS, conceived the project, designed and performed the  
25 experiments, analyzed the data, prepared figures and wrote manuscript; DKP,  
26 designed and performed experiments and analyzed the data and wrote the  
27 manuscript; PT wrote the manuscript; EK initiated and supervised the project  
28 and wrote the manuscript; all authors critically reviewed the manuscript and  
29 approved it for submission.

30

## 31 **Abstract**

32 Most organs of multicellular organisms are built from epithelial tubes. To exert  
33 their functions, tubes rely on apico-basal polarity, on junctions, which form a  
34 barrier to separate the inside from the outside, and on a proper lumen,  
35 required for gas or liquid transport. Here we identify *apnoia* (*apn*), a novel  
36 *Drosophila* gene required for tracheal tube elongation and lumen stability at  
37 larval stages. Larvae lacking Apn show abnormal tracheal inflation and  
38 twisted airway tubes, but no obvious defects in early steps of tracheal  
39 maturation. *apn* encodes a transmembrane protein, primarily expressed in the  
40 tracheae, which exerts its function by controlling the localization of Crumbs  
41 (Crb), an evolutionarily conserved apical determinant. Apn physically interacts  
42 with Crb to control its localization and maintenance at the apical membrane of  
43 developing airways. In *apn* mutant tracheal cells, Crb fails to localize apically  
44 and is trapped in retromer-positive vesicles. Consistent with the role of Crb in  
45 apical membrane growth, RNAi-mediated knockdown of Crb results in  
46 decreased apical surface growth of tracheal cells and impaired axial  
47 elongation of the dorsal trunk. We conclude that Apn is a novel regulator of  
48 tracheal tube expansion in larval tracheae, the function of which is mediated  
49 by Crb.

50

## 51 **Author summary**

52 Tubular organs, such as the fruitfly airways, comprise essential functional  
53 pipes through which gas and liquid are transported. They consist of highly  
54 polarized epithelial cells that form a barrier between air and the larval body.  
55 However, during larval development, these tubes, though very rigid due to the

56 presence of cuticle, need to rapidly grow in size within short time-windows. To  
57 control their growth, cells have to regulate the apical membrane surface of the  
58 epithelial cells. In this work we have discovered a new gene called *apnoia*,  
59 which is important for tracheal growth and inflation at larval stages. We  
60 demonstrate that Apnoia is expressed in the apical membrane of tracheal  
61 epithelia cells, where it is required for apical membrane expansion. This  
62 function is mediated by regulating the proper localization and maintenance of  
63 the well-known apical determinant Crumbs. Both Apnoia and Crumbs proteins  
64 are required for expansion of the apical cell surface and, thereby, tube  
65 elongation. Such a mechanism is required to support the complex  
66 morphogenetic events that the tracheal system undergoes during  
67 development.

68

## 69 **Introduction**

70 Animal organs consist of epithelial tissues, which form the boundaries  
71 between internal and external environment [1-3]. During development,  
72 epithelia are instrumental to shape the various organs. Many epithelial tissues  
73 form tubular organs, such as the gut, the kidney or the pulmonary system. A  
74 fundamental feature of epithelial tubes and sheets is to keep the balance  
75 between the maintenance of structural integrity and tissue rigidity during  
76 organ growth and morphogenesis. To understand how this balance is  
77 achieved during rapid, temporally regulated developmental transitions from  
78 juvenile to adult body shapes, several studies in various animal models have  
79 focused on elucidating how cell proliferation, cell polarity, cell shape changes  
80 and trafficking contribute to the formation of tubular lumen length and

81 diameter [4-7]. The correct coordination of these processes is crucial for  
82 normal organ function. This is reflected in the fact that several human  
83 diseases are linked to defects in epithelial tube formation and maintenance,  
84 such as polycystic kidney disease or cystic fibrosis [8-11].

85         The developing tracheae of *Drosophila melanogaster*, a network of  
86 branched epithelial tubes that ensure oxygen supply to the cells of the body,  
87 has emerged as an ideal system to study cell fate determination and  
88 morphogenesis of epithelial tubes. The available genetic tools as well as the  
89 ease to image the tracheal system in the fly embryo has provided detailed  
90 insights into the developmental processes required to form tubular structures  
91 with defined functional lumens and have contributed to elucidate the interplay  
92 between tissue growth, differentiation and cell polarity [12-15].

93         The stereotypically branched tracheal system of *Drosophila* is set up at  
94 mid-embryogenesis. Once a continuous tubular network has formed, the tube  
95 expands to warrant increased oxygen supply to all tissues during animal  
96 growth. Tube expansion occurs by growth along the diameter and along the  
97 anterior-posterior axis. Growth is accompanied by the formation of a transient  
98 cable, comprised of a chitinous apical extracellular matrix (aECM), which fills  
99 the lumen of the tube. The generation of this cable requires the secretion of  
100 chitin and chitin-modifying enzymes. Mutations in genes affecting secretion or  
101 organization of the chitin cable result in excessively elongated tracheal tubes  
102 or tubes with irregular diameter (with constricted and swollen areas along the  
103 tube length) [15-17]. Axial growth, on the other hand, depends on the proper  
104 elongation of tracheal cells along the anterior- posterior axis. At later stages of

105 embryogenesis, the lumen becomes cleared and filled with air.

106         After hatching, the larvae undergo two molts, a process during which  
107 animals rapidly shed and replace their exoskeleton with a new one, bigger in  
108 size. For this, new chitinous aECM is secreted apically, thus surrounding the  
109 old tube. Remodeling of this aECM permits tissue growth between larval  
110 molts. The molting process is initiated by the separation of the old aECM from  
111 the apical surface of the epithelial cells and the secretion of chitinases and  
112 proteinases, which partially degrade the old cuticle. The remnants of the old  
113 cuticle in each metamer are shed through the spiracular branches. This  
114 process, called ecdysis, is followed immediately by clearance of the molting  
115 fluid and air filling [18-20]. Interestingly, while the diameter of the dorsal trunk  
116 only increases at each molt, tube length increases continuously throughout  
117 larval life, particularly during intermolt periods [14]. Despite the importance of  
118 tube expansion and elongation for larval development [18], the underlying  
119 mechanisms that control tracheal growth at this stage remain poorly  
120 understood.

121         A well-established regulator of apical domain size in developing  
122 epithelia is Crumbs (Crb). Crb is a type I transmembrane protein, which acts  
123 as an apical determinant of epithelial tissues [21]. It has a large extracellular  
124 domain, a single transmembrane and a short cytoplasmic domain. Loss- and  
125 gain-of-function experiments have shown that apical levels of Crb are  
126 important for proper cell polarity, tissue integrity and growth. For instance,  
127 absence of Crb in embryonic epithelia can result in loss of apical identity and  
128 disruption of epithelial organization [22-24]. In contrast, overexpression of Crb  
129 can trigger apical membrane expansion, which can lead to a disordered



130 epithelium, abnormal expansion of tracheal tubes and/or tissue overgrowth  
131 [21,25-33]. These results underscore the importance of Crb levels for  
132 epithelial development and homeostasis.

133         Several mechanisms have been uncovered that ensure proper levels of  
134 apical Crb. These include: stabilization of Crb at the membrane, mediated  
135 through interactions of its cytoplasmic domain with scaffolding proteins, e.g.  
136 Stardust (Sdt) or by homophilic interactions between Crb extracellular  
137 domains [26,34-36], regulation of Crb trafficking, including endocytosis by AP-  
138 2, Rab5 or Avalanche, membrane delivery by Rab11, recycling by the  
139 retromer and endocytic sorting by the ESCRT III component Shrub/Vps32  
140 [29,33,37-41].

141         To gain further insight into the molecular mechanisms that regulate Crb  
142 and its activity during epithelial growth, we set out to identify novel interacting  
143 partners of Crb by using the yeast two-hybrid system. One of the candidates  
144 identified, CG15887, encodes a transmembrane protein, which localizes to  
145 the apical surface of tracheal tubes. We found that the CG15887 protein  
146 physically interacts with Crb. Based on the phenotype of mutations in  
147 CG15887, which is characterized by defects in tracheal growth and inflation  
148 during larval stages, we named this gene *apnoia* (*apn*). *apn* mutant animals  
149 die as second instar larvae with dorsal trunks displaying reduced axial growth  
150 and impaired apical surface area expansion, resulting in shorter tubes. This  
151 phenotype is correlated with the absence of Crb from the apical surface. RNAi  
152 knock-down of *crb* phenocopies the *apn* mutant phenotype of impaired  
153 longitudinal growth. These results identify Apn as the first regulator of tracheal

154 tube growth in the larvae, which acts through Crb to control tube axial tube  
155 expansion.

156

157

158 **Results**

159 **Apnoia is an apical transmembrane protein expressed in *Drosophila***  
160 **tracheae**

161 To identify novel interactors of Crb, we searched for binding partners  
162 using a modified yeast two-hybrid screen (MBmate Y2H) [42,43] allowing bait  
163 and prey to interact at the yeast plasma membrane. The bait consisted of the  
164 most C-terminal extracellular EGF (epidermal growth factor)-like repeat, the  
165 transmembrane domain and the cytoplasmic tail of *Drosophila* Crb. One of the  
166 Crb interacting clones contained a 414bp cDNA insert representing the full-  
167 length transcript encoded by CG15887. Based on the tracheal inflation  
168 phenotype described below we named the gene *apnoia* (*apn*) (ἀπνοια, Greek  
169 for: lack of air).

170 The *apn mRNA* encodes a single protein isoform of 137 amino acids.  
171 Apn is predicted to contain a signal peptide at the amino terminus (1-23 aa)  
172 and two transmembrane domains (amino acids 50-72 and 79-101), based on  
173 the TMHMM transmembrane algorithm [44] prediction. Both the amino and  
174 carboxy terminus are located extracellularly, separated by a small intracellular  
175 loop (Suppl. Fig. S1). The PFAM algorithm (PFAM domains database 27.0)  
176 predicts that Apn contains two LPAM domains (47-56 aa and 78-90 aa),  
177 known as prokaryotic membrane lipoprotein lipid attachment site. Apn is  
178 highly conserved within the insect order (Suppl. Fig. S1) but does not appear  
179 to have a true orthologue in vertebrates.

180 To determine the tissue distribution and subcellular localization of *apn*  
181 mRNA and protein we performed *in situ* hybridizations and immunostainings  
182 of wild-type or transgenic animals, which either carried the *fosapn<sub>stGFP</sub>*, a

183 fosmid encoding the Apn protein C-terminally tagged with superfolded (sf)  
184 GFP [45], or a UAS-transgene encoding fluorescently-tagged Apn (UAS-  
185 *apn<sub>mCitrine</sub>*). In addition, anti-Apn antibodies were raised in rabbits against a  
186 peptide of the N-terminal extracellular domain (aa 24-40). Expression of both  
187 *apn* mRNA (Fig. 1 A-C) and Apn protein (Fig. 1D-F and Suppl. Fig. S2A, B)  
188 was first detected in embryos at stage 13 in tracheal fusion cells. During  
189 embryonic stages 15 and 16, expression could also be detected in the dorsal  
190 and lateral trunks, in the visceral and dorsal branches and in the transverse  
191 connective branches. In the larvae, Apn is continuously expressed in the  
192 entire tracheal system (Suppl. Fig. S2C, D). As shown by antibody staining or  
193 *Apn<sub>mCitrine</sub>* fluorescence, Apn is restricted to the apical plasma membrane,  
194 where it co-localizes with the apical markers Stranded at second (Sas) (Fig.  
195 1G-G'' and cross section in G''') and Uninflatable (Uif) (Fig. 1H-H'' and cross  
196 section in H'''). Apn co-localizes with Crb in the subapical region, a small  
197 region of the apical membrane apical to the adherens junctions (AJ) (Fig. 1I-I''  
198 and cross section in I''').

199         This co-localization and the interaction in the yeast 2-hybrid system  
200 (Suppl. Fig. S2E) prompted us to further analyze the interaction between Apn  
201 and Crb in co-immunoprecipitation experiments. Full-length Apn (*Apn<sup>FL</sup>*)  
202 expressed in S2R<sup>+</sup> cells co-immunoprecipitated full-length Crb (*Crb<sup>FL</sup>*) (Fig.  
203 1J). *In situ* interactions between Crb and Apn were corroborated by Proximity  
204 Ligation Assays (PLA) [46] using the fosmid line (*fosapn<sub>sfGFP</sub>*). We found that  
205 Crb and Apn-sfGFP interact in the larval tracheae (Fig. 2A-A'', D-D'' and C),  
206 whereas no interaction between Apn and *DEcad*-GFP (negative control) was  
207 detected (Fig. 2B-B'', E-E'' and C), indicating that the observed signal was

208 specific for the Crb-Apn interaction. To exclude any random interactions  
209 between Crb and Apn in the apical domain we have tested a different apical  
210 protein (SAS-Venus)[47] for its interaction with Apn and found no increased  
211 PLA signal as compared to the signal between Crb and Apn (Fig. 2F-F”and G-  
212 G”).

213

#### 214 ***apnoia* is required for tracheal tube growth**

215 To address possible functions of *apn* in tracheal development, we  
216 generated a knockout line by CRISPR-Cas9, in which the open reading frame  
217 of *apn* was replaced by DsRed (*apn*<sup>1</sup>). No Apn protein could be detected with  
218 the anti-Apn antibody in homozygous *apn*<sup>1</sup> mutant larvae and embryos  
219 (Suppl. Fig. S2B, D, F). In addition, no interaction between Crb and Apn was  
220 detected in *apn*<sup>1</sup> mutant tracheae in PLA assays as compared to wild type  
221 tracheae (Suppl. Fig. S2G-G’).

222 *apn*<sup>1</sup> mutant tracheae displayed wild type morphology in all embryonic  
223 stages, even in embryos derived from *apn*<sup>1</sup> mutant germ line clones (Suppl.  
224 Fig. S2H). However, *apn*<sup>1</sup> mutant larvae died at second instar with reduced  
225 body size and unusually twisted and uninflated tracheal tubes (compare Fig.  
226 3A and B, Suppl. Fig. S2I, K). The phenotype is mostly manifested in the  
227 posterior tracheal metameres 9 (Tr9) and 10 (Tr10). Similar phenotypes were  
228 observed in larvae that carry *apn*<sup>1</sup> in trans to Df(3R)Exel8158 (Suppl. Fig.  
229 S2J), a chromosomal deletion that includes the *apn* locus, as well as in larvae  
230 upon knock-down of *apn* by RNAi in the tracheae (Fig. 3C and Suppl. Fig.  
231 S2K and S3A, B). In addition, the length of the dorsal trunk was significantly  
232 reduced, as revealed by measurements of the posterior metamer length (Fig.

