

1 **Title:** Super-resolution imaging uncovers the nanoscopic segregation of polarity proteins in epithelia

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9

10 **Abstract:**

11 Epithelial tissues acquire their integrity and function through the apico-basal polarization of their
12 constituent cells. Proteins of the PAR and Crumbs complexes are pivotal to epithelial polarization, but the
13 mechanistic understanding of polarization is challenging to reach, largely because numerous potential
14 interactions between these proteins and others have been found, without clear hierarchy in importance.
15 We identify the regionalized and segregated organization of members of the PAR and Crumbs complexes
16 at epithelial apical junctions by imaging endogenous proteins using STED microscopy on Caco-2 cells,
17 human and murine intestinal samples. Proteins organize in submicrometric clusters, with PAR3
18 overlapping with the tight junction (TJ) while PALS1-PATJ and aPKC-PAR6 β form segregated clusters that
19 are apical of the TJ and present in an alternated pattern related to actin organization. CRB3A is also apical
20 of the TJ and weakly overlaps with other polarity proteins. This organization at the nanoscale level
21 significantly simplifies our view on how polarity proteins could cooperate to drive and maintain cell
22 polarity.

23 Introduction

24 In epithelial tissues, cells coordinate their organization into a polarized sheet of cells. Each cell
25 acquires an apico-basal organization and specialized lateral junctions, namely tight junctions (TJs, also
26 known as zonula occludens), adherens junctions and desmosomes (Farquhar & Palade, 1963). This
27 organization is key to the development, the maintenance, and the function of epithelial tissues. How this
28 organization is orchestrated remains largely unknown.

29 Over the past two decades, a number of proteins have been discovered to be pivotal to epithelial
30 polarization, such as PAR3, PAR6 and aPKC (PAR complex), Crumbs, PATJ and PALS1 (Crumbs complex)
31 and Scribble, LGL and DLG (Scribble complex) in mammals (for review see (Assémat et al., 2008; Pickett et
32 al., 2019; Rodriguez-Boulan & Macara, 2014). These proteins are remarkably well conserved over the
33 animal kingdom (Belahbib et al., 2018; Le Bivic, 2013). Deletion or depletion of one of these proteins
34 usually results in dramatic developmental defects (Alarcon, 2010; Charrier et al., 2015; Hakanen et al.,
35 2019; Lalli, 2012; Park et al., 2011; Sabherwal & Papalopulu, 2012; Tait et al., 2020; Whiteman et al., 2014).

36 In the quest to understand the role of polarity proteins, numerous genetic and biochemical studies
37 have been carried out. We and others have found that these proteins interact to form multiprotein
38 complexes. Pioneering studies defined three core complexes based on the discovery of protein
39 interactions or localization: the PAR complex consisting of PAR3, PAR6, and aPKC proteins (Joberty et al.,
40 2000; Lin et al., 2000), the Crumbs complex consisting of CRUMBS, PALS1, and PATJ (Bhat et al., 1999;
41 Makarova et al., 2003; Roh, Makarova, et al., 2002), and the Scribble complex consisting of Scrib, Lgl, and
42 Dlg (Bilder et al., 2000). However, this view became more complex over the years as many interactions
43 between proteins of different complexes can occur (Assémat et al., 2008; Hurd et al., 2003; Lemmers et
44 al., 2004), and interactions of polarity proteins with cytoskeleton regulators and lateral junction proteins
45 are common (Assémat et al., 2008; Chen & Macara, 2005; Itoh et al., 2001; Médina et al., 2002; Michel et

46 al., 2005; Roh, Liu, et al., 2002; Takekuni et al., 2003; Tan et al., 2020). A current limitation in the
47 understanding of polarization is that there is no clear hierarchy in the importance of these numerous
48 interactions. Potential interactions revealed through biochemical assays do not necessarily reflect relevant
49 interactions in cells, and do not specify when nor where in the cell these interactions could be relevant.

50 Polarity proteins have been localized with classical light microscopy and remarkably, they are often
51 found concentrated at the apical junction, a key organizational landmark of epithelial cells. To understand
52 how polarity proteins cooperate to orchestrate cell polarization, one needs to understand how precisely
53 polarity proteins organize with respect to apical junctions or to the cytoskeleton. However, except from a
54 few limited cases (Hirose et al., 2002; Izumi et al., 1998; Tan et al., 2020), the precise localization of polarity
55 proteins at these organizational landmarks is missing. Moreover, knowing how polarity proteins organize
56 in relation to each other in the cell should enable us to decipher from their plentiful known potential
57 interactions, which ones are more relevant in specific sub-regions of the cell.

58 To tackle these challenges, we decided to systematically localize with STED microscopy, the
59 polarity proteins that are key to the establishment of the apical pole of epithelia: PAR3, aPKC, PAR6 β , PATJ,
60 PALS1 and CRB3A. These proteins localize at the apical junction region of epithelial cells. Because how
61 proteins interact and localize is likely to depend on cell differentiation, we decided to focus here on mature
62 epithelia, a state where we hypothesize that protein interactions and localization are stationary. Using
63 human and murine intestine and Caco-2 cells, we first imaged endogenous polarity proteins with respect
64 to the TJ, to appreciate their overall organization in the region. Second, we localized these proteins two-
65 by-two, to uncover relevant apical polarity protein sub-cellular associations. Finally, we focused on polarity
66 proteins organization with respect to the actin cytoskeleton. We find that polarity proteins localize in
67 distinct sub-regions that do not reflect the canonical definition of polarity proteins complexes. In addition,
68 their localization with respect to the cytoskeleton emphasizes some emerging roles of polarity proteins as
69 regulators of actin organization.

70 Results

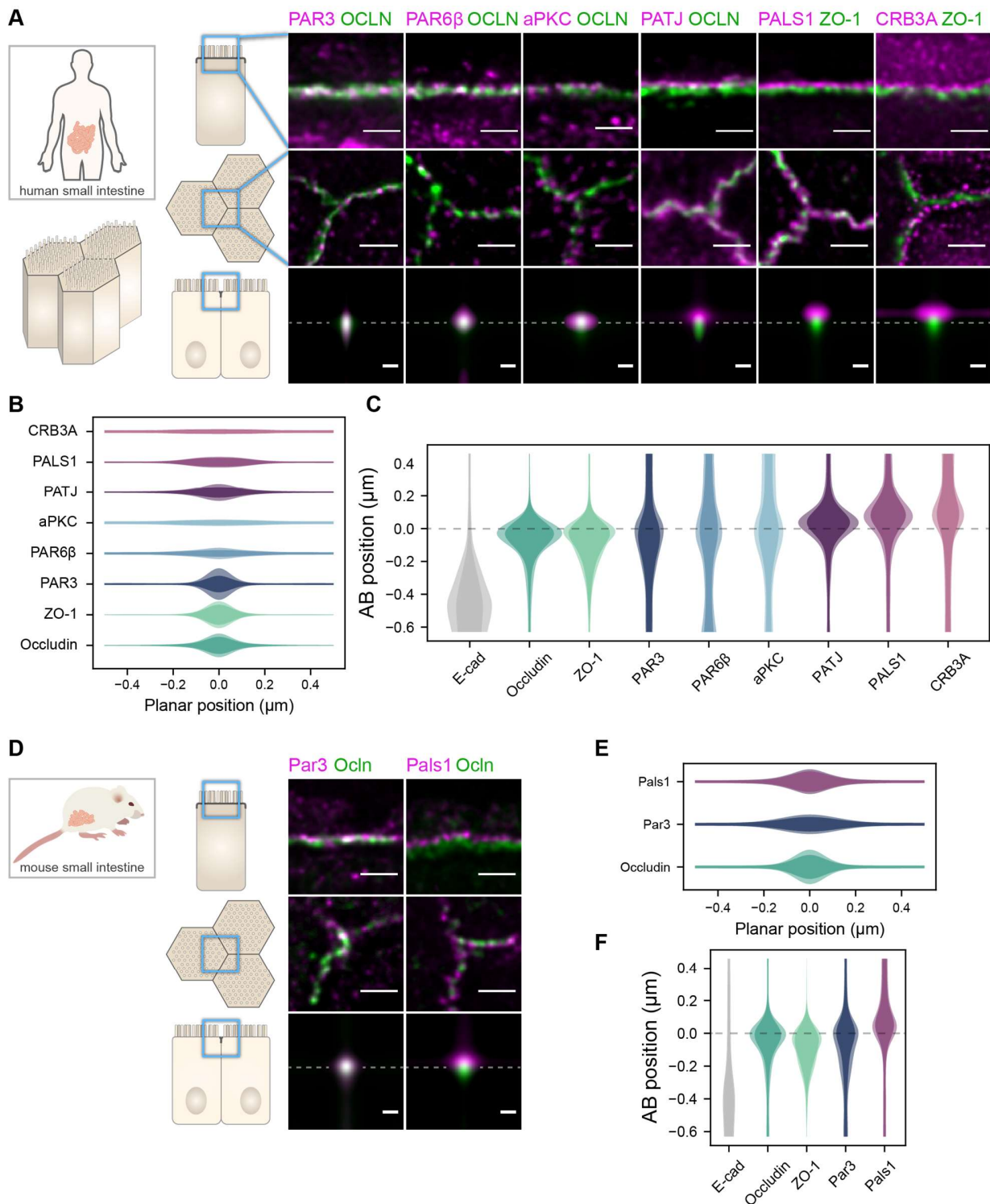
71 Polarity proteins are localized in separate subdomains in the apical junction region

