1   **Interplay between cortical actin and E-cadherin dynamics regulates cell shape in the**

2   ***Drosophila* embryonic epidermis.**

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11 **Abstract**

12 Precise regulation of cell shape is vital for building functional tissues. Here, we study the

13 mechanisms which lead to the formation of highly elongated anisotropic epithelial cells in the

14 *Drosophila* epidermis. We demonstrate that this cell shape is the result of two counteracting

15 mechanisms at the cell surface: actomyosin, which inhibits cell elongation downstream of

16 RhoA signalling, and intercellular adhesion, modulated via clathrin-mediated endocytosis of

17 E-cadherin, which promotes cell elongation downstream of the GTPase Arf1. We show that

18 these two mechanisms are interconnected, with RhoA signalling activity reducing Arf1

19 recruitment to the plasma membrane. Additionally, cell adhesion itself regulates both

20 mechanisms: p120-catenin, a regulator of intercellular adhesion, promotes the activity of both

21 Arf1 and RhoA. Altogether, we uncover a complex network of interactions between cell-cell

22 adhesion, the endocytic machinery, and the actomyosin cortex, and demonstrate how this

23 network regulates cell shape in an epithelial tissue *in vivo*.

24

Introduction

The structure of all biological organisms is the product of the tissues of which they are composed. The morphogenesis of these tissues requires precise control over the shape of individual cells. In epithelial tissues, which outlines all cavities and surfaces of animal bodies, a variety of shapes and functions is observed. Cell shape is determined by mechanical properties, which define cell geometry based on intracellular and intercellular forces (Chalut and Paluch, 2016; Lecuit and Lenne, 2007). At the cell surface, mechanical properties are determined by an interplay of two factors: cortical actin and intercellular adhesion (Lecuit and Lenne, 2007; Winklbauer, 2015).

The first factor, cortical actin, is a meshwork of actin filaments crosslinked by specific cross-linking proteins and myosin motors (Chugh and Paluch, 2018). Myosin motors, such as non-muscle Myosin II (MyoII), generate contractile forces and cortical tension, which acts to minimize the contact area between cells (Blankenship et al., 2006; Clark et al., 2014). The control of cortical contractility is required to establish and maintain specific cell shapes. One of the best documented regulators of cortical contractility is the GTPase RhoA (Spiering and Hodgson, 2011). A key effector of RhoA is the enzyme Rho-Kinase (Rok), which is recruited to membranes by the activated form of RhoA, where it phosphorylates the myosin light chain, leading to activation of MyoII and an increase of actin contractility (Amano et al., 2010; Kawano et al., 1999; Kureishi et al., 1997; Leung et al., 1995).

The second factor, intercellular adhesion, is the property of one cell binding to it neighbours using specialized proteins on its surface. In epithelia, this is mediated by Adherens Junctions (AJs), with the principle component being E-cadherin (E-cad), a transmembrane protein which binds to E-cad molecules on adjacent cells (Takeichi, 1977; van Roy and Berx, 2008). Intercellular adhesion often opposes cortical tension by increasing the contact surface between cells (De Vries et al., 2004; Lecuit and Lenne, 2007), and its

50   strength is proportional to both the levels and dynamics of E-cad at the cell surface (Foty and

51   Steinberg, 2005; Troyanovsky et al., 2006). The latter largely relies on the processes of

52   endocytosis and recycling, which constantly remodel AJs (Kowalczyk and Nanes, 2012).

53   The p120-catenin (p120ctn) protein family are the key regulators of E-cad

54   endocytosis in mammalian cells, through directly binding the juxtamembrane domain of E-

55   cad (Daniel and Reynolds, 1995; Garrett et al., 2017; Ireton et al., 2002; Oas et al., 2013;

56   Shibamoto et al., 1995; Yap et al., 1998; Yu et al., 2016). This catenin family is represented

57   by a single gene in invertebrates, such as *Drosophila*, whereas p120ctn has 7 members in

58   humans with different expression patterns and functional requirements, reflecting an increase

59   in tissue types and complexity (Carnahan et al., 2010; Gul et al., 2017; Hatzfeld, 2005). Most

60   studies have focused on the founding family member, p120ctn, however other members, δ-