233 3E, F, H) The morphological and growth defects were rescued by one copy of  
234 a fosmid containing the complete *apn* locus (*fosapn<sub>mCherry.NLS</sub>*) (Fig. 3D, G, H),  
235 whereas a cDNA of Apn expressed in the tracheae rescued the phenotype in  
236 only 20% of the larvae (compare Suppl. Fig. S3A, D and C, F).

237 The uninflated tubes observed in *apn<sup>1</sup>* deficient animals suggested  
238 defects in tracheal maturation. In wild-type embryos as well as in each molting  
239 step of larval development, tracheal maturation is characterized by distinct  
240 sequential processes: i) secretion of a chitinous apical extracellular matrix  
241 (aECM) into the lumen, which confers rigidity to the tube and is responsible  
242 for tube expansion; ii) a pulse of endocytosis, resulting in the removal of  
243 luminal proteins, and iii) liquid clearance and air filling [16,48]. Electron  
244 micrographs of *apn<sup>1</sup>* mutant larvae revealed a disorganized lumen with  
245 “tongues” of cellular protrusions into the lumen (Suppl. Fig. S3G, H). This  
246 phenotype is probably a consequence of the irregularly twisted tubes  
247 (compare Fig. 3I, J) and not due to defects in cuticle organization, since the  
248 two different cuticular layers, epicuticle and procuticle, were normally formed  
249 and the spaced thickenings formed by the aECM (taenidia) [49] appeared  
250 similar to that of wild type tubes (Fig. 3I, J and Suppl. Fig. S3G’, H’). This  
251 conclusion is further supported by the normal expression of Dumpy (Dp) and  
252 Piopio (Pio), two zona pellucida (ZP) domain proteins secreted into the lumen  
253 [50,51] (Suppl. Fig. S3I-J’). The second maturation step, endocytosis of  
254 luminal proteins, was also not impaired in *apn<sup>1</sup>* mutant tubes either. Using the  
255 heterologous secreted mCherry-tagged protein ANF (UAS-ANF-mCherry, a  
256 rat Atrial Natriuretic Factor) [52] revealed normal secretion and endocytosis in  
257 tracheal cells deficient for *apn<sup>1</sup>* (compare Suppl. Fig. S3 K-L’). However, the

258 last maturation steps, liquid clearance and gas filling, were strongly affected in  
259 *apn*<sup>1</sup> mutant tracheae (compare Fig. 3K, K' and L, L'). We could exclude  
260 leakage of the septate junctions (SJ) and hence loss of paracellular barrier as  
261 a cause of this phenotype, since Contactin (Con) and Discs Large (Dlg), two  
262 SJ components [53,54] were properly localized in the tracheae of *apn*<sup>1</sup>  
263 mutants (Suppl. Fig. S3M-N').

264 Taken together, our data demonstrate that loss of *apn* affects late  
265 steps of tracheal tube maturation, including liquid clearance and gas filling,  
266 and impairs growth and morphology of the dorsal trunk at the second larval  
267 stage.

268

269

## 270 **Apn supports apical membrane growth in larval tracheae**

271 A striking defect observed in *apn*<sup>1</sup> mutant larvae was a reduction in the  
272 length of the dorsal trunk (Fig. 3E-H). To determine the cellular basis of this  
273 phenotype we stained for *Drosophila* E-cadherin (*DE-Cad*) to visualize the cell  
274 outline. We could not detect significant differences in cell number within  
275 different metameres (data not shown). This led us to hypothesize that  
276 shortening of tracheal tubes is caused by defective apical cell surface  
277 expansion. Therefore, we measured the long and the short axes of cells  
278 (referred to as axial and circumferential length, respectively) (see Fig. 4A) as  
279 well as their cell surface area. While the circumferential cell length was not  
280 significantly different, the axial cell length of *apn*<sup>1</sup> mutants was reduced in  
281 comparison to that of wild type cells (Fig. 4A, B and E, F). This difference was  
282 also reflected by a reduced aspect ratio of the two axes (axial to

283 circumferential length) (Fig. 4G) and the overall reduction of the apical surface  
284 area (Fig. 4C, D, H). From these results we conclude that Apn is required for  
285 anisotropic apical surface expansion and hence tracheal tube elongation.

286

287 **Apnoia is required for maintenance of Crumbs on the apical membrane**  
288 **of tracheal cells**

289 Regulation of apical cell surface area during axial growth of tracheal  
290 tubes has been shown to require junctional and polarity proteins as well as  
291 the apical protein Uif [55-58]. Therefore, to better understand the mechanism  
292 by which *apn* ensures apical membrane growth, we examined the subcellular  
293 distribution of junctional and polarity proteins in the tracheae of *apn<sup>1</sup>* mutants.  
294 The AJ markers Armadillo (Arm), the *Drosophila*  $\beta$ -catenin [59] (Fig. 5A, B),  
295 Polychaetoid (Pyd), the single *Drosophila* ZO-1 orthologue [60] (Fig. 5C, D)  
296 and DE-Cad (Fig. 5E, F) localized similar as in wild type tracheae. *apn<sup>1</sup>*  
297 mutant tracheal cells also showed normal distribution of Uif (Fig. 5G, H).  
298 These results indicate no major defects in apico-basal polarity and epithelial  
299 integrity of the tracheal tube in *apn<sup>1</sup>* mutant larvae. The physical interaction  
300 between Apn and Crb motivated us to analyze the expression of Crb in *apn<sup>1</sup>*  
301 mutants. In wild type tracheal cells of second instar larvae, Crb is localized in  
302 the subapical region, outlining the cell (Fig. 5I). In contrast, Crb strongly  
303 accumulated in cytoplasmic vesicles of multicellular, autocellular and  
304 seamless tubes in *apn<sup>1</sup>* mutant tracheae and upon knock-down of *apn* (Fig.  
305 5J, R and Suppl. Fig. S4A-D'). Consistent with these results, not only  
306 multicellular, but also autocellular and seamless tubes were twisted and  
307 uninflated (Suppl. Fig. S4E-F'). However, the total protein levels of Crb were



308 unchanged as revealed by western blotting (Suppl. Fig. S2F). To investigate  
309 whether *apn* is required for Crb apical localization only in the trachea we  
310 analyzed another epithelial tube, the salivary glands. A uniform apical  
311 localization of Crb was observed in both wild type and *apn*<sup>1</sup> mutant salivary  
312 glands indicating a tracheae-specific role of *apn* (Fig. S4H-I').

313         Similar as Crb, Stardust (Sdt) (Fig. 5K, L) and Moesin (Moe) (Fig. 5M,  
314 N), whose subapical localization depends on Crb in many epithelia [61-64],  
315 are found in the same vesicular compartments as Crb. The introduction of one  
316 copy of the *apn* genomic locus (*fosapn*<sub>mCherry.NLS</sub>) into the *apn*<sup>1</sup> mutant  
317 background restored Crb membrane localization and suppressed the  
318 accumulation of Crumbs loaded vesicles (CLVs) (Fig. 5O-Q). These results  
319 indicate that Apn is required for Crb trafficking to or maintenance at the  
320 plasma membrane of tracheal cells.

321         In order to distinguish between these two possibilities, we blocked  
322 endocytosis in *apn*<sup>1</sup> mutant tracheae chemically and genetically. After 2 hours  
323 incubation with dynasore, an inhibitor of Dynamin [65], Crb was mostly  
324 localized at the plasma membrane in *apn*<sup>1</sup> mutant tracheal cells (Fig. 6A). In  
325 contrast, *apn*<sup>1</sup> mutant cells incubated with dynasore-free medium showed only  
326 punctate staining of Crb (Fig. 6B). To corroborate this result, we blocked  
327 endocytosis by using *shibire*<sup>ts1</sup> (*shi*<sup>ts1</sup>), a temperature sensitive allele of *shi*,  
328 which encodes Dynamin. When incubated at the restrictive temperature  
329 (34°C) *shi*<sup>ts1</sup>;*apn*<sup>1</sup> double mutant tracheae retained Crb at the apical plasma  
330 membrane (Fig. 6C), as compared to *shi*<sup>ts1</sup>;*apn*<sup>1</sup> mutant tracheae, incubated  
331 at the permissive temperature (25°C) (Fig. 6D). From these results we  
332 concluded, that Apn is required for Crb maintenance at the apical membrane.

333 A striking feature of the *apn*<sup>1</sup> mutant phenotype is the accumulation of  
334 Crb in intracellular vesicles (Fig. 5I, P). To determine their identity, we  
335 analyzed components of the trafficking machinery, including markers for  
336 endosomes, lysosomes and retromer. No major co-localization was observed  
337 between CLVs and the early endosomal markers Rab5 (Fig. 7A-A'') and Hrs  
338 (Suppl. Fig. S5A-A'') or Rab11, a marker for the recycling endosome (Fig. 7B-  
339 B''). Interestingly, 25% of CLVs were also positive for the late endosomal  
340 marker Rab7 (Fig. 7C-C''). No major overlap was found between vesicular Crb  
341 and Lamp1 [66] or Arl8 [67], two markers of the lysosome (Suppl. Fig. S5B-B''  
342 and C-C''). Strikingly, about 79% of CLVs co-localized with the retromer  
343 component Vps35 (Fig. 8A-A'').

344 We noticed that the majority of Vps35-positive vesicles were  
345 significantly larger in *apn*<sup>1</sup> mutants, measuring around 0.7  $\mu\text{m}$  (n=194  
346 vesicles) in diameter, as compared to 0.27  $\mu\text{m}$  (n=152 vesicles) in control  
347 larval tracheal cells (Fig. 8B). No significant size differences in two other  
348 trafficking compartments, such as the Arl8- (lysosomal) and the Golgin245-  
349 (*trans*-Golgi) [68] positive vesicles, were observed between the two genotypes  
350 (Fig. 8C, D). This result suggests that the size increase specifically in the  
351 Vps35-positive compartment is an aspect of the *apn*<sup>1</sup> mutant phenotype.

352 Taken together, these results suggest that Apn maintains apical Crb by  
353 preventing its clathrin-dependent endocytosis. Loss of *apn* results in Crb  
354 accumulation in Vps35/retromer-positive vesicles of increased size.

355

356 **Tracheal defects caused by *apn* depletion are mediated by *crb***

357           Since loss of apical Crb is often associated with reduced apical  
358 membrane [28,69,70] and Crb is depleted from the apical membrane in *apn<sup>1</sup>*  
359 mutant tracheal cells, we asked whether the impaired apical surface growth  
360 observed in *apn<sup>1</sup>* mutant tracheae is due to its effect on apical Crb. Since  
361 homozygous *crb* mutant embryos die with severe defects in many epithelia,  
362 including the tracheae [24,71], we knocked-down *crb* in tracheal tubes by  
363 expressing *crb* RNAi ubiquitously (using *da-Gal4*) or specifically in the  
364 tracheae (using *btl-Gal4*). This resulted in a strong depletion of Crb and its  
365 binding partner Sdt (Fig. 9A, B and A', B'), but had no effect on *Apn*  
366 expression and localization (Fig. 9C, D and C', D'). RNAi-mediated  
367 downregulation of *crb* reproduced several aspects of the *apn<sup>1</sup>* mutant  
368 phenotypes, such as twisted tracheal tubes, lack of gas filling (Fig. 9E-H) and  
369 reduced apical surfaces of tracheal tube cells (Fig. 9I-K). No defect in apico-  
370 basal polarity was observed upon knockdown of Crb (Fig. 9I, J and I', J"). In  
371 addition, most animals died at L2 (larval stage 2) with some surviving until L3  
372 (larval stage 3) instar larvae.

373           To assess whether the increased size of Vps35 positive vesicles in  
374 *apn<sup>1</sup>* mutants are due to Crb accumulation in these vesicles, we knocked-  
375 down *crb* in *apn<sup>1</sup>* tracheae using *btl-Gal4*. We found a small, yet significant  
376 reduction in the size of Vps35 positive vesicles in *apn<sup>1</sup>* tracheal cells upon *crb*  
377 RNAi expression, compared to that of *apn<sup>1</sup>* single mutants (Fig. 10A, B, D and  
378 Suppl. Fig. S6A-C). In contrast, Vps35 positive vesicles in tracheal cells  
379 expressing *crb* RNAi in otherwise wild-type animals are comparable in size to  
380 those of wild type Vps35 vesicles (compare Fig. 10C, D and Fig. 8B).

381           These results are the first to show that loss of *crb* results in a reduction  
382 of the apical surface area of larval tracheal cell, which in turn prevents proper  
383 tube elongation. In addition, they identify Apn as a novel regulator of apical  
384 Crb in the developing tracheae, which controls dorsal trunk maturation and  
385 expansion. Absence of *apn* leads to accumulation of Crb in Vps35 positive  
386 vesicles, which may contribute to the increase in vesicular size.

387

## 388 **Discussion**

389           This work identifies Apn as the first protein essential for airway  
390 maturation in *Drosophila* larval stages. Apn is expressed apically in tracheal  
391 epithelial cells, where it co-localizes and physically interacts with Crb. *apn*<sup>1</sup>  
392 mutant larvae exhibit loss of tracheal tissue structure, manifested by tube size  
393 defects and impaired gas filling, resulting in body size reduction and lethality  
394 at second instar. At the cellular level, exclusion of Crb from the apical  
395 membrane in *apn*<sup>1</sup> mutant larval tracheae goes along with apical cell surface  
396 reduction and an overall tracheal tube shortening. Absence of *apn* leads to  
397 Crb inhibition and accumulation in enlarged, Vps35/retromer-positive vesicles.