72 To obtain a first estimate of polarity protein localization in the TJ region, we systematically imaged
73 each polarity protein with respect to a marker of the TJ. To this end, each apical polarity protein and a
74 tight junction marker (ZO-1 or occludin) were immunostained and imaged together using Stimulated-
75 Emission-Depletion (STED) microscopy (Hell & Wichmann, 1994) (Figure 1 and 2). STED images were
76 acquired in the TJ region both in the apico-basal and the planar orientations of cells in human and mouse
77 intestinal biopsies (Figure 1) and Caco-2 cells (Figure 2). To optimize the sample orientation, samples were
78 cryo-sectioned when needed, in particular to obtain apico-basal orientation. Since we focused on mature
79 epithelia, intestinal cells were observed exclusively in villi and Caco-2 cells were seeded on filters and
80 grown over 14 days to allow differentiation (Pinto et al., 1983). Because the resolution of STED microscopy
81 followed by deconvolution was, in our hands, about 80 nm in each color channel, the gain of resolution
82 compared to classical confocal microscopy approaches was 3-fold in the planar orientation, and 7-fold
83 along the apico-basal axis.

84 We found that the localization of each polarity protein was conserved across all samples and
85 species (Figure 1 and 2). All proteins were concentrated in the TJ region as clusters of typically 80 to 200
86 nm in size (the smallest cluster size found is likely due to the imaging resolution limit), but their precise
87 localization was protein dependent. We could group proteins in three main localization types. While we
88 mostly found PAR3 at the TJs (Figure 1A,C,D,F and 2A,C), PAR6 β and aPKC were at the TJ level and apical
89 of the TJ (Figure 1A,C and 2A,C). We found CRB3A, PALS1 and PATJ almost exclusively apical of the TJ
90 (Figure 1A,C,D,F and 2A,C). Interestingly, we often found PAR6 β , aPKC, CRB3A, PALS1 and PATJ separated
91 laterally from the TJ, since we frequently detected clusters of these proteins 100 to 200 nm away from the
92 TJ (Figure 1A,B,D,E and 2A,E). There were some slight differences between intestinal samples and Caco-2

93 cells that may originate from sample preparation or from differences in cell organization due to tissue
94 maturation. These first results show that polarity proteins organize in separate subdomains in the TJ
95 region, namely PAR3 at the TJ and the other polarity proteins studied mostly apical of the TJ.

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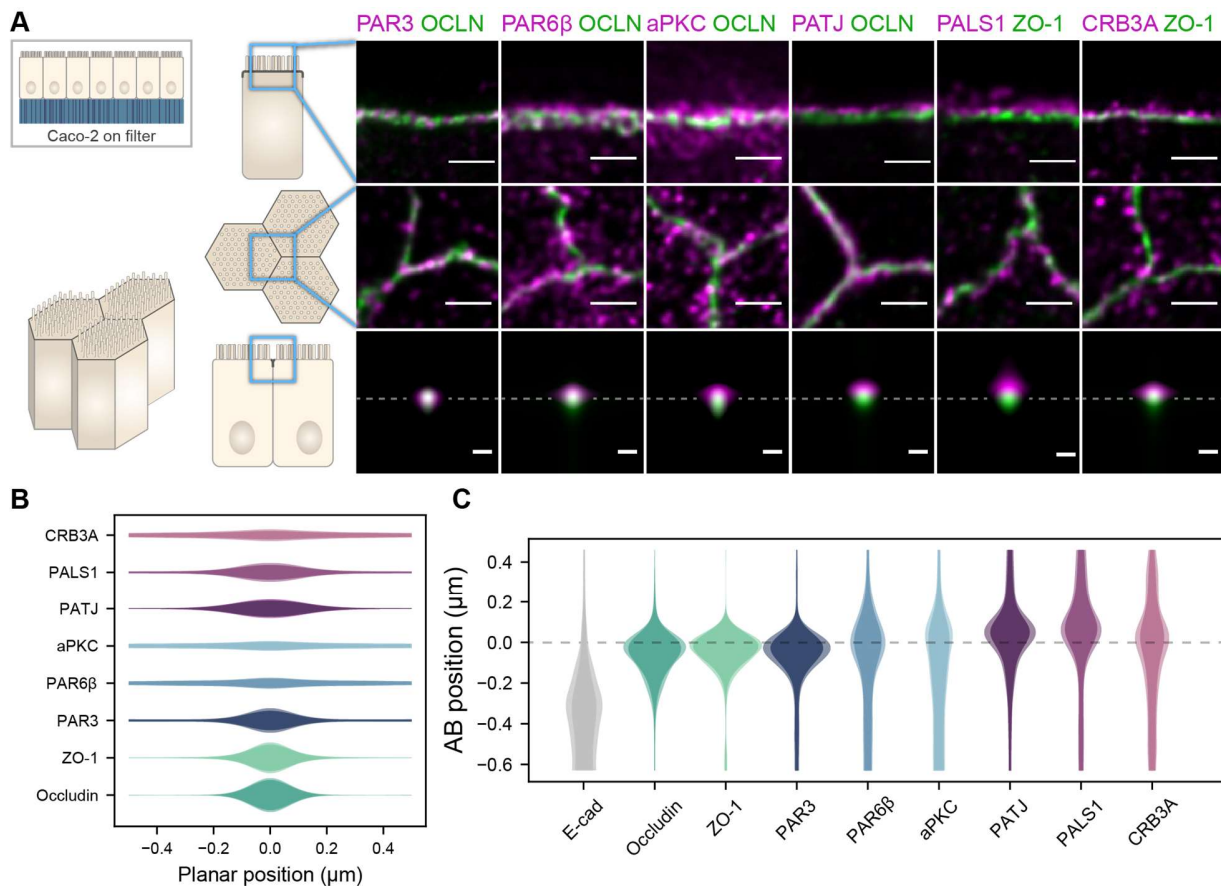


97

98 **Figure 1.** Polarity proteins localize in separate subdomains in the TJ region in human (A-C) and murine (D-F) small
 99 intestine biopsies. (A,D) STED images of protein localization in the TJ area. TJ proteins in green, polarity proteins in

100 magenta. Top row, apico-basal orientation. Middle row, planar orientation. Bottom row, estimate of average protein
101 localization in the apico-basal orientation perpendicular to the junction, obtained by multiplying average localizations
102 estimated in (B) and (C) for human biopsies and (E) and (F) for murine biopsies. Top row and middle row, scale bar 1
103 μm ; bottom row scale bar 200 nm. **(B,E)** Average localization of polarity proteins in the planar orientation, obtained
104 by measuring the intensity profile of proteins perpendicular to the junction, using the TJ protein position as a
105 reference. **(C,F)** Average localization of polarity proteins in the apico-basal orientation, obtained by measuring the
106 intensity profile of proteins along the apico-basal orientation, using the TJ protein position as a reference. In (B,C,E,F),
107 on a given position dark colors represent average intensity values, and lighter colors the average added with the
108 standard deviation. The number of junctions used in quantification and details of the analysis are specified in the
109 Material and Methods section.