61   catenin and ARVCF, seem to have similar functions (Davis et al., 2003). In mammalian cells,

62   p120ctn is required to maintain E-cad at the plasma membrane: uncoupling p120ctn from E-

63   cad or reducing expression results in complete internalization of E-cad (Davis et al., 2003;

64   Ireton et al., 2002; Ishiyama et al., 2010), as the binding of p120ctn to E-cad conceals

65   endocytosis triggering motifs (Nanes et al., 2012; Reynolds, 2007). This model of p120ctn

66   activity has recently been augmented in mammalian cells, when it was found that p120ctn

67   can also promote endocytosis of E-cad through interaction with Numb (Sato et al., 2011).

68   By contrast, in *Drosophila* and *C. elegans,* p120ctn was thought to play only a

69   supporting role in adhesion as genetic ablation failed to replicate the effects observed in

70   mammalian systems (Myster et al., 2003; Pacquelet et al., 2003; Pettitt et al., 2003). This was

71   thought to be due to the greater similarity of invertebrate p120ctn to mammalian δ-catenin,

72   ablation of which is similarly viable in mice (Carnahan et al., 2010; Israely et al., 2004).

73   However, δ-catenin expression is restricted to neural and neuroendocrine tissues (Ho et al.,

74   2000), which is likely to explain the mildness of knockout phenotypes, whereas invertebrate

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75   p120ctn is broadly expressed in both epithelia and neurons (Myster et al., 2003), suggesting

76   potential functional similarity with mammalian p120ctn which shares the broad expression

77   pattern (Davis et al., 2003). Furthermore, it has recently been reported that *Drosophila*

78   p120ctn is required to stabilize E-cad in the pupal wing (Iyer et al., 2019) and promotes the

79   endocytosis and recycling of E-cad in the embryo and larval wing discs (Bulgakova and

80   Brown, 2016). This indicates an evolutionary conservation of p120ctn function, where

81   depending upon the context, p120ctn either inhibits or promotes E-cad endocytosis.

82   Another important protein family regulating E-cad endocytosis is the Arf GTPases,

83   which recruit coat proteins to facilitate the intracellular trafficking of vesicles (Donaldson

84   and Jackson, 2011). The first member of the family, Arf1, is classically viewed as Golgi

85   resident and responsible for anterograde transport from the Golgi to the plasma membrane

86   (Donaldson and Jackson, 2011; McMahon and Boucrot, 2011). Recently, however, Arf1 was

87   detected at the plasma membrane, where it participates in trafficking by co-operating with

88   Arf6-dependent endocytosis (Humphreys et al., 2013; Padovani et al., 2014). In *Drosophila*,

89   Arf1 is required for facilitating endocytosis in the early syncytial embryo (Humphreys et al.,

90   2012; Lee and Harris, 2013; Rodrigues et al., 2016). Indeed, Arf1 interacts with E-cad and

91   another component of AJs, Par-3 (Shao et al., 2010; Toret et al., 2014).

92   Cortical actin and intercellular adhesion do not exist in isolation but are intimately

93   interconnected. Intracellularly E-cad interacts with other catenin proteins: at its distal C-

94   terminus E-cad binds β-catenin, with α-catenin binding β-catenin and actin, thus directly

95   linking E-cad to cortical actin (Ozawa et al., 1990; Shapiro and Weis, 2009). In addition to

96   direct linkage, cortical actin and intercellular adhesion share common regulators. In in

97   mammalian epithelial cells, RhoA localizes to AJs, where E-cad adhesion complexes create

98   local zones of active RhoA by recruiting Ect2 GEF through α-catenin (Priya et al., 2013;

99   Ratheesh et al., 2012). In these cells, p120ctn has a context-dependent role in RhoA

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100   regulation: it can directly inhibit RhoA, indirectly via p190RhoGAP, direct its spatiotemporal

101   activity, or activate it (Anastasiadis et al., 2000; Derksen and van de Ven, 2017; Lang et al.,

102   2014; Taulet et al., 2009; Yu et al., 2016; Zebda et al., 2013). The role of p120ctn in the