398           Elongation of the tracheal tube has been extensively studied in  
399 embryos where it has been shown to rely on different mechanisms, such as  
400 the organization of the aECM and cell shape changes [33,72-76]. Anisotropic  
401 growth of the apical plasma membrane is an additional mechanism to achieve  
402 proper longitudinal tube expansion. However, only few proteins have been  
403 described so far to regulate this process. One of these, the protein kinase  
404 Src42A, is required for the expansion of the cells in the axial direction, and  
405 loss of *Src42A* function results in tube length shortening, which is associated

406 with an increased tube diameter [72,75,77]. Src42A has been suggested to  
407 exert its function, at least in part, by controlling DE-cadherin recycling and  
408 hence adherens junctions remodeling [72] and/or by its interaction with the  
409 Diaphanous-related formin dDAAM (*Drosophila* Dishevelled-associated  
410 activator of morphogenesis), loss of which results in reduced apical levels of  
411 activated pSrc42A [75]. More recently, Src42A has been suggested to control  
412 axial expansion by inducing anisotropic localization of Crb along the  
413 longitudinal junctions in comparison to transverse junctions [78]. However, we  
414 never observed any anisotropic distribution of Crb in wild type larval tracheal  
415 cells, making it unlikely that at this developmental stage, axial expansion is  
416 regulated by a Src42A-dependent mechanism. This assumption is  
417 corroborated by the observation that, unlike in *Src42A* mutants, the lack of  
418 longitudinal expansion in *apn<sup>1</sup>* mutant larval tubes is not associated with  
419 circumferential expansion. Another protein regulating tube elongation in the  
420 embryo is the epidermal growth factor receptor, EGFR. Expressing a  
421 constitutively active EGFR results in shortened tracheal tubes with smaller  
422 apical cell surfaces, but with increased diametrical growth. In this condition,  
423 Crb shows altered apical distribution [78,79]. This phenotype differs from the  
424 *apn<sup>1</sup>* phenotype, where apical localization of Crb is almost completely lost and  
425 only longitudinal tube growth is affected. This suggests that Apn executes  
426 tube length expansion by a different mechanism.

427         How does decrease in tubular growth lead to loss of tracheal structure?  
428 During development, the larval body, including the tracheal tissue, elongates  
429 about 8-fold [18]. Impaired axial tracheal cell growth in *apn<sup>1</sup>* mutants thus may  
430 affect the balance between the forces exerted by the apical membrane growth

431 on the one hand and the resistance provided by the luminal aECM on the  
432 other, an important mechanism described previously to control tube shape in  
433 the embryo [33]. This could lead to physical rupture of tubes mutant for *apn*<sup>1</sup>,  
434 allowing fluid entry. The presence of fluid would, in turn, disrupt proper gas  
435 filling, resulting in hypoxia and, consequently, in impaired body growth.

436 Several studies have shown that in some tissues Crb accumulation on  
437 the apical membrane is mediated by the retromer complex, which controls  
438 either the retrograde transport of Crb to the *trans*-Golgi [80] or the direct  
439 trafficking from the endosomes to the plasma membrane [39,41,79]. The  
440 physical interaction of Apn and Crb, the functional requirement of Apn for Crb  
441 apical localization and the fact that in *apn*<sup>1</sup> mutants Crb is trapped in Vps35-  
442 positive/retromer vesicles all suggest that Apn is required for trafficking and/or  
443 maintenance of Crb at the apical membrane (Fig. 11).

444 However, the increase in the size of Vps35-positive vesicles in *apn*<sup>1</sup>  
445 mutant cells, which is, to some extent, due to the accumulation of Crb,  
446 suggests defects in retromer function, which may prevent Crb lysosomal  
447 degradation. Further studies will help to elucidate at which level Apn controls  
448 Crb trafficking in larval tracheae.

449

## 450 **Materials and Methods**

### 451 **Fly stocks**

452 Flies were maintained at 25°C with 50% humidity unless stated  
453 otherwise. The *fosapn*<sub>sfGFP</sub> (tagging with 2XTY1-SGFP-V5-preTEV-BLRP-  
454 3XFLAGdFRT was done C-terminally) and *fosapn*<sub>mCherry.NLS</sub> (tagging with ubi-  
455 mCherry-NLS-T2A was done N-terminally) were provided by the Flyfos library

456 at MPI-CBG [45]. The following fly lines were used: Rab5-YFP, Rab7-YFP  
457 and Rab11-YFP ([http://rablibrary.mpi-cbg.de/cgi-bin/rab\\_overview.pl](http://rablibrary.mpi-cbg.de/cgi-bin/rab_overview.pl)) [81]  
458 (kindly provided by Marko Brankatschk), UAS-moe-GFP (kindly provided by  
459 Brian Stramer) [82], UAS-ANF-mCherry (modified from [52]), and w; DE-  
460 Cad::GFP [83], w; Sas:: Venus [47], w; btl-Gal4 [84]. The following fly stocks  
461 were obtained from the Bloomington *Drosophila* Stock Center (BDSC): y[1]  
462 M{w[+mC]=nos-Cas9.P}ZH-2A w[\*] (BDSC, #54591), *shi<sup>ts1</sup>* (BDSC, #7068),  
463 y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.HMS01842}attP40 (*crb<sup>RNAi</sup>*) (BDSC,  
464 #38373), w[1118];da<sup>G32</sup>-Gal4 (BDSC, #55851) [21], w[1118];  
465 Df(3R)Exel8158/TM6B, Tb (BDSC, #7974) and UAS-lamp1-GFP (BDSC,  
466 #42714). The CG15887 (*apn<sup>RNAi</sup>*) (VDRC, #9070) was obtained from the  
467 Vienna *Drosophila* Resource Center.

468

#### 469 **Generation of *apn*<sup>1</sup> mutant allele by CRISPR/Cas9**

470 Target sites were designed using the settings of the flyCRISPR  
471 Optimal Target Finder (<http://tools.flycrispr.molbio.wisc.edu/targetFinder/>), to  
472 guide Cas9 to two target sites, one at the 5'UTR (gagggctctgggcccggcttacTGG)  
473 and one at the 3'UTR end (gcaaagtcacggagaaatctGGG) (UPPERCASE:  
474 PAM) of CG15887 (*apn*), with the aim to delete the whole Open Reading  
475 Frame. The following phosphorylated DNA oligomers were used as primers  
476 for PCR, using 10ng of pCFD4-U6:1U6:3tandem gRNAs as template  
477 (Addgene #49411;[85]): forward primers 5'-[P]-  
478 tatataggaaagatatccgggtgaacttcgGCAAAGTCACGGAGAAATCTgttttagagctaga  
479 aatagcaag-3', reverse primer 5'-[P]-  
480 attttaacttgctatttctagctctaaaacGTAAGCCGGCCCAGACCCTCcgacgttaaattgaa

481 aataggtc-3'. The resulting DNA fragment was cloned into pCFD4-  
 482 U6:1U6:3tandemgRNAs via Gibson Assembly after linearization of the vector  
 483 with BbsI. To replace the CG15887 ORF with 3XP3-dsRed we used the pHD-  
 484 DsRed-attP vector (Addgene #51019;[86]). The homology arms necessary to  
 485 obtain Homology Directed Repair were sequences covering 1kb regions of  
 486 upstream and downstream of 5'UTR and 3'UTR gRNA cut sites, respectively.  
 487 Cloning into the vector was obtained with AarI for the 5'-homology arm (5'-HA)  
 488 and SapI for the 3'-homoly arm (3'-HA). Primer sequences are: Forward  
 489 primer 5'-HA (5'-  
 490 TGTACACCTGCGAATTCGCCACACTGTTTGGCATCTGGCGGCGCTCCT  
 491 CC-3'), Reverse primer 5'-HA (5'-  
 492 TGTACACCTGCAGATCTACTTTCTCCGTGACTTTGCTCATAGCTCATTAT  
 493 GG-3'), Forward primer 3'-HA (5'-  
 494 GCTAGCTCTTCGTATTACTGGGCGGCTACTTGAAATTCGGGAGCC-3'),  
 495 Reverse primer 3'-HA (5'-  
 496 GCTAGCTCTTCGGACCCCAATAACATGTCCGTCCGCACTACG-3').  
 497 Homology arm sequences were amplified from the BAC genomic clone  
 498 BACR05K08 (obtained from BACPAC resources center (BPRC)). The two  
 499 plasmids were injected in a concentration of 400ng each, into nos::Cas9  
 500 embryos [85].

501

## 502 **Generation of transgenic flies**

503 To generate the UAS-*apn* transgenic line, the CG15887 (*apn*) cDNA  
 504 (RE53127; obtained from DGRC) was cloned into pJFRC-MUH-mCitrine[87]



505 by BglII-NotI using standard molecular biology techniques. Plasmid constructs  
506 were injected by BestGene.

507

#### 508 **Dynasore treatment of larval tracheae**

509 *apn*<sup>1</sup> mutant tracheae from second instar larvae were dissected in  
510 Grace's medium supplemented with Pep/Strep. Tissues were incubated in  
511 60µM dynasore (Enzo Life Sciences) in Grace's medium containing Pep/Strep  
512 and 2.5% FCS at room temperature for 2hr. The dynasore was washed out  
513 and tracheae were fixed in 4% FA in Grace's medium for 30min.

514

#### 515 **Yeast-two-Hybrid screen**

516 Part of the coding sequence of a *Drosophila melanogaster crb* cDNA  
517 (encoding aa: 2034-2189) (GenBank accession number NM\_001043286.1)  
518 was PCR-amplified and cloned in pB102, in frame with the STE2 leader  
519 sequence at the N-terminus and ubiquitin (Cub) at the C-terminus of the bait  
520 which is coupled to the artificial transcription factor LexA-VP16 (STE2-Crb-  
521 Cub-LexA-VP16). The construct was checked by sequencing. Prey fragments  
522 were isolated from an MBmate screen with *Drosophila melanogaster* Crb as  
523 bait against a *Drosophila* Embryo NubG-x (D3DE\_dT) library (NubG stands  
524 for the N-terminal domain of mutated ubiquitin and x for the prey fragment).  
525 Interaction pairs were tested in duplicate as two independent clones. For each  
526 interaction, several dilutions (undiluted, 10<sup>-1</sup>, 10<sup>-2</sup>, 10<sup>-3</sup>) of the diploid yeast  
527 cells (culture normalized at 5Å~10<sup>7</sup> cells) and expressing both bait and prey  
528 constructs were spotted in selective media. The DO-2 selective medium  
529 lacking tryptophan and leucine was used to control for growth and to verify the

530 presence of both the bait and prey plasmids. The different dilutions were also  
531 spotted on a selective medium without tryptophan, leucine and histidine (DO-  
532 3). Six different concentrations of 3-AT, an inhibitor of the HIS3 gene product,  
533 were added to the DO-3 plates to increase stringency and reduce possible  
534 auto activation. The following 3-AT concentrations were tested: 1, 5,10, 50,  
535 100 and 200 mM. The 1-by-1 Yeast two-hybrid assays were performed by  
536 Hybrigenics Services, S.A.S., Paris, France (<http://www.hybrigenics.com>)

537

### 538 **Cell Culture and Transfection**

539 *Drosophila* S2R<sup>+</sup> cells were cultured at 25 °C in Schneider's *Drosophila*  
540 medium (Sigma) supplemented with 10% fetal bovine serum. pAct5-Gal4  
541 together with UAS-Apn<sup>FL</sup> and/or UAS-Crb<sup>FL</sup> [21] (encoding full-length Apn and  
542 Crb, respectively) was transfected into S2R<sup>+</sup> cells using FuGENE HD  
543 (Promega) according to the manufacturer's protocol.

544

### 545 **Immunoprecipitation**

546 Transfected cells were harvested after 48 h, washed with ice-cold PBS  
547 (120 mM NaCl in phosphate buffer at pH 6.7), resuspended in lysis buffer  
548 (containing 10% glycerol; 1% Triton X-100; 1.5 mM MgCl<sub>2</sub>; 120 mM NaCl; 100  
549 mM PIPES, pH 6.8; 3 mM CaCl<sub>2</sub>; 1 mM PMSF and Complete™). Cells were  
550 lysed on ice for 20min and lysates were spun at 14.000 rpm for 20min at  
551 4°C. The supernatant was incubated with the antibody for 2 hours. In the  
552 meantime 50µl of Protein G were washed 3 times with blocking solution and  
553 incubated with the antibody solution overnight at 4°C. Protein G beads were  
554 collected by centrifugation for 2 min at 3.000rpm and washed 4 times with

555 lysis buffer. Beads were resuspended in 1.5x SDS sample buffer and heated  
556 for 5min at 95°C.

557

#### 558 **Western Blot**

559 Wild-type and *apn*<sup>1</sup> mutant embryos and dissected larval tracheae  
560 were homogenized on ice using a Dounce tissue grinder in 1ml of lysis buffer  
561 containing 130mM NaCl, 50mM Tris-HCL pH=8, 0,5% Triton-X and protease  
562 inhibitor (Roche). After 30min at 4°C under rotation the homogenate was  
563 centrifuged for 20min at 14.000rpm. Sample buffer 3x SDS was added to the  
564 supernatant and boiled for 5min at 95°C.

565 Proteins were separated by SDS-PAGE and blotted onto nitrocellulose  
566 0.45 membrane (Amersham). After blocking in 5% BSA+TBST, the  
567 membrane was incubated overnight with rabbit anti-Apn diluted 1:1000, rat  
568 anti-Crb[88] diluted 1:1000 and mouse anti-alpha-tubulin (Sigma) diluted  
569 1:5000 in blocking buffer. Peroxidase antibodies were used for detection.

570

#### 571 **Proximity Ligation assay (PLA)**

572 Tracheae from *fosapn<sub>stGFP</sub>* third instar larvae were dissected and fixed  
573 in ice cold 4% FA in PBS. Primary antibodies against GFP (rabbit anti-GFP  
574 1:250; Invitrogen A11122) and Crb (rat anti-Crb 1:500 [88]) were added and  
575 incubated overnight at 4°C. The Duolink PLA Kit (Sigma Aldrich) was used to  
576 incubate the tissue with the PLA probes PLUS and MINUS at 37°C for 1 hour.  
577 Ligation of the PLA oligonucleotides and amplification were performed at  
578 37°C for 30 min and 100min, respectively. Samples were mounted in Duolink  
579 mounting media and imaged using Zeiss LSM 880.

580

581 **Generation of Apn antiserum**

582 Polyclonal antibodies against CG15887 were raised in rabbits using the  
583 KLH-conjugated synthetic peptide QQAANSSDSDSDVAESC (from the N-  
584 terminal extracellular part) for immunization. Antibodies were subsequently  
585 affinity-purified using the same peptide immobilized on SulfoLink Coupling Gel  
586 (ThermoFisher #20401) and following recommendations by the manufacturer.  
587 The work was performed by the MPI-CBG Antibody Facility.