110



111
 112 **Figure 2.** Polarity proteins localize in separate subdomains in the TJ region in Caco-2 cells. **(A)** STED images of protein
 113 localization in the TJ area. TJ proteins in green, polarity proteins in magenta. Top row, apico-basal orientation
 114 (obtained from cryo-sectioning cells grown on filter). Middle row, planar orientation. Bottom row, estimate of
 115 average protein localization in the apico-basal orientation perpendicular to the junction, obtained by multiplying
 116 average localizations estimated in **(B)** and **(C)**. Top row and middle row, scale bar 1 μm ; bottom row scale bar 200
 117 nm. **(B)** Average localization of polarity proteins in the planar orientation obtained by measuring the intensity profile
 118 of proteins perpendicular to the junction, using the TJ protein position as a reference. **(C)** Average localization of
 119 polarity proteins in the apico-basal orientation obtained by measuring the intensity profile of proteins along the
 120 apico-basal orientation, using the TJ protein position as a reference. In **(B,C)**, on a given position dark colors represent
 121 average intensity values, and lighter colors the average added with the standard deviation. The number of junctions
 122 used in quantification and the details of the analysis are specified in the Material and Methods section.

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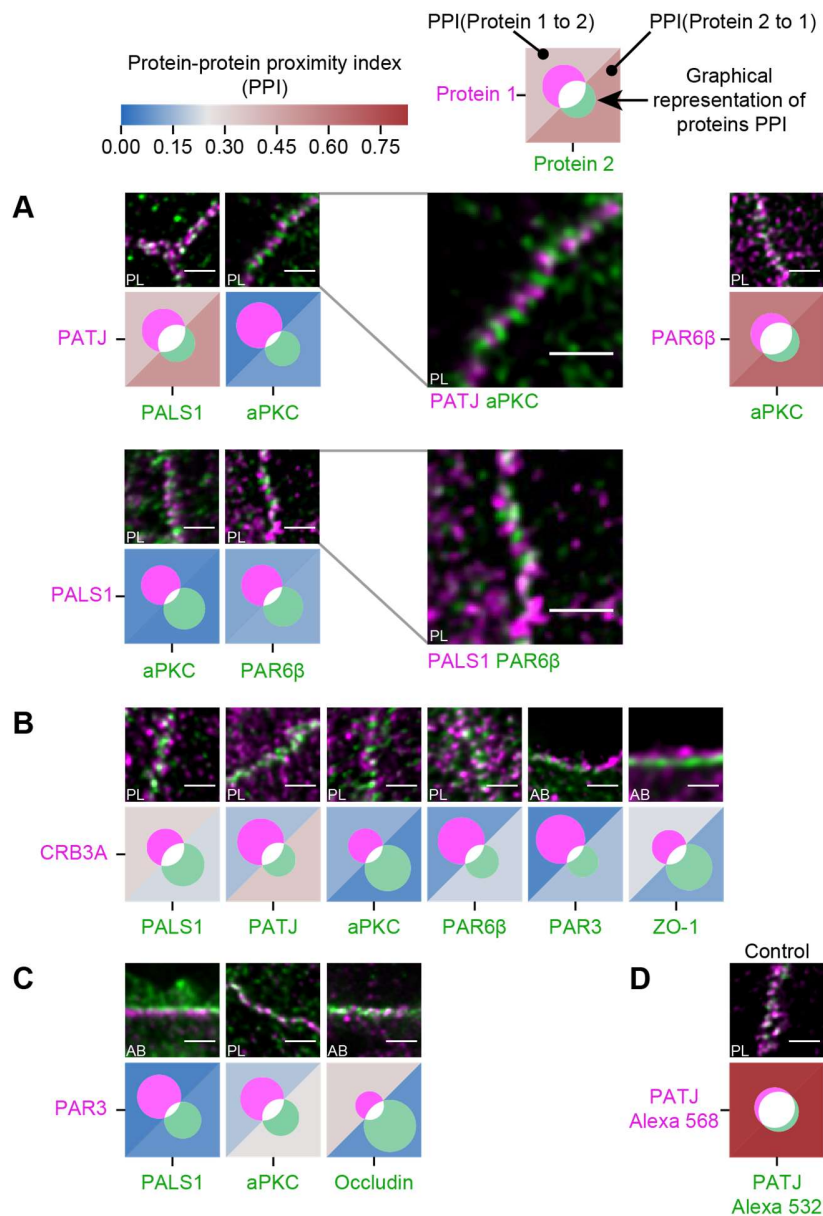
124 Redefining relevant interactions between polarity proteins from colocalization analysis

125 The organization of proteins in separate subdomains led us to investigate how polarity proteins
126 were organized within these subdomains, and more specifically how clusters of polarity proteins were
127 localized with respect of each other. To tackle this question, we imaged polarity proteins two-by-two in
128 Caco-2 cells and quantified the extent of their colocalization, using the protein-protein proximity index
129 developed in (Wu et al., 2010), providing a quantitative estimate of protein proximity (Figure 3). Because
130 of the organization of protein clusters, different proteins that localize at the same level on the apico-basal
131 axis may appear as overlapping “more” when observed in the apico-basal orientation rather than when
132 they are observed in the planar orientation; this is due to the fact that the axial-resolution (about 550 nm)
133 is 7-fold lower than the planar resolution (about 80 nm). To circumvent this limitation, we minimized the
134 apparent colocalization for each protein pair by orienting our sample either in the planar or apico-basal
135 orientation, wherever apparent colocalization was lowest.

136 First, we found that some of the proteins colocalize strongly: PALS1 and PATJ seem to reside in the
137 same clusters, similarly to aPKC and PAR6 β that also colocalize strongly, presumably in both cases forming
138 a complex, as the literature suggests (Joberty et al., 2000; Lin et al., 2000; Roh, Makarova, et al., 2002)
139 (Figure 3A). Surprisingly, we found PALS1-PATJ and aPKC-PAR6 β well segregated from each other when
140 we observed them in the planar orientation. They sometimes appeared as alternating bands along the
141 junction with a spatial repeat in the range of 200 nm to 300 nm (zooms in Figure 3A). In some cases, these
142 bands seemed formed by clusters facing each other in neighboring cells, indicating a potential coordination
143 of polarity protein organization between adjacent cells. Second, we found that only a minority of CRB3A
144 colocalized with any of the other polarity proteins (Figure 3B). These observations are also surprising,
145 because CRB3A has been reported to strongly interact both with PALS1 and PAR6 (Hayase et al., 2013;
146 Lemmers et al., 2004; Li et al., 2014; Makarova et al., 2003). This could mean that these interactions are
147 mostly transient or that they are not prominent in the TJ area. This result questions the stability and

148 functional cellular meaning of the canonical Crumbs-PALS1-PATJ complex and of the CRB3-PAR6
149 interaction. Finally, when localizing PAR3 along with PALS1 or aPKC, we found that PAR3 is hardly found
150 with either of these proteins (Figure 3C). These data show that PAR3, aPKC and PAR6 β do not associate in
151 a static complex as it has been suggested in several non-mammalian models (Afonso & Henrique, 2006;
152 Harris & Peifer, 2005; Morais-de-Sá et al., 2010; Rodriguez et al., 2017). It appears, in our conditions, that
153 aPKC and PAR6 β are likely linked in the apical TJ region, whereas PAR3 is mostly not associated to them.
154 Again, it is possible that the interaction between PAR3 and PAR6 β -aPKC is mostly transient or that it is not
155 relevant in the TJ area. We conclude that PAR3 is mostly isolated from other polarity proteins at the TJ,
156 and that PALS1-PATJ, PAR6 β -aPKC and CRB3 form three spatially separated entities in the apical region of
157 the TJ.