103   RhoA pathway in *Drosophila* is unclear (Fox et al., 2005; Magie et al., 2002). However,

104   RhoA itself regulates E-cad and is required for establishment of E-cad-mediated adhesion

105   (Braga et al., 1997). In *Drosophila*, RhoA promotes the regulated endocytosis of E-cad by

106   Dia and AP2 (Levayer et al., 2011b). Conversely, RhoA activity antagonises E-cad

107   endocytosis in the early embryo (Lee and Harris, 2013), indicating a context-dependent role

108   of RhoA in E-cad endocytosis. Finally, E-cad endocytosis can direct actin remodelling

109   (Hunter et al., 2015). However, how this interplay between cortical actin and intercellular

110   adhesion produces cell shape has yet to be fully elucidated.

111   Here, we investigated the interplay between cortical actin and E-cad-mediated

112   adhesion in the highly elongated cells of the late embryonic epidermis in *Drosophila.* We

113   demonstrate that this cellular elongation is accompanied by a reciprocal anisotropy of cortical

114   tension relative to E-cad adhesion. We determined that the prototypical member of the

115   p120ctn family is directly involved in shaping these cells. p120ctn achieves this by both

116   influencing the endocytosis of E-cad and by modulating cortical tension. We characterised

117   the mechanism of this p120ctn dual function and found that it is dependent on interactions

118   with two GTPase signalling pathways: RhoA to increase cortical tension and inhibit

119   endocytosis, and Arf1 to promote endocytosis and enable the remodelling of the AJs. Finally,

120   we show that the interplay between these two GTPase pathways downstream of p120ctn

121   directly participates in shaping the morphology of cells *in vivo*.

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125   **Results**

126   *1) Anisotropy of cortical actin and adhesion in the epidermis of stage 15 Drosophila*

127   *embryos*

128   The epidermal cells of stage 15 *Drosophila* embryos have a distinct rectangular morphology

129   in their apical plane (Fig. 1A-C). These cells are elongated in the direction of the dorsal-

130   ventral axis of the embryo and have the average aspect ratio of 6.1 ± 1.3 (ratio between

131   lengths of the long and short cell axes, mean ± SD, Fig. 1D). Consistent with the elongated

132   shape, these cells have two distinct types of cell border: long borders, which are orthogonal to

133   the anterior-posterior axis of the embryo (AP borders), and short borders, which are

134   orthogonal to the dorsal-ventral axis (DV borders, Fig. 1B-C).

135   These AP and DV cell borders differ in their actin cortex, with MyoII enriched at the

136   AP borders in these cells (AP:DV≅2:1, Fig 1E-F), consistent with previous reports

137   (Bulgakova et al., 2013; Simoes et al., 2010). As the accumulation of MyoII has been linked

138   to cortical tension (Priya et al., 2015; Scarpa et al., 2018; Yu and Fernandez-Gonzalez, 2016),

139   we compared tension between the AP and DV borders using microablation and measured the

140   initial recoil as it is proportional to the tension in the system rather than other variables

141   (Liang et al., 2016; Mao et al., 2013). We used an E-cad tagged at its endogenous locus (E-

142   cad-GFP, Huang et al., 2009) to label cell outlines and quantify the initial recoil (Fig. 1G-H).

143   We found that while the initial recoil was positive for the AP borders, showing that they are

144   under tension, it was negative for the DV borders, suggesting that they are under compression

145   (Fig. 1H).

146   Further to the difference in MyoII distribution and tension properties, the AP and DV

147   borders display a substantial difference in the distribution of intercellular adhesion

148   components, specifically, an asymmetry in the levels and dynamics of E-cad. In these

149   differentiated cells E-cad localizes in a narrow continuous band of fully formed mature AJs

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150   (Adams et al., 1996; Tepass and Hartenstein, 1994). E-cad-GFP localized asymmetrically

151   with a 1:2 (AP:DV) ratio between the two cell borders (Fig. 1I, Table S1, and Bulgakova et

152   al., 2013). This asymmetry is due to an accumulation at the DV borders of a specific pool of

153   E-cad, which is highly dynamic due to its endocytic trafficking (Bulgakova et al., 2013).

154   Overall, the elongated shape of these epidermal cells coincides with anisotropies in both

155   cortical actin and tension, and the levels and dynamics of adhesion complexes. Therefore, we

156   investigated how the interplay between cortical tension and adhesion at the cell surface

157   produces cell shape, and how the interaction between these two processes is mediated.