588

589 **Immunohistochemistry**

590 Immunostainings on embryos were done as follows: embryos were  
591 dechorionated in 50% bleach for 2min and fixed for 20min in  
592 formaldehyde/heptane mixture. After devitellinization in methanol, embryos  
593 were permeabilized in 0.1% Triton X-100/PBS except for rabbit anti-Apn  
594 staining, for which embryos were permeabilized in 0.2% Saponin/ PBS. After  
595 washing, embryos were incubated for 1h at RT in blocking solution [(0.5%w/v  
596 BSA in PBST/S (0.1%v/v Triton X-100) or (0.2%w/v Saponin)]. Second instar  
597 larvae were opened in PBS and fixed in 4% formaldehyde for 20min. After  
598 washing in either 0.1% Triton X-100/PBS or 0.2% Saponin/ PBS (for anti-Apn  
599 antibody staining), tracheae were dissected and incubated in blocking solution  
600 for 1h at RT. Embryos and tracheae were incubated in primary antibodies  
601 overnight at 4°C, washed and incubated with secondary antibody for 2h at  
602 RT. Samples were mounted in Vectashield (Vector Laboratories) and imaged  
603 with LSM 880 Laser Scanning Confocal Microscope (Carl Zeiss). Unless  
604 otherwise indicated, images shown are z-stack projections of sections.

605 Images were processed with Fiji software [89]. Cell area measurements were  
606 obtained by using the Fiji Freehand selection tool.

607 The following primary antibodies were used: rabbit anti-*Apn* (1:500-1:1000)  
608 (this study), rabbit or rat anti-*Crb* (1:1000) [21,24,88], rabbit anti-*Sdt* (1:1000)  
609 [61], guinea pig anti-*Cont* (1:1500; gift from Manzoor Bhat), guinea pig anti-*Uif*  
610 (1:20; gift from Robert Ward), mouse anti-*Pyd* (1:1000; gift from Alan  
611 Fanning), rabbit anti-*Pio* (1:50; gift from Markus Affolter), guinea pig anti-*HRS*  
612 and anti-*Vps26* (1:20 and 1:1000, respectively; gift from Hugo Bellen), rabbit  
613 anti-*SAS* (1:500 gift from Douglas Cavener), goat anti *Golgin245* (1:200,  
614 DSHB), mouse anti-*Arm* (1:50, DSHB, N27A1), rabbit anti-*Arl8* (1:100,  
615 DSHB), rat anti-*DE-Cad* (1:50, DSHB, DCAD2), mouse anti-*Dlg* (1:500,  
616 DSHB, 4F3), rabbit anti-GFP A11122 (1:250, Thermo Fischer), mouse anti-  
617 GFP (1:250, Roche), Chitin binding probe-633 (1:20; gift from Maria  
618 Leptin)[90]. The secondary antibodies Alexa Fluor 488, 568 and 633  
619 (Molecular Probes) were used at 1:400 dilution.

620

### 621 **RNA *in situ* hybridization**

622 DIG-labelled RNA probes were synthesized from PCR templates amplified  
623 from for a full-length *apn* (RE53127) cDNA clone. Sequence specific primers  
624 for pFLC-I vector (BDGP resources) were: M13 (-21) 5'-  
625 TGTAACGACGGCCAGT-3' and M13 (REV) 5'-  
626 GGAAACAGCTATGACCATG-3'. PCR products were purified by PCR  
627 purification columns (Promega, PCR CleanUp system). *In vitro* transcription  
628 reactions were performed by mixing the PCR product with the polymerase  
629 mix, which includes T3 RNA polymerase. RNA was labelled with digoxigenin-

630 UTP (Roche Applied Science # 11277073910). Eggs were collected on apple  
631 juice plates for 12h. Embryos were dechorionated in 50%bleach for 2min and  
632 fixed for 20min in formaldehyde/heptane mixture. After devitellinization in  
633 methanol embryos were processed for hybridization modified from [91].

634

635

636 **Electron microscopy analysis**

637 Larvae were fixed in 2% Glutaraldehyde in 0.1M PB buffer pH 7.2 for  
638 20min at room temperature. Larvae were transferred in microcentrifuge tubes  
639 and fixed in 1%OsO<sub>4</sub>/2% Glutaraldehyde and then 2% OsO<sub>4</sub>. Further  
640 procedures were done according to the protocol described [92]. Ultrathin  
641 sections of 0.1µm were prepared and analyzed with Tecnai 12 BioTWIN (FEI  
642 Company).

643

644 **Image analysis**

645 We developed a Fiji script to quantify the co-localization of proteins of  
646 the trafficking machinery (e.g. retromer, lysosome, Golgi) with Crb-positive  
647 vesicles. Two channel images showing fluorescent Crb signal and protein X  
648 signal were imported into a script for the freely available Fiji software [89] and  
649 characterized for their overlap. The plugin was tested on Fiji current version:  
650 (Fiji is just ImageJ) ImageJ 2.0.0-rc-65/1.51w. The code of the scripts and its  
651 documentation are available on the project repository ([https://git.mpi-](https://git.mpi-cbg.de/bioimage-informatics/Skouloudaki_et_al_Crumbs_overlap_analysis)  
652 [cbg.de/bioimage-informatics/Skouloudaki\\_et\\_al\\_Crumbs\\_overlap\\_analysis](https://git.mpi-cbg.de/bioimage-informatics/Skouloudaki_et_al_Crumbs_overlap_analysis)).

653

654

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668

669

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- 928
- 929

930 **Figure Legends**

931

932 **Figure 1. Apn is a small transmembrane protein detected on the apical**  
933 **membrane of the tracheal epithelium.**

934 **(A-F)** Expression of *apn* as detected by whole mount *in situ* hybridization (A-  
935 C) and *fosapn<sub>sfGFP</sub>* expression (D-F) in lateral views of stage 13, 15, 16, 17  
936 embryos. *apn* RNA and protein appear first in fusion cells (FC) and later in the  
937 dorsal trunk (DT), visceral branches (VB) and dorsal branches (DB). Scale  
938 bar: 50µm.

939 **(G-H''')** Apn-mCitrine (green) localizes on the apical plasma membrane  
940 together with the apical markers SAS (G, G', G'') and Uif (H, H', H''), as  
941 revealed by z-projections of stage 17 tracheal tubes and optical cross  
942 sections (G''' and H'''). Scale bars: (G-G'' and H-H'') 10µm, (G''') 5µm, (H''')  
943 5µm. Arrows in G''' and H''' point to protein overlap.

944 **(I-I''')** Tracheae of *btl>apn-mCitrine* stage 17 embryos stained with anti-Crb  
945 (magenta) and anti-GFP (green). Cross section indicates the apical  
946 localization of Apn as compared to subapical localization of Crb. Arrows in I'''  
947 point to protein overlap. Scale bars: (I-I'') 10µm, (I''') 3µm.

948 **(J)** Co-immunoprecipitation experiments from S2R<sup>+</sup> cells expressing the  
949 following constructs: UAS-Crb<sup>FL</sup>+UAS-Apn<sup>FL</sup> or UAS-Apn<sup>FL</sup>+UAS-GFP or  
950 UAS-Crb<sup>FL</sup>+UAS-GFP. Crb is co-immunoprecipitated with Apn, but not with  
951 GFP (used as negative control).

952

953 **Figure 2. Apnoia interacts with Crb.**

954 **(A-B'')** Proximity ligation assay (PLA) shows a robust increase in number of  
955 interactions between Crb and Apn (spots in magenta) in *fosapn<sup>sfGFP</sup>* larval  
956 tracheae (A-A'') as compared to control tracheae (*DEcad-GFP*) (B-B''). Scale  
957 bar: 20µm.

958 **(C)** Quantification of the PLA signal produced between Crb and Apn as  
959 compared to Crb and *DEcad*.

960 **(D-E'')** Proximity ligation assay (PLA) shows increased numbers of  
961 interactions between Crb and Apn (spots in magenta) in *fosapn<sup>sfGFP</sup>* larval  
962 tracheae (D-D'') when Crb and GFP antibodies were used as compared to  
963 *fosapn<sup>sfGFP</sup>* larval tracheae (E-E'') when *DEcad* and GFP antibodies were  
964 used. Scale bar: 50µm.

965 **(F-G'')** Proximity ligation assay (PLA) shows a robust increase in number of  
966 interactions between Crb and Apn (spots in magenta) in *fosapn<sup>sfGFP</sup>* larval  
967 tracheae (F-F'') as compared to a control apical protein SAS-Venus (G-G'').  
968 Scale bar: 50µm.

969

970 **Figure 3. Apn is essential for tracheal tube maturation.**

971 **(A-D)** Brightfield images showing the tracheal tubes of second instar larvae.  
972 Rigid and gas filled tracheal tube are present in wild type (WT) larvae (A),  
973 whereas absence of *apn* causes twisted and gas deficient tubes (B). *apn*  
974 down-regulation (*apn* RNAi) recapitulates *apn<sup>1</sup>* mutant tracheal defects (C).  
975 Tube morphology defects are rescued by one genomic copy of *apn*  
976 (*fos.apn<sup>mCherry.NLS</sup>*) (D). Anterior is to the left. Scale bar: 500µm.

977 **(E-G)** The 9<sup>th</sup> metamer of the dorsal trunk (yellow dotted line) of *apn<sup>1</sup>* mutant  
978 second instar larvae (F) is shorter than that of wild type larvae (WT) (E).

979 Expression of one genomic copy of *apn* (*fos.apn<sub>mCherry.NLS</sub>*) significantly  
980 rescues the metamer elongation defects of *apn* mutant larvae (G). Anterior is  
981 to the left. Scale bar: 200  $\mu$ m.

982 **(H)** Quantification of the length of the 9<sup>th</sup> metamer of second instar tracheal  
983 tubes of wild type (WT), *apn*<sup>1</sup> mutants and *apn*<sup>1</sup> mutants rescued with one  
984 extra *apn* copy (*fos.apn<sub>mCherry.NLS</sub>;apn*<sup>1</sup>). Measurements were pooled from 6  
985 larvae.

986 **(I-J)** Chitin binding probe (CBP) allows the visualization of the tracheal tube  
987 structure. Note the wrinkled and twisted tube of *apn*<sup>1</sup> mutants (J) as compared  
988 to that of wild type (WT) (I) larvae. Scale bar: 50 $\mu$ m.

989 **(K-L')** Brightfield images to show dorsal trunk diameter expansion (K, L) and  
990 gas filling (K', L') of the newly formed tracheal tube of wild type (WT, K, K')  
991 and *apn*<sup>1</sup> mutant (L, L') second larval instar. Yellow indicates the tracheal cells  
992 that line along the newly formed lumen (green). Note the bubble filling the  
993 newly formed lumen in wild type (WT, white arrow) (K'), which is absent in the  
994 tube of *apn*<sup>1</sup> mutants (L'), which fails to fill with gas. Scale bar: 50  $\mu$ m.

995

996 **Figure 4. Apn is required for longitudinal elongation of tracheal cells.**

997 **(A-D)** Tracheal tubes of wild type (WT; A, C) and *apn*<sup>1</sup> mutant (B, D) second  
998 instar larvae stained with anti-DE-Cad. The long and short axes of the apical  
999 surfaces (A, B) are indicated by red and green arrows, respectively, and the  
1000 cells by yellow rectangles (C, D). Note that the number of cells occupying  
1001 identical areas in the tracheal tube is higher (~ 4 cells) in *apn*<sup>1</sup> mutants (D)  
1002 than in WT (~ 1 cell) (C). Scale bars: 20  $\mu$ m.



1003 (E-H) The median length of the long (E) axis is significantly smaller in *apn*<sup>1</sup>  
1004 mutant tracheal cells than in wild type (WT) cells as compared to the short  
1005 axis, which is not affected (F). The median aspect ratio (long/short axis) (G)  
1006 and the median apical surface area (H) are significantly smaller in *apn*<sup>1</sup>  
1007 mutant tracheal cells than in wild type (WT) cells. For box plot measurements,  
1008 *n* values were calculated from 48 cells (WT) and 58 cells (*apn*<sup>1</sup>).

1009

1010 **Figure 5. Apn-dependent tube growth correlates with defects in**  
1011 **localization of Crb complex components**

1012 (A-N) Projections of confocal sections of tracheal dorsal trunks of second  
1013 instar larvae. *apn*<sup>1</sup> mutants stained for the adherens junction proteins Arm (B),  
1014 Polychaetoid (Pyd; D), DE-Cad (F) and for the apical protein Uif (H) do not  
1015 display any significant changes compared to the respective wild type (WT)  
1016 tissues (A, C, E, G). In contrast, localization of the Crb complex proteins, Crb  
1017 (I, J) and Sdt (K, L), and the FERM protein, Moe (M, N) are mis-localized in  
1018 *apn*<sup>1</sup> mutants. Scale bars: 20 μm.

1019 (O-R') Tracheae of second instar larvae stained for Crb (O-R) reveals strong  
1020 reduction of Crb from the apical membrane and its accumulation in  
1021 cytoplasmic vesicles in *apn*<sup>1</sup> mutants (P) and upon tracheal knockdown of *apn*  
1022 (R). Staining for chitin binding probe (CBP; O'-R') reveals twisted tracheal  
1023 tube in *apn*<sup>1</sup> mutants (P') and upon tracheal knockdown of *apn* (R'), though  
1024 less severe. Expression of an additional copy of *apn* (*fos.apn*<sub>mCherry.NLS</sub>)  
1025 rescues Crb apical localization (Q) and tubular structure defects (Q'). Scale  
1026 bar: 20 μm.

1027

1028 **Figure 6. Blocking endocytosis prevents internalization of apical Crb in**  
1029 ***apn*-mutant tracheae.**

1030 **(A, B)** *apn*<sup>1</sup> mutant tracheal tubes stained for Crb, after 2 hours incubation in  
1031 60μM dynasore (A) or in the absence of dynasore (B).

1032 **(C, D)** Tracheal tubes hemizygous for the temperature sensitive *dynamain*  
1033 allele, *shibire*<sup>ts1</sup> stained for Crb, after 4 hours incubation at 34°C (C) and 25°C  
1034 (D, as control). Scale bars: 20 μm.

1035

1036 **Figure 7. Crb subcellular accumulation in *apn*<sup>1</sup> mutant tracheal cells.**

1037 Projections of confocal sections of second instar tracheal tubes of transgenic  
1038 lines expressing the respective YFP-tagged Rab protein stained for Crb  
1039 (magenta) or YFP (green). (A'', B'', C'') show magnifications of the boxed area  
1040 in (A-A', B-B', C-C') respectively.

1041 **(A, A')** *apn*<sup>1</sup> mutant tracheal tubes immunostained for Crb and Rab5-YFP.  
1042 (A'') Magnification shows vesicular Crb, most of which does not colocalize with  
1043 the Rab5.

1044 **(B, B')** *apn*<sup>1</sup> mutant tracheal tubes immunostained for Crb and Rab11-YFP.  
1045 Magnification (B'') shows vesicular Crb, most of which does not colocalize with  
1046 Rab11.