158



159

160 **Figure 3.** Proximity analysis of polarity proteins redefines protein complexes. The analysis is carried out in Caco-2
 161 cells, where we used the concept of protein-protein proximity index (PPI) introduced in (Wu et al., 2010), indicating
 162 the proximity of two different proteins populations. PPI of 0 indicates no proximity (or no colocalization), and PPI of
 163 1 indicates perfect proximity (or perfect colocalization); intermediate values give an estimate of the fraction of a
 164 given protein being in close proximity (or colocalize) with another one. Here the result of the proximity analysis
 165 is represented graphically with color-coded values and Venn diagrams as depicted on the top of the figure (details in
 166 Material and Methods). The analysis has been carried out on apico-basal (AB) or planar (PL) orientation images to
 167 minimize apparent colocalization due to overlapping in different planes; this is reported in the representative image
 168 of each experiment. **(A)** Proximity analysis for PATJ, PALS1, aPKC and PAR6β and corresponding representative
 169 images. Zoomed images (PATJ/aPKC and PALS1/PAR6β) illustrate the segregation of these proteins. **(B)** Proximity
 170 analysis for CRB3A and the other polarity proteins. **(C)** Proximity analysis for PAR3 with PALS1, aPKC and occludin. **(D)**
 171 Control experiment with PATJ labelled with an Alexa 532 secondary antibody and an Alexa 568 tertiary antibody. The
 172 number of junctions used in quantification and the details of the analysis are specified in Material and Methods.
 173 Scale bars: 1 μm.

174 PATJ localization in the TJ region with electron tomography

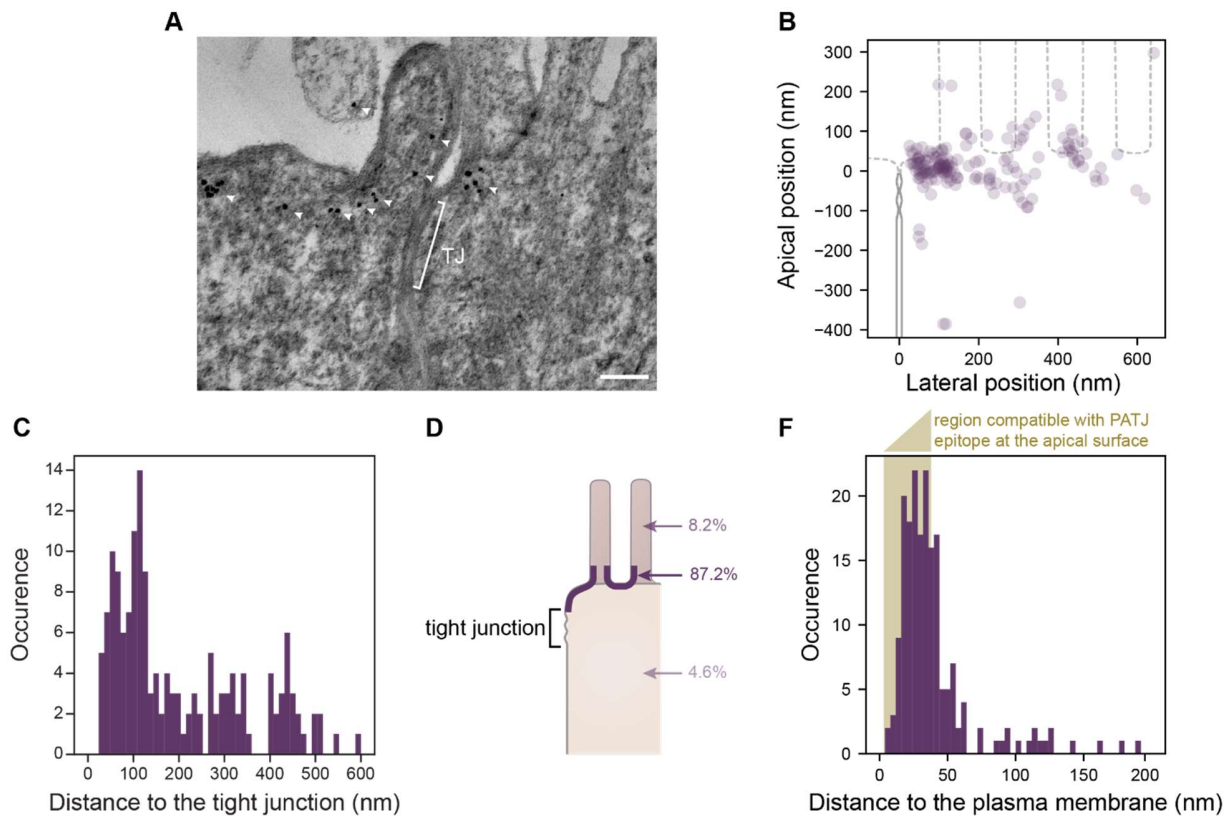
175 In the generally accepted description of the canonical Crumbs complex, PALS1 binds to the
176 transmembrane protein CRB3A and PATJ binds to PALS1 (Roh, Makarova, et al., 2002). Therefore, PALS1
177 and PATJ are thought to be in close vicinity of the membrane since CRB3A is a short transmembrane
178 protein. Moreover, it was proposed that PATJ links CRB3A-PALS1 to the TJ area (Michel et al., 2005)
179 because of PATJ direct interaction with the TJ protein ZO-3 and Claudin1 (Roh, Liu, et al., 2002). Our
180 protein-proximity analyses, however, raise the question of whether PALS1/PATJ interact with CRB3A in
181 the TJ region (Figure 3), and our localization of PATJ with STED suggests that most PATJ proteins are often
182 too far from the TJ to interact with this structure (Figure 1 and 2). Therefore, to obtain a more complete
183 understanding of PATJ localization in the TJ region, we observed PATJ with electron tomography using
184 immunogold labelling in Caco-2 cells (Figure 4).

185 Consistent with what we observed with STED, we often found PATJ organized in clusters apical of
186 the TJ (Figure 4A). We started by quantifying PATJ position with respect to the TJ, using as a reference the
187 most apical part of the TJ (defined morphologically as the most apical position of contact between
188 neighboring cells plasma membranes) (Figure 4B). We found that most PATJ proteins were about 80 nm
189 away from the TJ (Figure 4C). Although PATJ molecular structure is not known, given its sequence including
190 multiple potent unstructured domains, it is likely to be a globular protein, which size cannot fill the 80 nm
191 gap we find, with the nanometer-sized proteins of the TJ. Therefore, our data suggest that most PATJ
192 molecules do not interact directly with TJ proteins. We found instead most PATJ proteins close to the apical
193 membrane and that only a small fraction was present in microvilli or in the cytoplasm (Figure 4D). Previous
194 observations that PATJ associate with ZO-3 or Claudin1 might depend on the cellular state or these
195 interactions could be transient.

196 CRB3A is thought to anchor PALS1 and PATJ to the plasma membrane. However, given our results
197 showing a minor colocalization of PATJ and PALS1 with CRB3A, it is unlikely to be the case for most PALS1

198 and PATJ molecules. Therefore, the localization of PATJ close to the apical membrane led us to wonder
199 whether PATJ together with PALS1 could be associated with the apical plasma membrane via interactors
200 that remain to be discovered. Thus, we measured the distance of the immunogold label of PATJ to the
201 plasma membrane (Figure 4E) and found that the distance of the gold label is in most cases compatible
202 with the association of PATJ and PALS1 with the apical plasma membrane (123/169 \approx 73% of gold particles
203 were less than 38 nm away from the plasma membrane, corresponding to the size of the primary and gold-
204 labelled secondary antibody combination added with the size of PALS1). We conclude that PATJ and PALS1
205 are likely to be anchored to the apical membrane not by CRB3A but by yet unknown apical membrane
206 proteins.

207



208
209 **Figure 4.** Electron tomography shows that PATJ localize as clusters at the plasma membrane apically of the TJ in Caco-
210 2 cells. **(A)** Representative image of PATJ labelled with gold particles (arrowheads pointing at single particles or cluster
211 of particles). Bracket with TJ indicate the tight junction. Minimum intensity projection of a 150 nm thick tomogram,
212 scale bar: 100 nm. **(B)** Localization of gold particles labelling PATJ with respect to the TJ both in the apico-basal and
213 lateral directions. **(C)** Distance between the center of gold particle labels and the TJ. **(D)** Summary of gold particles
214 localization in the microvilli, in the vicinity of the plasma membrane and the cytoplasm. **(E)** Distance between gold
215 particles and the apical surface. In amber, the region of distances compatible with PATJ epitope being at the apical
216 surface, between 3 nm (radius of gold particles) and 37 nm (size of the primary and gold-labelled secondary antibody
217 combination added with the presumed size of PALS1 (Li et al., 2014)).

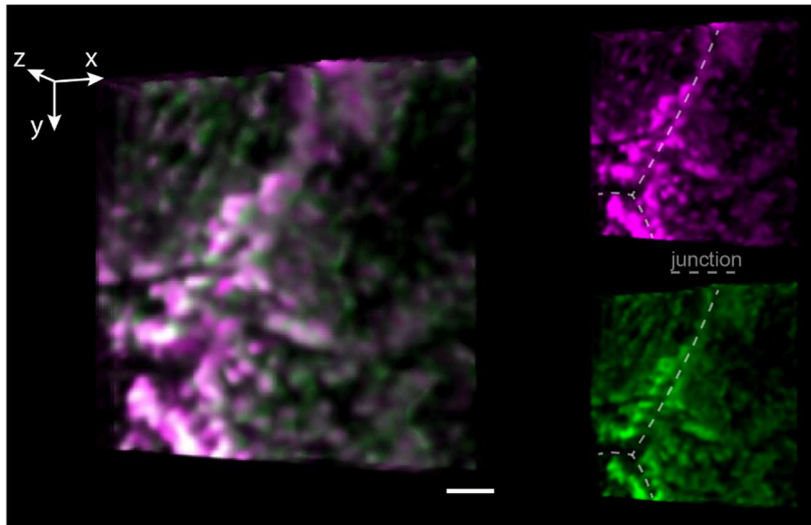
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219 Organization of PATJ-PALS1, PAR6 β -aPKC and the actin cytoskeleton

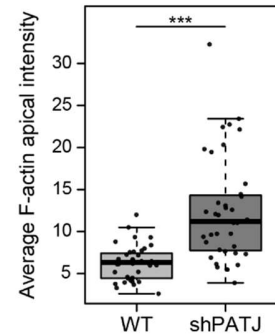
220 Because polarity proteins play a key role in epithelial organization, we wondered how these
221 proteins were organized with respect to the actin cytoskeleton. When labelling aPKC together with the
222 actin-staining phalloidin, we found with 3D STED that aPKC labelled actin in microvilli localized in the direct
223 vicinity of the junction (Figure 5A and supplement movie 1). Since we observed that PALS1-PATJ and
224 PAR6 β -aPKC complexes localize above the TJ in an alternated pattern (Figure 3A), and because of PATJ
225 localization (Figure 4D), it appeared that the labelling pattern corresponded to the alternation of PAR6 β -
226 aPKC at microvilli and PALS1-PATJ at the plasma membrane in between microvilli. As a result, the patterns
227 of PALS1-PATJ and PAR6 β -aPKC complexes seem to follow the organization of actin just above the TJ.

228 How PALS1-PATJ and PAR6 β -aPKC complexes interact with actin is unknown. As an attempt to
229 uncover a potential role of these complexes in the organization of actin in the area, we used a
230 downregulated PATJ stable clonal line of Caco-2 cells (clone 4 from (Michel et al., 2005)) and mixed them
231 with WT Caco-2 cells to compare protein localizations in both cell types grown in the same conditions. We
232 and others have already shown that the depletion of PATJ impairs the TJ and depletes both PALS1 and
233 CRB3 from the TJ region (Michel et al., 2005; Shin et al., 2005). In the apical part of WT Caco-2 cells, actin
234 is present in microvilli and in an apparent belt at the adherens junction level (Mangeol et al., 2019). In
235 contrast, we found in PATJ KD cells that the distribution of apical actin was strongly affected (Figure 5B).
236 In downregulated PATJ cells, the intensity of apical actin was doubled on average in comparison to WT
237 cells (Figure 5C). Moreover, while the actin belt was easy to identify in WT cells, it was sometimes difficult
238 to discern it in PATJ downregulated cells. Similarly, aPKC intensity was increased towards the apical
239 membrane in many PATJ knock-down cells (Figure 5D). These results show that PATJ influences the
240 regulation of the actin cytoskeleton organization in the apical region of Caco-2 cells.

A F-actin aPKC



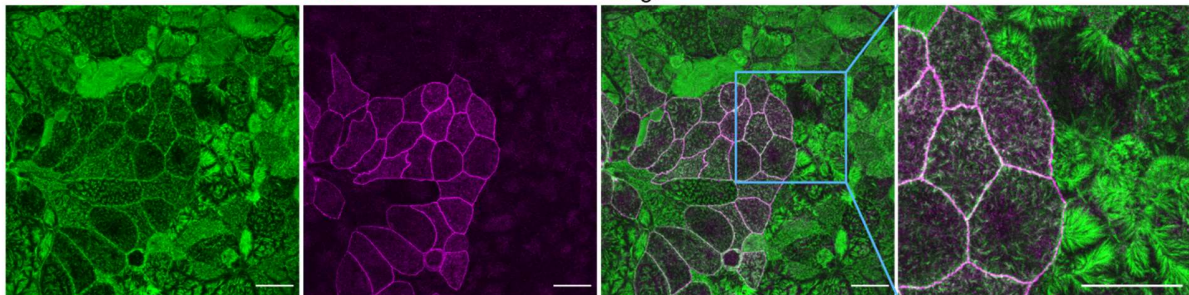
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B F-actin

PATJ

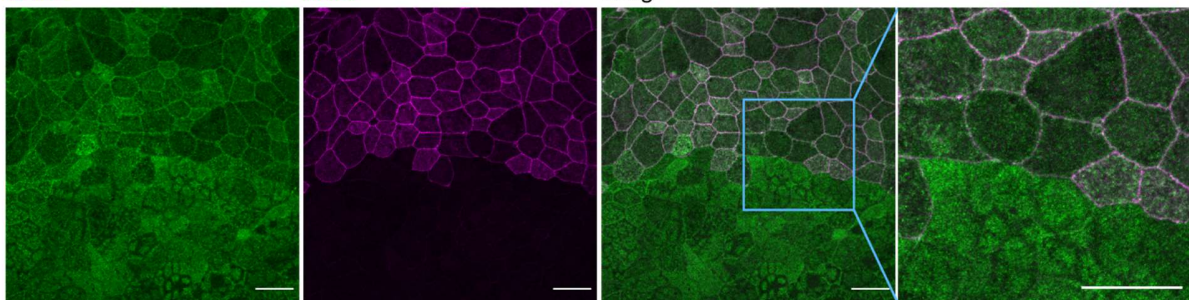
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D aPKC