1047 **(C, C')** *apn*<sup>1</sup> mutant tracheal tubes immunostained for Crb and Rab7-YFP.  
1048 Magnification (C'') shows vesicular Crb colocalization with Rab7 of approx. 25  
1049 %. Scale bars: A, A', B, B', C, C': 20 μm and A'', B'', C'': 5 μm.

1050

1051 **Figure 8. Crb is localized in abnormally large, Vps35-positive vesicles in**  
1052 ***apn*<sup>1</sup> mutant tracheal cells.**

1053 (A-A'') Projections of confocal sections of second instar tracheal tubes,  
1054 stained for Crb (magenta) and Vps35 (green). (A'') shows a magnification of  
1055 the boxed area in A and A'. A substantial number (approx. 79%) of Crb-  
1056 positive vesicles are also positive for Vps35 in *apn*<sup>1</sup> mutant tracheal  
1057 cells. Scale bars: A, A': 20 μm and A'': 5 μm.

1058 (B-D) Quantification of the size of Vps35-positive vesicles (B), Arl8-positive  
1059 vesicles (C) and Golgin245-positive vesicles in *apn*<sup>1</sup> and wild type (WT)  
1060 tracheal cells. Only Vps35-positive vesicles show a significant increase in  
1061 size.

1062

1063 **Figure 9. Downregulation of *crb* induces tracheal tube defects similar to**  
1064 **those of *apn*<sup>1</sup> mutant tubes.**

1065 (A-D') RNAi- mediated downregulation of Crb (B, B' and D, D') results in  
1066 strong depletion of Crb (B, D) and Sdt (B'), but does not affect Apn expression  
1067 (D') in the dorsal trunk of second instar larvae. Projections of confocal  
1068 sections of second instar tracheal tubes, stained for Crb (A-D), Sdt (green; A',  
1069 B') and Apn (green; C', D'). Nuclear staining in C' and D' is due to an  
1070 unspecific staining of Apn antibody. Scale bars: 20 μm.

1071 (E-H) Brightfield images of second instar larvae showing the tracheal tubes.  
1072 Tracheal tubes are rigid and gas filled in wild type (WT) (E). Reduction of Crb  
1073 using either a ubiquitous (F) or a tracheal-specific (G) Gal4 recapitulates the

1074 characteristic *apn*<sup>1</sup> mutant tracheal defects (compare F, G and H), with  
1075 constricted and gas-deficient tubes. Scale bars: 20 μm.

1076 (I-J'') RNAi-mediated downregulation of *crb* strongly reduces Crb (compare I'  
1077 and J'), but does not affect the localization of Arm or DE-Cad (compare I' with  
1078 J' and I'' with J'', respectively). Scale bars: 20 μm.

1079 (K) Quantification of the median apical surface area of *crb* RNAi tracheal  
1080 cells. The surface area is significantly reduced compared to that of wild type  
1081 (WT) tracheae.

1082

1083 **Figure 10. RNAi-mediated knockdown of *crb* reduces the size of Vps35-**  
1084 **positive vesicles in *apn*<sup>1</sup> mutant tracheal cells**

1085 (A-C) Projections of confocal sections of second instar tracheal tubes,  
1086 stained for Vps35 (green). Scale bars: A-C: 20 μm.

1087 (D) Quantification of the size of Vps35-positive vesicles in tracheal *crb*  
1088 knockdown, *apn*<sup>1</sup> mutants and double *apn*<sup>1</sup>/*crb* knockdown tracheal cells.

1089

1090 **Figure 11. Model to explain Apn-mediated larval tracheal tube growth.**

1091 (A) Top: In the wild type (WT) dorsal trunk, the apical membrane grows along  
1092 the axial axis (indicated by red arrows) and pulls the apical extracellular matrix  
1093 (aECM), until the aECM resistance (indicated by green arrows) balances the  
1094 forces provoked by tube elongation [33]. Bottom: In wild type tracheal cells  
1095 Crb (blue) is enriched at the apical membrane where it controls apical surface  
1096 growth. Apn (green) in the apical membrane is responsible for Crb  
1097 maintenance and therefore ensures tube elongation. Crb trafficking involves  
1098 recycling by the retromer to either the trans-Golgi network (TGN; blue

1099 vesicles) or to the plasma membrane (red vesicles), or by Rab11, or its  
1100 degradation in the lysosome.

1101 **(B)** Top: In the *apn*<sup>1</sup> mutant dorsal trunk, the pulling forces of the apical  
1102 membrane expansion are decreased due to decreased growth of the apical  
1103 membrane (thin red arrows), whereas the forces mediated by the aECM are  
1104 likely to remain unchanged, causing breakage of the tube. Bottom: In the  
1105 absence of Apn, Crb is depleted from the apical surface due to increased  
1106 endocytosis. Crb is trapped in enlarged, Vps35 (retromer)-positive vesicles. It  
1107 fails to be recycled (as shown by the lack of colocalization with Rab11), but is  
1108 also not degraded, pointing to a functional defect of the retromer.

1109

## 1110 **Supporting information**

1111

### 1112 **Suppl. Figure 1. Amino acid sequence alignment of the protein encoded** 1113 **by CG15887 (*apn*).**

1114 Prank software of the homologous sequences within the insect order was  
1115 used. Colored bars indicate the protein domains. SP: Signal Peptide, N'-ECD:  
1116 N-terminal Extracellular Domain, TM: Transmembrane domain, ICD:  
1117 Intracellular Domain, C'-ECD: C-terminal Extracellular Domain.

1118

### 1119 **Suppl. Figure 2. Apn expression in the embryonic and larval tracheae.**

1120 **(A-D)** Immunostaining of embryonic (A-B') and larval (C, D) tracheae with  
1121 anti-Apn antibody shows specific tracheal staining in wild type (WT) stage 17

1122 embryos (A) and second instar (L2) larva (C). No Apn protein can be detected  
1123 in *apn*<sup>1</sup> mutant embryos (B) and second instar (L2) larva (D). Embryos/larvae  
1124 in A', B' were additionally stained for chitin binding probe (CBP) to highlight  
1125 the luminal matrix. Scale bars: 20 μm.

1126 (E) Yeast-two-hybrid analysis detecting interaction between the bait (Crb) and  
1127 the prey (Apn). The top plate shows growth on media lacking Tryptophan and  
1128 Leucine used to verify co-transformation of plasmids as well as a growth  
1129 control. The bottom plate shows the same dilutions spotted on medium  
1130 lacking additionally Histidine and is used to confirm the interaction between  
1131 bait and prey. Column 1 is the positive control whereas columns 2,3,4  
1132 represent the negative controls (2: pB102/pP55 empty vectors, 3: pB102  
1133 empty vector/CG15887, 4: Crb/pP55 empty vector), column 5 represents the  
1134 interaction between Crb-Apn. For each interaction several dilutions (undiluted,  
1135 10<sup>-1</sup>, 10<sup>-2</sup>, 10<sup>-3</sup>) were spotted. Interactions were tested with two independent  
1136 clones (A and B).

1137 (F) Western blot from lysates of larval tracheae showing the expression levels  
1138 of Crb and Apn in wild type (WT) and *apn*<sup>1</sup> mutants. Tubulin is used as  
1139 loading control. The 15 kDa, Apn-positive band is absent in the *apn*<sup>1</sup> mutant  
1140 extract. The higher molecular weight bands are unspecific.

1141 (G, G') Proximity ligation assay (PLA) between WT and *apn*<sup>1</sup> mutant larval  
1142 tracheae using Apn and Crb antibodies shows that the interaction is abolished  
1143 in mutants lacking *apn* as compared to wild type. Scale bar: 20μm.

1144 (H, I) *apn*<sup>1</sup> mutant embryo (H) and larva (I) derived from germline clones (M/Z;  
1145 maternal/zygotic).

1146 (H) No defects were observed in the tracheal tubes of mutant embryos. Scale  
1147 bar: 100  $\mu\text{m}$ .

1148 (I) Defects appear at second larval instar with irregular and twisted tracheal  
1149 tubes (I). Scale bar: 500 $\mu\text{m}$

1150 (J) Brightfield image of a hemizygous second instar larva transheterozygous  
1151 for *apn*<sup>1</sup> and a deficiency that removes *apn*. Scale bar: 500 $\mu\text{m}$ .

1152 (K) Body size reduction in *apn*<sup>1</sup> mutants and tracheal knockdown larvae as  
1153 compared to wild type larvae (bottom).

1154

1155 **Suppl. Figure 3. *apn* controls tube elongation independent of the aECM**  
1156 **and septate junction pathway.**

1157 (A-C) Brightfield dorsal views of second instar larvae, showing the structure of  
1158 tracheal tubes of wild type (WT) (A) and tracheal-specific *apn* down-regulation  
1159 (*btl*>*apn* RNAi), which recapitulates *apn*<sup>1</sup> mutant tracheal defects (B). Tube  
1160 morphology defects are partially rescued by tracheal expression of Apn (*apn*<sup>1</sup>;  
1161 *btl*>Apn). Scale bar: 500 $\mu\text{m}$ .

1162 (D-F) The 9<sup>th</sup> metamer of the dorsal trunk (yellow dotted line) of *apn*<sup>1</sup> mutant  
1163 second instar larvae (E) is shorter than that of wild type larvae (WT) (D).  
1164 Tracheal expression of a transgene (*apn*<sup>1</sup>;*btl*>*apn*) rescues the metamer  
1165 elongation defects of *apn* mutant larvae (F). Anterior is to the left. Scale bar:  
1166 200 $\mu\text{m}$ .

1167 (G-H') Transmission electron micrographs of cross sections through a wild  
1168 type (WT; G, G') and *apn*<sup>1</sup> mutant (H, H') second instar. (G, H) are axial views  
1169 of the dorsal trunk (DT), G' and H' are higher magnifications to depict the

1170 larval cuticular ECM (epi- and procuticle) and the taenidial ridges. Scale bars:  
1171 (G,H) 7,5  $\mu\text{m}$ , (G',H') 700 nm.

1172 (I-J') Immunostaining of larval tracheal tubes with antibodies against the  
1173 apical extracellular matrix (aECM) proteins Dumpy (Dp) (I,J) and Piopio (I',J').  
1174 Scale bars: 20  $\mu\text{m}$ .

1175 (K-L') Tracheal maturation of wild type (WT) (K, K') and *apn*<sup>1</sup> mutant (L, L')  
1176 second instar larvae. Secretion of the luminal protein ANF-Cherry (E, F) as  
1177 well as its clearance from the luminal space (K', L') are comparable between  
1178 wild type (WT) and *apn*<sup>1</sup> mutants. Scale bars: 50  $\mu\text{m}$ .

1179 (M-N') Immunostaining of wild type (WT) and *apn*<sup>1</sup> mutant tracheal tubes of  
1180 second instar larvae with antibodies against the septate junction proteins  
1181 Contactin (Cont) (M, N) and Discs Large (Dlg) (M', N'). Scale bar: 20  $\mu\text{m}$ .

1182

1183 **Suppl. Figure 4. Distribution of Crb in tracheal branches of distinct**  
1184 **cellular architecture and in salivary glands.**

1185 (A-B''') Confocal projections showing tracheal tubes of wild type (WT, A-A''')  
1186 and *apn*<sup>1</sup> mutant (B-B''') second larval instar larvae, stained with anti-Crb. Crb  
1187 localization is affected in multicellular tubes (MT), lateral branches  
1188 (autocellular (AT) and seamless tubes (ST) of mutant larvae. Scale bars: (A,  
1189 B, A'', B'') 20  $\mu\text{m}$  and (A', B', A''', B''') 10  $\mu\text{m}$ .

1190 (C-D') RNAi-mediated knockdown of *apn* by *daughterless*-Gal4 (*da*-Gal4)  
1191 results in accumulation of Crb-positive cytoplasmic punctae (compare C and  
1192 D) and strong reduction of Apn (compare C' and D'). Nuclear Apn signal is  
1193 considered to be unspecific. Scale bars: 20  $\mu\text{m}$ .



1194 **(E-F')** Brightfield lateral views of second instar larvae showing the structure of  
1195 multicellular tubes (MT), autocellular (AT) and seamless tubes (ST) of wild  
1196 type (WT, E, E') and *apn*<sup>1</sup> mutants (F, F'). Scale bars: (E, F) 200 μm and (E',  
1197 F') 1000 μm.

1198 **(G-H')** Confocal projections showing the salivary gland of wild type (WT, G,  
1199 G') and *apn*<sup>1</sup> mutants (H, H') second instar larvae, stained for Crb and Dlg.  
1200 Scale bars: 20 μm.

1201

1202 **Suppl. Figure 5. Endosomal sorting components in *apn*<sup>1</sup> mutants.**

1203 **(A-A'')** *apn*<sup>1</sup> mutant tracheal tubes of second instar larvae immunostained for  
1204 Crb (magenta) and Hrs (green). Magnification in A'' shows hardly any co-  
1205 localization of vesicular Crb and Hrs.

1206 **(B-B'')** *apn*<sup>1</sup> mutant tracheal tubes of second instar larvae immunostained for  
1207 Crb (magenta) and Lamp1 (green). Magnification in B'' shows hardly any co-  
1208 localization of vesicular Crb and Lamp1.

1209 **(C-C'')** *apn*<sup>1</sup> mutant tracheal tubes of second instar larvae immunostained for  
1210 Crb (magenta) and Arl8 (green). Magnification in C'' shows hardly any co-  
1211 localization of vesicular Crb and Arl8 staining. Scale bars: A, A', B, B', C, C':  
1212 20 μm and A'', B'', C'': 5 μm.

1213

1214 **Suppl. Figure 6. Expression of Crb of *apn*<sup>1</sup> and *crb* depletion tracheal  
1215 cells.**

1216 **(A-C')** RNAi-mediated downregulation of Crb (A, C) results in depletion of  
1217 Crb, but does not affect Dlg expression (A', C') in the dorsal trunk of second  
1218 instar larvae. In *apn*<sup>1</sup> mutants, Crb is detected in cytoplasmic punctae (B),

1219 whereas Dlg is properly localized baso-laterally in tracheal cells (B').