PATJ

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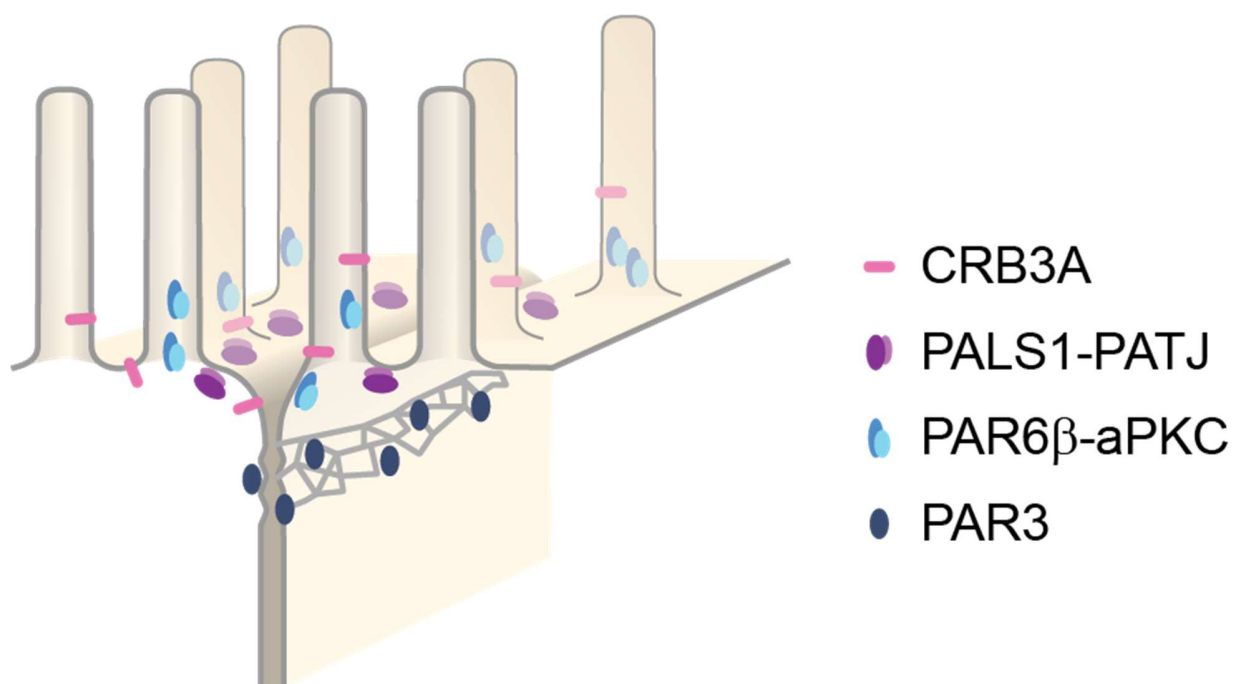


241

242 **Figure 5.** Actin organization and PATJ-PALS1 / aPKC-PAR6 β complexes. (A) Localization of aPKC with respect to F-
243 actin. 3D rendering of a 3D STED image stack extracted from the supplemental movie 1. The orientation of this image
244 is slightly tilted from the planar orientation to ease visualization. aPKC in magenta, phalloidin staining in green. Scale
245 bar (for the merged color image) 1 μ m. (B-D) Effect of PATJ depletion on the organization of apical actin and aPKC.
246 To evaluate the effect of PATJ depletion, we used a mix of WT and KD PATJ Caco-2 cells. (B) Effect of PATJ depletion
247 on the actin organization at the apical surface (PATJ in magenta, phalloidin staining in green). (C) Quantification of
248 the average apical intensity in WT versus shPATJ cells. Junctions were excluded from the analysis. Because of the
249 non-normality of data, we used the Wilcoxon rank sum test to test for the difference of median between WT and
250 shPATJ samples average apical phalloidin staining intensity; we obtained p-value = 7.9e-09. (D) Effect of PATJ
251 depletion on aPKC organization at the apical surface (PATJ in magenta, aPKC staining in green). Scale bars 20 μ m.

252 Discussion

253 In this study, we have systematically localized polarity proteins with super-resolution microscopy in
254 epithelial cells. We observed endogenous PAR3, aPKC, PAR6 β , PATJ, PALS1 and CRB3A in human intestine
255 and Caco-2 cells, and PAR3 and PALS1 in mouse intestine. We found the following (Figure 6). **(1)** All these
256 polarity proteins organize as submicrometric clusters concentrated in the TJ region. PAR3 localizes at the
257 TJ, aPKC and PAR6 β localize at the tight junction level, but mostly apically of the TJ, while PATJ, PALS1 and
258 CRB3A are apical of the TJ (Figures 1,2). **(2)** PAR6 β -aPKC and PATJ-PALS1 form two pairs that are often
259 respectively found in the same clusters (Figure 3A), strongly indicating that these respective proteins form
260 a stable and major complex in this region of the cells (*i.e* the PAR6-aPKC complex and the PALS1-PATJ
261 complex). **(3)** Unexpectedly, PALS1-PATJ and PAR6 β -aPKC clusters are segregated from each other (Figure
262 3A). Our data suggest that the PAR6 β -aPKC complex is localized at the base of the first row of microvilli in
263 the direct vicinity of the TJ, whereas PALS1-PATJ is localized between the TJ and these microvilli, as well as
264 in between these microvilli (Figure 4,5). This direct link between actin organization and polarity protein
265 localization led us to probe the effect of PATJ on actin organization. **(4)** We found that PATJ regulates the
266 organization of filamentous actin in the area, as the depletion of the PATJ affects both microvilli and the
267 apical actin belt (Figure 5). **(5)** CRB3 shows little association with any of the other polarity proteins (Figure
268 3B), questioning how PALS1-PATJ and PAR6 β -aPKC are mechanistically recruited to the plasma membrane
269 and localized to the apical surface.



270

271 **Figure 6.** Organizational model of polarity proteins in the TJ region.

272

273 Previous studies were largely based on biochemical approaches. The first interactions found
274 defined canonical polarity protein complexes, while subsequent studies highlighted the numerous
275 potential interactions that can be found with such approach, between polarity proteins of different
276 complexes, (Assémat et al., 2008; Bhat et al., 1999; Hurd et al., 2003; Joberty et al., 2000; Lemmers et al.,
277 2004; Lin et al., 2000; Makarova et al., 2003; Roh, Makarova, et al., 2002) as well as between polarity
278 proteins and other interactors (Chen & Macara, 2005; Itoh et al., 2001; Médina et al., 2002; Michel et al.,
279 2005; Roh, Liu, et al., 2002; Takekuni et al., 2003; Tan et al., 2020). Altogether these studies provide a
280 complex potential model of molecular interactions. However, in most cases, we do not know to what
281 extent and where these interactions do occur in cells and whether they are transient or permanent.
282 Notably, most of these previous studies used overexpression to identify the interactors of a given protein;
283 this methodological limitation might have introduced false-positive in some cases. In an attempt to reduce
284 the complexity of the current view, our study proposes a snapshot in the mature intestinal epithelia to
285 simultaneously localize endogenous proteins two-by-two with unprecedented spatial resolution. Our
286 results may bring a new light to the understanding of polarity proteins interactions, as it defines polarity
287 complexes as they occur in the apical epithelial junction region. In particular, we question the existence of
288 the canonical Crumbs and PAR complexes as previously described and propose that only PAR6 β -aPKC and
289 PALS1-PATJ can be defined as major structural complexes. The other numerous possible interactions that
290 have been claimed previously may exist transiently and our approach cannot rule out that they occur at
291 other locations in the cell, but it questions their relevance to the understanding of the epithelia cell
292 junction.

293 The interaction between PAR3, PAR6 and aPKC is key to epithelial polarization (Horikoshi et al.,
294 2009; Joberty et al., 2000) but the permanence of these interactions has been discussed in the past. In
295 mammalian epithelial cells, PAR3, PAR6 and aPKC have been thought to interact at apical junctions as
296 these proteins concentrate there, but only PAR6 and aPKC are found at the apical surface (Martin-

297 Belmonte et al., 2007; Satohisa et al., 2005). Moreover in a few non-mammalian systems, PAR3 was
298 observed as segregated from PAR6 and aPKC at epithelial apical junctions: when observed with confocal
299 microscopy, PAR3 is clearly basal of PAR6 and aPKC in the apical junctions of *Drosophila melanogaster*
300 embryos during cellularization (Harris & Peifer, 2005), as well as in chick neuroepithelial cells (Afonso &
301 Henrique, 2006). Our data suggest that the segregation of PAR3 from PAR6-aPKC is likely to be a conserved
302 principle of organization in polarized epithelia. Even if the interaction of PAR3 with PAR6-aPKC is central
303 to polarization, it is not permanent. The mechanistic basis for the transient character of the interaction
304 between PAR3 and PAR6-aPKC in mammalian epithelia may be similar to the Cdc-42-dependant
305 mechanisms found in *Drosophila melanogaster* (Morais-de-Sá et al., 2010) or *Caenorhabditis elegans*
306 (Rodriguez et al., 2017).

307 Our finding that PAR3 localizes at the TJ confirms previous observations using electron microscopy
308 in rat small intestine (Izumi et al., 1998) and MDCK cells . One recent study found a small fraction of PAR3
309 at the level of the adherens junction (Tan et al., 2020). Even though STED allows for much larger volume
310 to be probed compared to electron microscopy, we did not observe PAR3 basal of the TJ. The localization
311 of PAR3 may depend on the cell type as well as its maturation state, but interestingly PAR3 is never found
312 in the region apical of the TJ, where we find the other polarity proteins.

313 Because CRB3 is a transmembrane protein and that several studies reported its interaction with
314 PALS1, it was thought to anchor PALS1 and PATJ to the apical membrane (Makarova et al., 2003; Roh,
315 Makarova, et al., 2002). Similarly, it is suggested in *Drosophila melanogaster* that Crb recruits PAR6 and
316 aPKC to the apical membrane (Morais-de-Sá et al., 2010). Our study suggests that the recruitment of
317 PALS1, PATJ, PAR6 β , and aPKC to the plasma membrane is unlikely to be due to CRB3A, because CRB3A
318 poorly colocalizes with these proteins. Nevertheless, our data suggest that PALS1-PATJ are localized at the
319 plasma membrane, perhaps confined in this area by another set of interactors to be uncovered. This last
320 observation is likely to be similar for PAR6-aPKC. We cannot rule out both for PALS1-PATJ and PAR6 β -aPKC

321 that the interaction with CRB3A could be transient, and that this transient interaction would be sufficient
322 to localize these proteins complexes in the apical surface area.

323 The importance of polarity proteins for the epithelial organization point at the fact that these
324 proteins are likely to play a key role in the organization of the cytoskeleton. Several proteins having a role
325 in actin regulation have been shown to interact with polarity proteins (Bazellières et al., 2018; Médina et
326 al., 2002), but how polarity protein could influence actin organization is largely unknown. The correlation
327 of organization between the actin cytoskeleton and PAR6-aPKC and PALS1-PATJ clusters points at a
328 potentially structural role of these proteins to the cytoskeleton organization. These findings call for further
329 investigations, including functional and structural approaches.

330 In this study, we define endogenous polarity protein organization and how polarity protein are
331 likely to interact. The early concept of polarity protein complexes introduced by biochemical studies is
332 impractical today because of the very large number of potential interactions between proteins discovered.
333 Additionally, it omits important features, such as the dynamics of interaction as well as their reality in
334 relation to cell sub-regions. Our study proposes a snapshot of the polarity organization in mature intestinal
335 epithelial cells that calls for novel, more dynamic definition of interactions between polarity proteins and
336 associated proteins that will be needed to uncover the mechanistic basis of cell apico-basal polarization.

337 [Materials and Methods](#)

338 [Cell culture](#)

339 A clone of Caco-2 cells, TC7, was used in this study because differentiated TC7 cells form a regular
340 epithelial monolayer (Chantret et al., 1994). Cells were seeded at a low concentration of 10^5 cells on a 24
341 mm polyester filter with 0.4 μm pores (3450, Corning inc., Corning, NY). Cells were maintained in
342 Dulbecco's modified Eagle's minimum essential medium supplemented with 20% heat-inactivated fetal

343 bovine serum and 1% non-essential amino acids (Gibco, Waltham, MA), and cultured in 10% CO₂/90% air.
344 The medium was changed every 48 hours.

345 *Sample preparation for immunostaining*

346 *Human sample preparation*

347 Human intestine biopsies were obtained under the agreement IPC-CNRS-AMU 154736/MB.
348 Intestinal samples were fixed in paraformaldehyde (PFA 32%, Fischer Scientific) 4% in phosphate buffer
349 saline (PBS, Gibco, Waltham, MA) for 1 hour at 20°C. Biopsies were embedded in optimal cutting
350 temperature compound (OCT compound, VWR) and frozen in liquid nitrogen.

351 *Mouse sample preparation*

352 Mouse intestine samples were obtained following ethical guidelines. After washing with PBS
353 intestinal samples were fixed in PFA 4% in PBS for 20 minutes at room temperature. Samples were then
354 embedded in OCT compound and frozen in liquid nitrogen.

355 *Cell culture preparation for optical microscopy*

356 Cells were washed in PBS and then fixed in PFA 4% in PBS for 20 minutes at room temperature.
357 When apico-basal orientation observations were needed, cells were sectioned along the apico-basal axis.
358 Prior sectioning, cells were embedded in OCT compound and frozen in liquid nitrogen.

359 *Samples sectioning*

360 When needed, samples were sectioned with a cryostat (Leica CM 3050 S, Leica Biosystems,
361 Germany). 10 µm sections were transferred to high precision 1.5H coverslips (Marienfeld, Germany)
362 previously incubated with Poly-L-lysine solution (P-4832, Sigma-Aldrich, St. Louis, MO).

363 Immunostaining for optical microscopy

364 Intestinal sections and cultured cells were prepared similarly. Intestinal sections were
365 permeabilized in 1% SDS (Sigma-Aldrich) in PBS for 10 minutes. In cultured cells, 10 minutes
366 permeabilization was achieved with 1% SDS in PBS for CRB3A antibody, as well as PAR6 β and aPKC
367 antibodies when used in combination with tight junction markers; otherwise all other protein labelling
368 were using 1% Triton X100 (Sigma-Aldrich) in PBS permeabilization for 10 minutes. After washing with PBS,
369 samples were saturated with 10% fetal bovine sera (Gibco) in PBS (“saturation buffer”) over an hour at
370 room temperature. Primary antibodies were diluted in the saturation buffer and incubated overnight at
371 4°C. In more details: rabbit anti-ZO-1 (1/500, 61-7300, Invitrogen), mouse anti-occludin (1/500, 331500,
372 Invitrogen), mouse anti-E-cadherin (1/500, 610181, BD Biosciences), rabbit anti-PAR3 (1/200, 07-330,
373 Sigma-Aldrich), rabbit anti-PAR6 β (1/200, sc-67393, Santa-Cruz), rabbit anti-PKC ζ (1/200, sc-216,
374 SantaCruz), mouse anti-PKC ζ (1/200, sc-17781, SantaCruz), chicken anti-PALS1 (1/200, gift of Jan Wijnholds
375 (Kantardzhieva et al., 2005)), rabbit anti-PATJ (1/200, (Massey-Harroche et al., 2007; Michel et al., 2005)),
376 rat anti-CRB3A (1/50 MABT1366, Merck). Secondary antibodies were incubated 1 hour at room
377 temperature. Alexa Fluor 568 conjugated to antibodies raised against mouse, rabbit and rat and Alexa
378 Fluor 532 conjugated to antibodies raised against mouse and rabbit (Invitrogen) were used at 1/200
379 dilution in the saturation media. Phalloidin Alexa Fluor A532 (Invitrogen) was mixed with secondary
380 antibodies and used at 1/100 dilution. After each incubation, samples were rinsed 4 times with PBS.
381 Samples were finally mounted in Prolong Gold antifade mountant (Invitrogen) at 37°C for 45 minutes.