1220 Projections of confocal sections of second instar tracheal tubes, stained for

1221 Crb (A-C), Dlg (green; A'-C'). Scale bars: 20  $\mu$ m.

1222

Figure 1

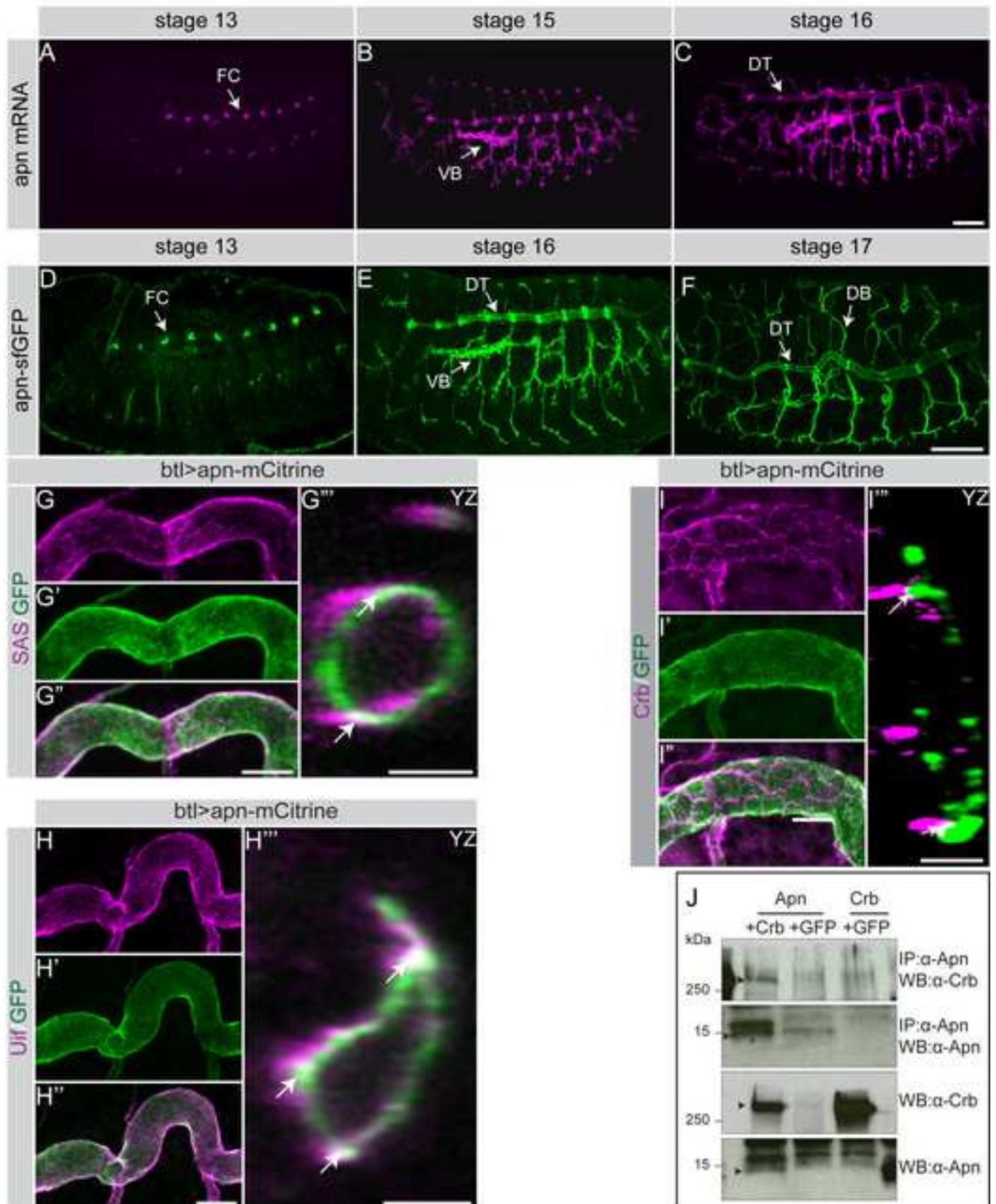


Figure 2

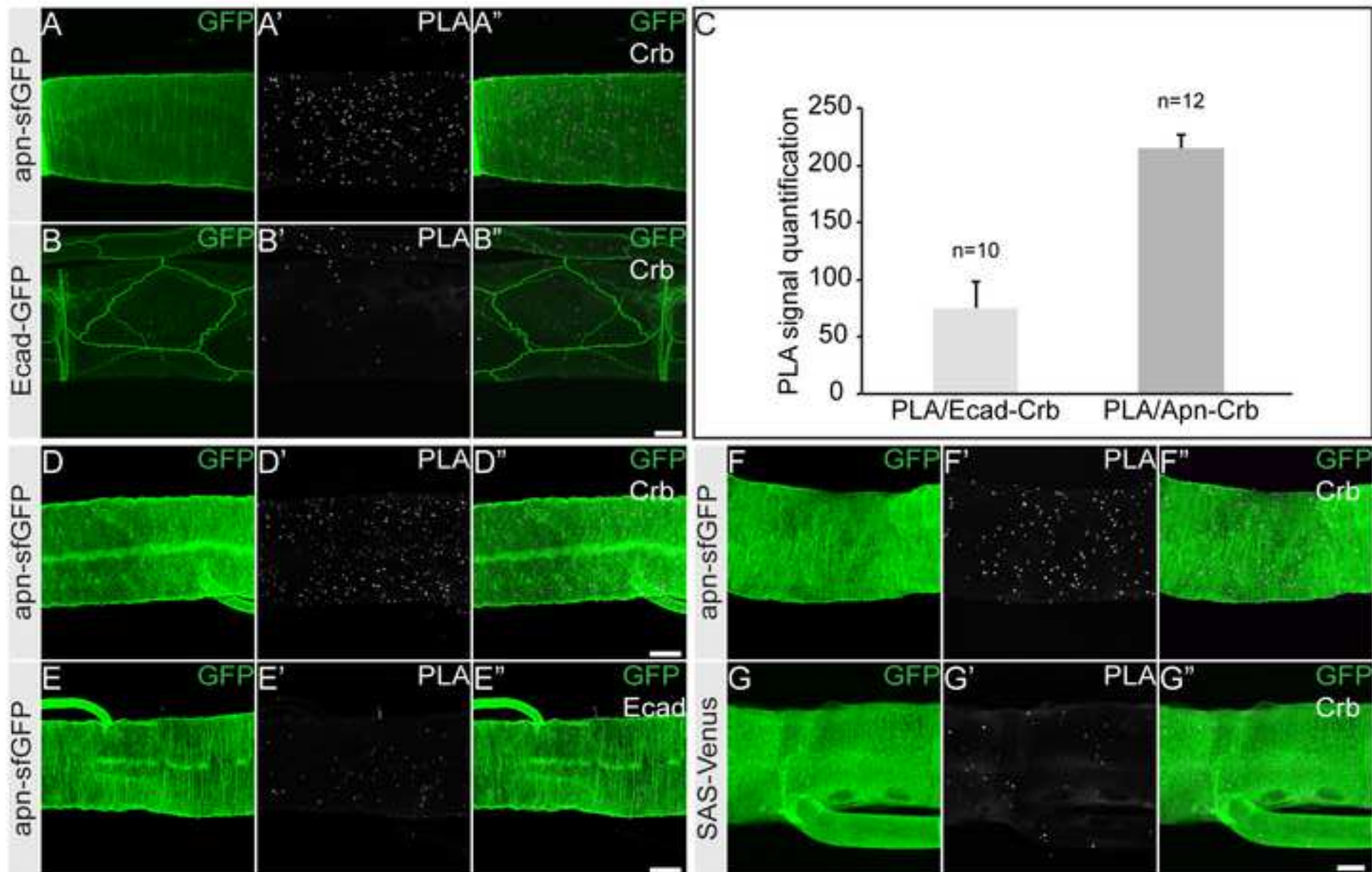


Figure 3

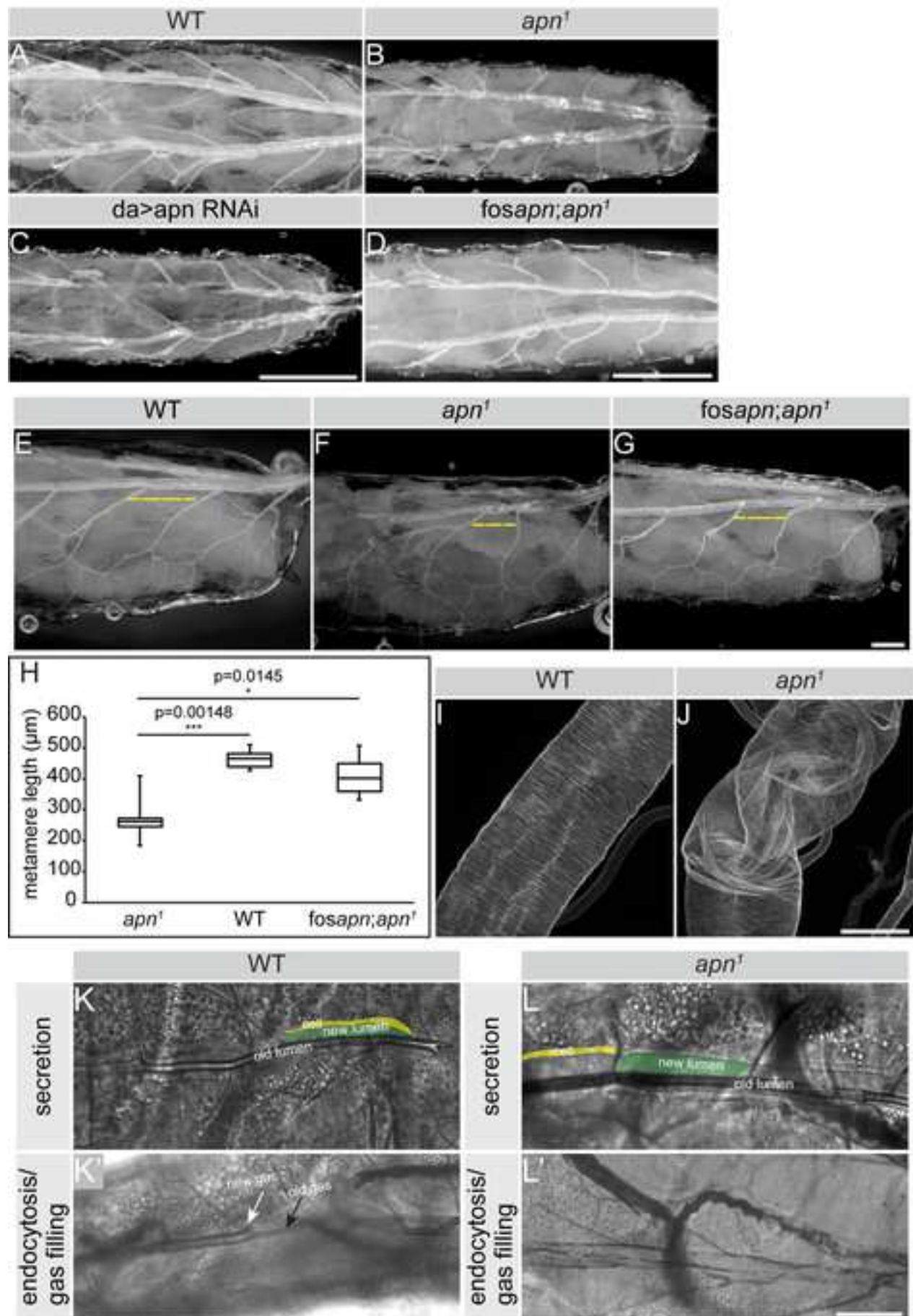


Figure 4