382 STED microscopy

383 Images of samples were acquired with a STED microscope (Leica TCS SP8 STED, Leica Microsystems
384 GmbH, Wetzlar, Germany), using a 100X oil immersion objective (STED WHITE, HC PL APO 100x/1.40, same
385 supplier). Two-color STED was performed with Alexa Fluor 532 excited at 522 nm (fluorescence detection
386 in the 532-555 nm window), and Alexa Fluor 568 excited at 585 nm (fluorescence detection in the 595-646

387 nm window). To minimize the effect of drifts on imaging, both dyes were imaged sequentially on each line
388 of an image and depleted using the same 660 nm laser. Detection was gated to improve STED signal
389 specificity.

390 Cultured cell preparation for electron microscopy

391 Cells were washed in PBS and then fixed in PFA 4% in PBS for 20 minutes at room temperature.
392 After rinsing with PBS, cells were put into a sucrose gradient to reach 30% sucrose overnight. Cells were
393 then frozen in liquid nitrogen and immediately thawed at room temperature. Immunostaining was carried
394 out without permeabilization step, directly with primary antibodies (rabbit anti-PATJ 1/100, for 3 hours at
395 room temperature). After washing steps, cells were incubated with secondary antibody carrying 6 nm gold
396 particles (goat anti-rabbit 1/20, 806.011, Aurion, The Netherlands). A tertiary antibody was used to
397 observe where gold particles were localized on a macroscopic level (Alexa 568 conjugated donkey anti-
398 goat 1/200 from Invitrogen, for 1 hour at room temperature).

399 Cells were then prepared specifically for electron microscopy. They were fixed in 2.5%
400 glutaraldehyde, 2% PFA, 0.1% tannic acid in sodium cacodylate 0.1M solution for 30 minutes at room
401 temperature. After washing steps, cells were post-fixed in 1% osmium in sodium cacodylate 0.1M solution
402 for 30 minutes at room temperature and contrasted in 2% uranyl acetate in water solution for 30 minutes
403 at room temperature. Cells were then dehydrated in ethanol and embedded in Epon epoxy resin.

404 Electron microscopy

405 Cells were observed with a transmission electron microscope, FEI Tecnai G2 200 kV (FEI, The
406 Netherlands), in an electron tomography mode. Tomograms were reconstructed using the Etomo tool of
407 the IMOD software.

408 Data analysis

409 *Analysis of protein density*

410 To quantify the density and positions of polarity proteins with respect to tight junction markers,
411 we used custom-made ImageJ macros and Python programs. In each case the reference protein was a tight
412 junction protein (ZO-1 or occludin) that was localized precisely, defining a reference position along the
413 junction from which intensity measurement was done. For planar orientations, the reference was the
414 maximum intensity of the tight junction marker along of the junction; intensity measurements consisted
415 in getting the intensity profiles of proteins perpendicular to the junction, all along the junction. For apico-
416 basal orientations, we measured intensity profiles on the apico-basal axis, all along the junction. On a given
417 profile, the reference was taken at the most apical point where the tight junction marker intensity was a
418 third of its maximum intensity; the reason for this choice is that tight junctions spread along the apico-
419 basal axis tended to vary up to three-fold from one cell to another and this definition of the reference
420 allowed us to define a reproducible apical edge of the tight junction. In the process, we used bilinear
421 interpolation to obtain sub-pixel quantification. Results of analyses were then normalized for intensity for
422 each junction to avoid junction-to-junction intensity variation. Because we used a reference protein for
423 each junction, we could then align all results based on the reference position of the reference protein and
424 pool all results into a single protein density plot.

425 *Protein-proximity analysis*

426 The principle of quantification of protein-proximity was proposed in (Wu et al., 2010). The authors of this
427 method observed that the autocorrelation of a given image or the cross-correlation between two images
428 coming from two different channels showed a peak at its center. The ratio of amplitude between the peaks
429 of the cross-correlated and autocorrelated images gave a good estimate of protein proximity, which they
430 coined the protein-protein proximity index. This index is similar to more classical colocalization

431 coefficients, but we found that the method of (Wu et al., 2010) was well suited for proteins distributed
432 along a junction.

433 In practice, we extracted junctions from two-color images, restricting the analysis to a band of 400 nm
434 centered on the reference given by the tight junction (as defined in the previous paragraph). As we found
435 the analysis to be dependent on orientation, when planar orientation was used, we excluded junctions
436 that were not straight. All extracted junctions of a given protein pair to be examined were then
437 concatenated into one large two-channel image on which we achieved autocorrelation and cross-
438 correlation analysis (autocorrelation is achieved on each channel, and cross-correlation is achieved with
439 both channels). We extracted the amplitude of peaks obtained in each of the autocorrelated and cross-
440 correlated images as proposed in (Wu et al., 2010). Therefore, when analyzing protein 1 and protein 2
441 proximity, we obtain the amplitude A_1 and A_2 from the autocorrelation of images of protein 1 and protein
442 2 respectively, and the amplitude C_{12} from the cross-correlation analysis. One evaluates the fraction of
443 protein 1 colocalizing with protein 2 with the protein-protein proximity index $P_1 = C_{12}/A_2$, and the fraction
444 of protein 2 colocalizing with protein 1 with the protein-protein proximity index $P_2 = C_{12}/A_1$.

445 In figure 2 we color coded the values of these indices. In order to obtain an absolute representation of
446 these values, we additionally used Venn diagrams to represent graphically for each protein the fraction of
447 colocalizing and non-colocalizing protein.

448

449 Number of junctions or cells used in the analysis

450 **Figure 1 and 2**

451 Number of junctions used in the analysis. PI: planar, AB: apico-basal

Sample	PAR3 Occl		aPKC Occl		PAR6 β Occl		PATJ Occl		PALS1 ZO-1		CRB3A ZO-1	
	PI	AB	PI	AB	PI	AB	PI	AB	PI	AB	PI	AB
Caco-2	29	8	16	9	18	11	33	8	12	9	16	12
Human	25	7	16	10	12	12	47	16	23	10	12	8
	PAR3 Occl								PALS1 Occl			
Mouse	10	9							10	8		

452

453 **Figure 3**

Label	PATJ PALS1	PATJ aPKC	PALS1 aPKC	PALS1 PAR6 β	PAR6 β aPKC	CRB3A PALS1	CRB3A PATJ
Number of junctions	21	42	36	27	31	25	25
Label	CRB3A aPKC	CRB3A PAR6 β	CRB3A PAR3	CRB3A ZO-1	PAR3 PALS1	PAR3 aPKC	PAR3 OCLN
Number of junctions	17	18	9	16	9	27	15
Label	PATJ-Alexa568 PATJ-Alexa532						
Number of junctions	15						

454

455 **Figure 4**

456 Tomograms of 300 nm in thickness of 12 junctions were used to extract the position of 169 gold particles

457 labelling PATJ proteins.

458 **Figure 5**

459 Number of cells used in Figure 4B quantification: 39 WT cells and 40 PATJ downregulated cells (from one

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