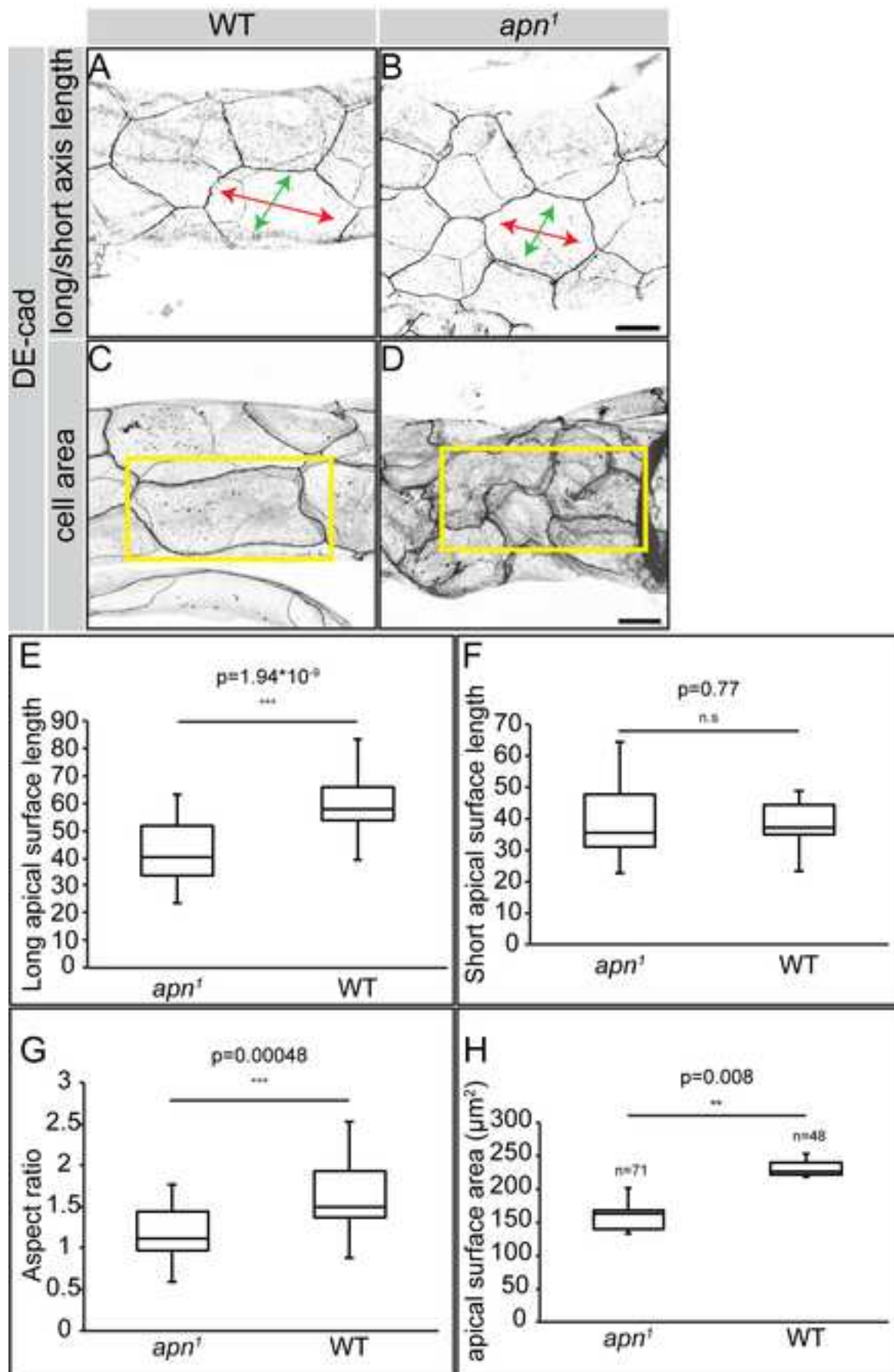


Figure 5

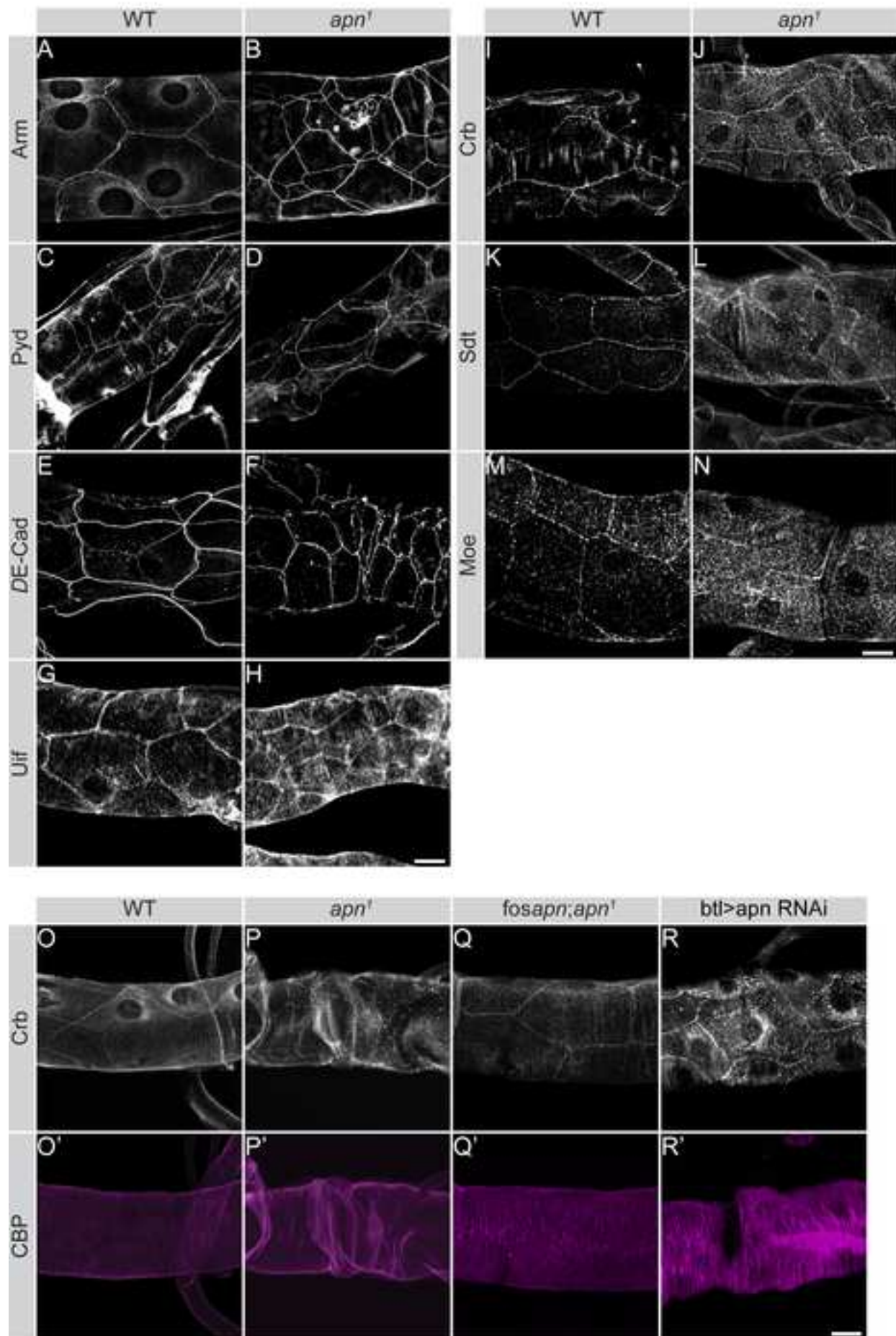


Figure 6

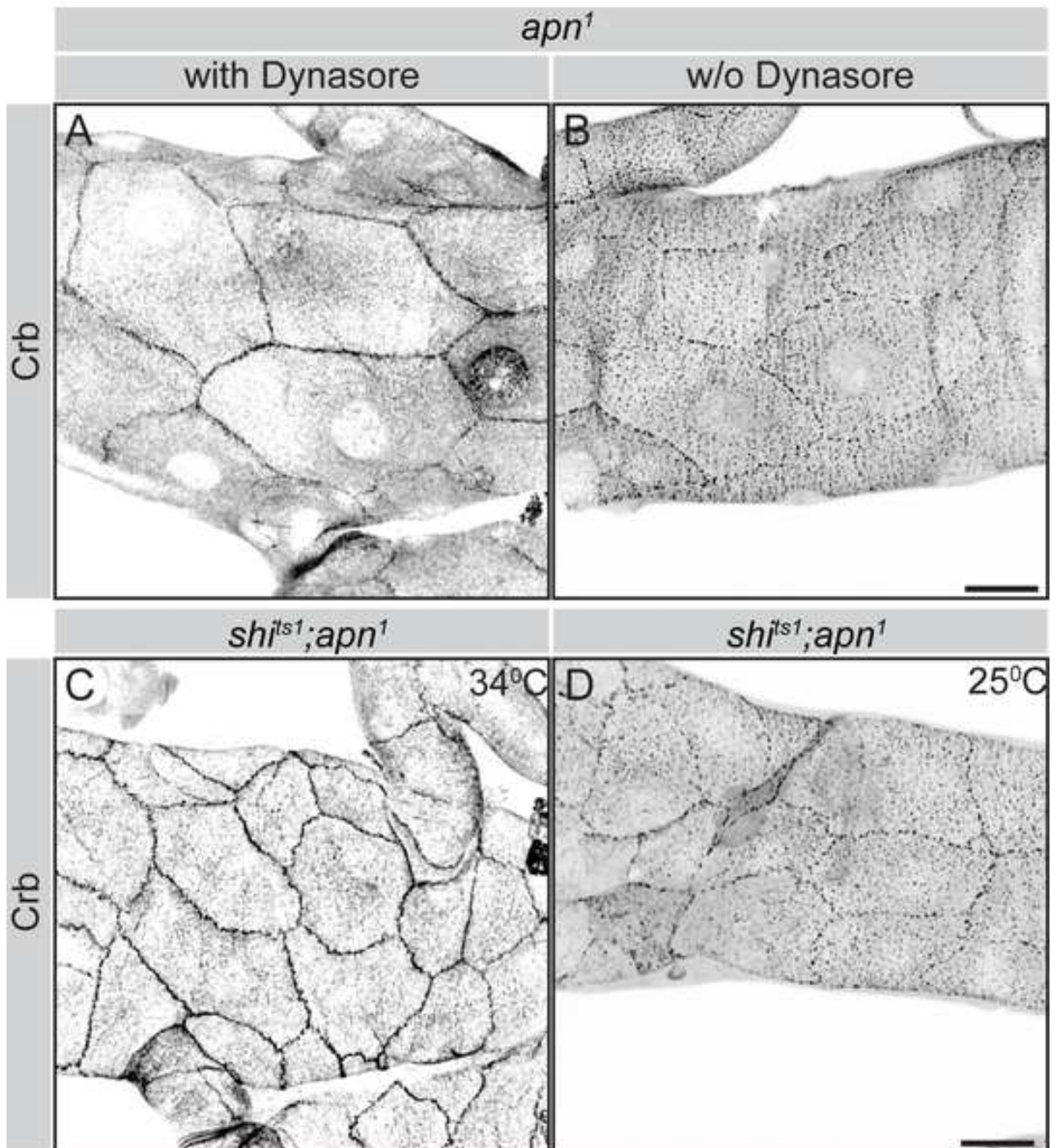




Figure 7

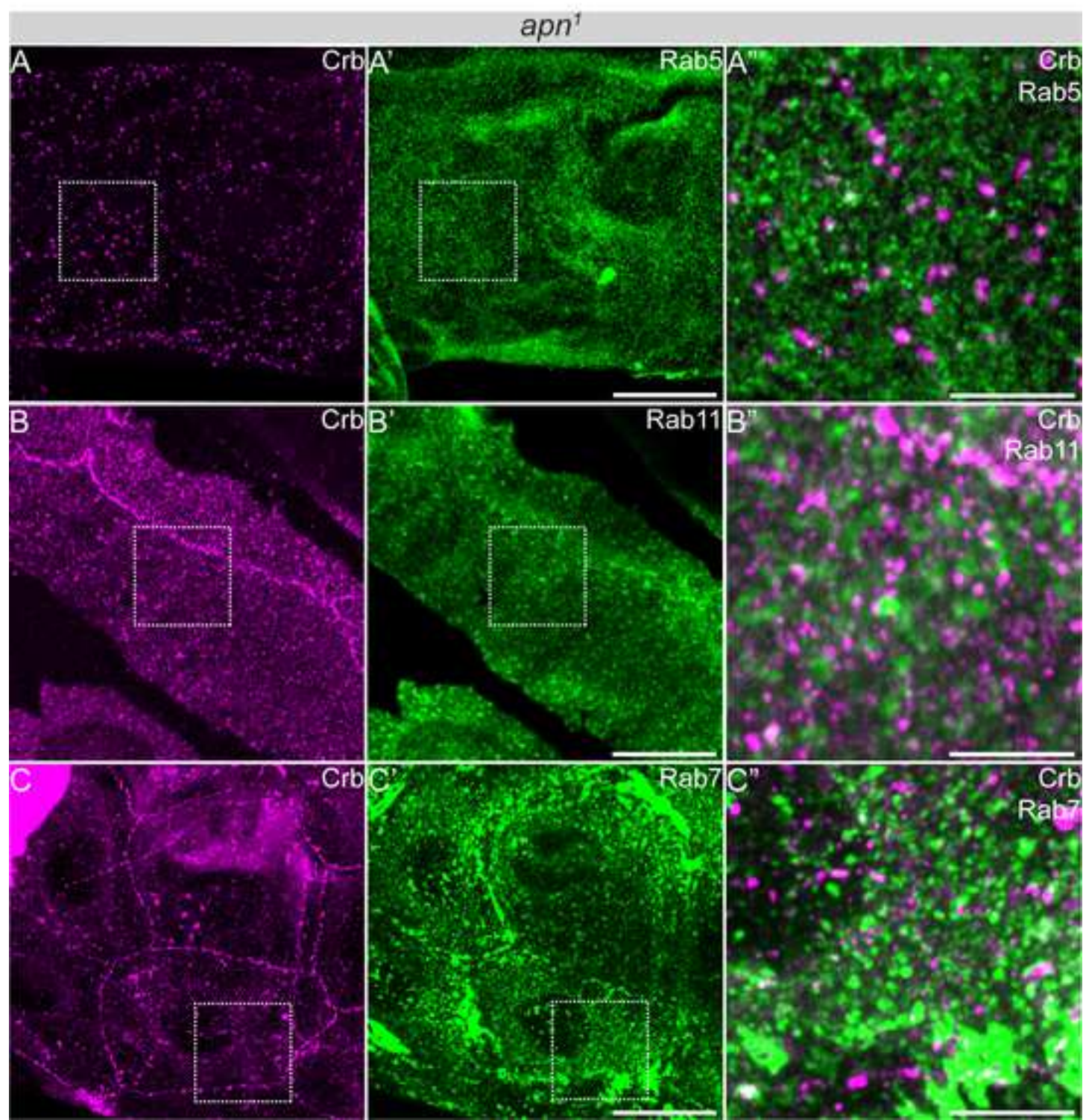


Figure 8

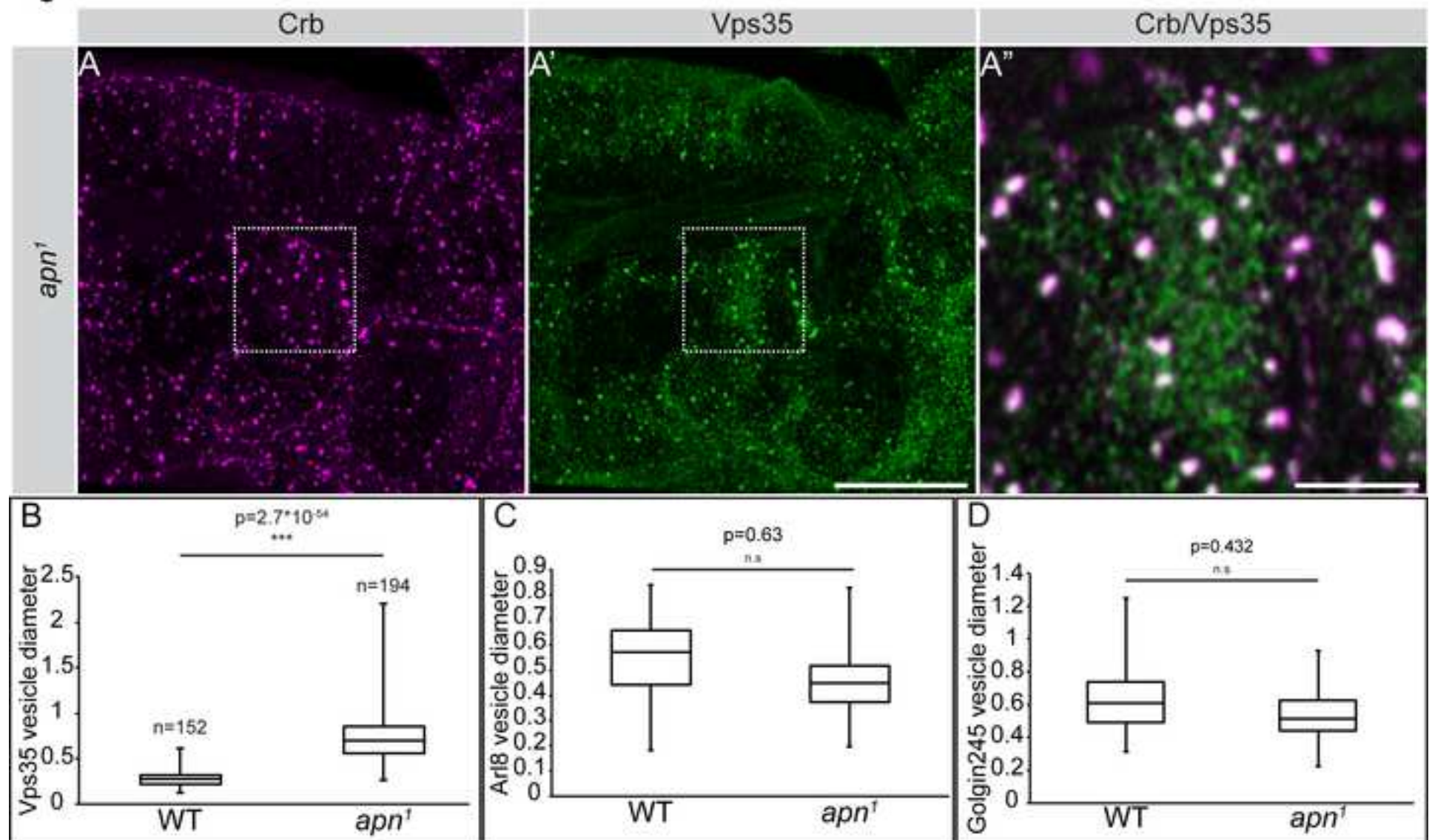


Figure 9

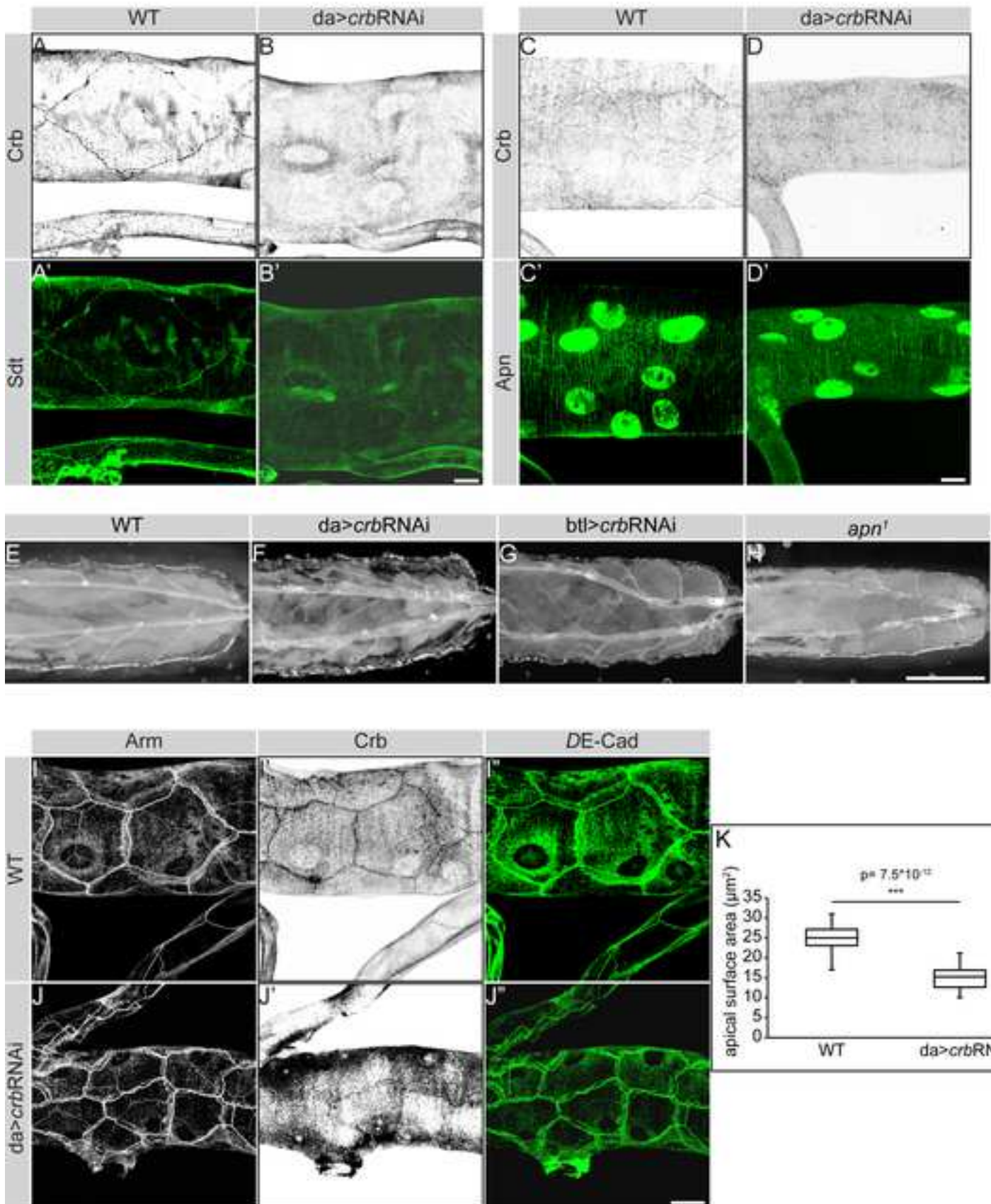


Figure 10

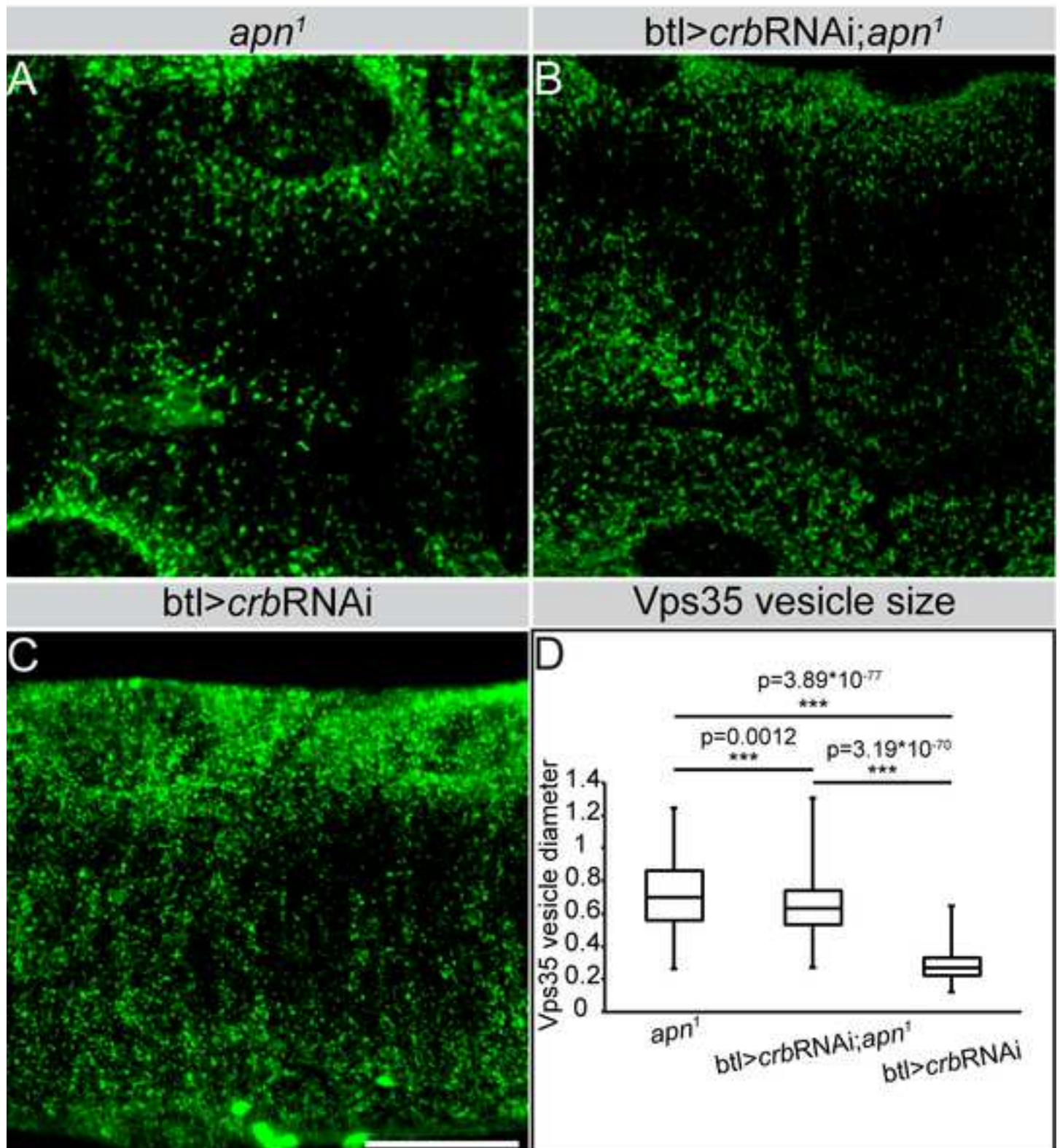
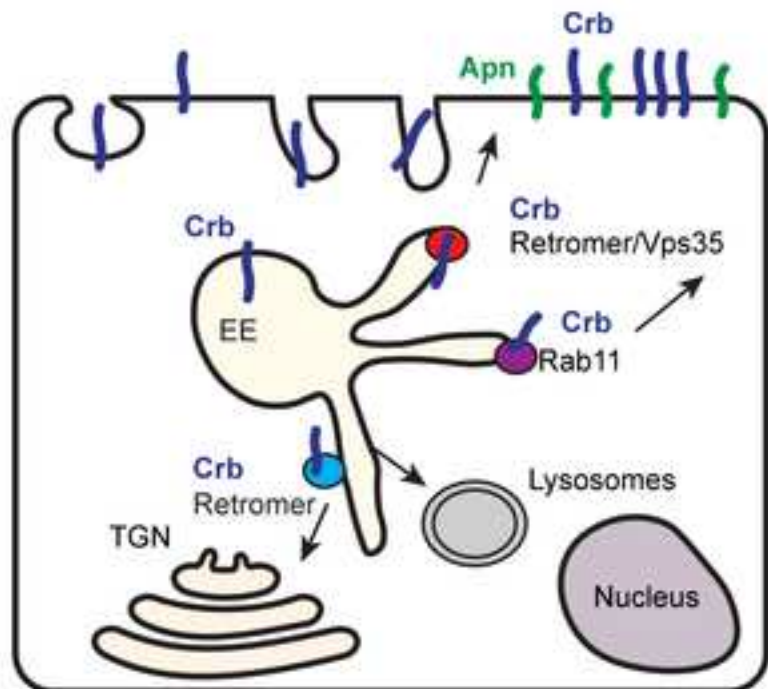
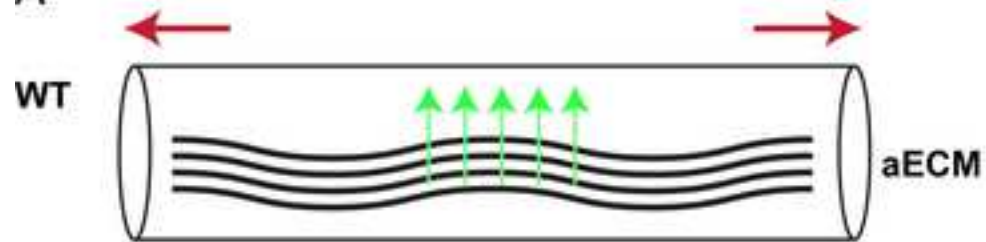
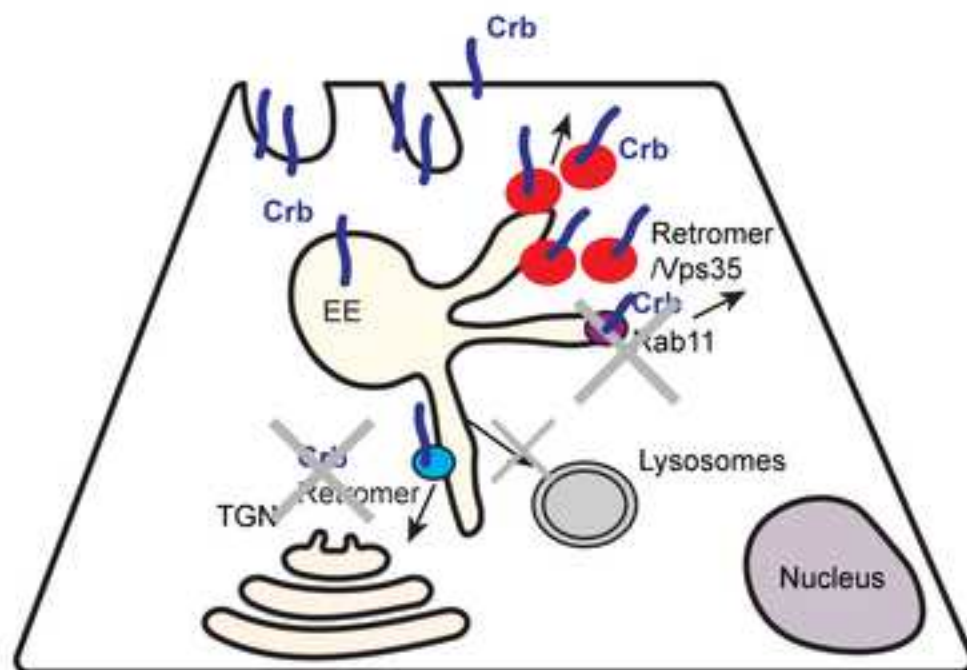
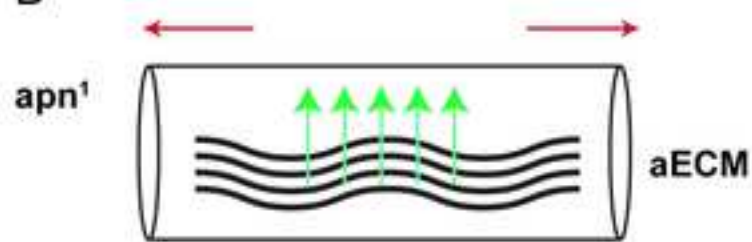


Figure 11

A

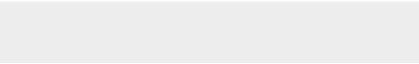


B





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