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159   *2) p120ctn influences cell shape, RhoA signalling and cortical tension*

160   Members of the p120ctn family regulate both the actin cytoskeleton and cadherin trafficking

161   and are thus good candidates to mediate the interplay between cortical tension and cell

162   adhesion. In this vein, we investigated if the function of p120ctn in *Drosophila*, which have

163   only one member of the family, affected the shape of cells. We created a p120ctn

164   overexpression construct under the control of a *UAS* promoter (*UAS*::p120ctn) and expressed

165   it in the posterior half of each embryonic segment using the *engrailed*::GAL4 (*en*::GAL4)

166   driver while marking the cells using *UAS::*CD8-Cherry (Fig. 2A). To exclude potential

167   differences between the compartments we used an external control: an *engrailed*

168   compartment expressing two copies of *UAS::*CD8-Cherry to balance the Gal4:UAS ratio

169   (Fig. 2A). We complemented this analysis by using a *p120ctn* mutant to examine the effect of

170   p120ctn loss (Fig. 2A, and Bulgakova and Brown, 2016). The cells expressing *UAS*::p120ctn

171   appeared distorted and had a smaller aspect ratio (p<0.0001, Fig. 2A-B), indicative of cell

172   rounding. By contrast the loss of p120ctn did not change the aspect ratio (p=0.27, Fig. 2A,C),

173   consistent with the previous report (Bulgakova and Brown, 2016). Therefore, increased

174   p120ctn levels have a distinct effect on the shapes of cells in the *Drosophila* epidermis,

175   altering their apparent elongation and anisotropy.

176   A well-established regulator of cell shape is cortical actin, which is highly

177   anisotropic in epidermal cells and is regulated by RhoA-signalling. As p120ctn family

178   members have been reported to both positively and negatively regulate RhoA-signalling, we

179   examined the interaction between p120ctn and RhoA in our system. We did so by examining

180   its downstream effectors: the enzyme Rok and its target MyoII. p120ctn overexpression

181   increased the amount of MyoII-YFP at the AP borders (p=0.006, Fig. 2D-E). Conversely, in

182   the *p120ctn* mutant embryos MyoII-YFP was reduced specifically at the AP borders (p=0.01,

183   Fig. 2D,F). The amount of MyoII-YFP at the DV borders were not affected in both cases

184   (p=0.47 and p=0.45, Fig. 2E-F). We complemented these experiments by using a tagged

185   kinase-dead variant of Rok (Venus-Rok^{K116A}, referred to as Rok-Venus, Simoes et al., 2010),

186   previously used as a readout of Rok localization and activity (Bulgakova et al., 2013; Simoes

187   et al., 2010). Rok-Venus had the same localization and was affected in the same way as

188   MyoII-YFP (compare Fig. S1 with Fig. 2E-F). p120ctn loss abolished Rok-Venus asymmetry

189   due to a reduction at the AP borders, and overexpression increased Rok-Venus at the AP

190   borders (p=0.013 and p=0.0043, respectively, Fig. S1). These results indicate that p120ctn

191   activates RhoA signalling specifically at the AP borders in a dose-dependent manner in these

192   epidermal cells.

193   Due to this positive correlation between p120ctn levels and RhoA signalling on

194   the long AP cell border, we decided to directly measure the cortical tension at the AP borders

195   in *p120ctn* mutant and overexpressing cells (Fig. 2G-H). The overexpression of p120ctn

196   increased the mean initial recoil (0.36 µm/sec in comparison to 0.13 µm/sec, p<0.0001, Fig.

197   2G-H). Conversely, in *p120ctn* mutant cells the recoil was decreased to a mean value of 0.07

198   µm/sec (p=0.022, Fig. 2G-H). This demonstrates that cortical tension correlates with p120ctn

199   levels. Overall, the changes in p120ctn levels altered the shape of the epidermal cells, with

200   the levels of p120ctn positively correlating with the activity of RhoA signalling and cortical

201   tension at the AP borders. The increased tension at the AP borders following p120ctn

202   overexpression is also consistent with the rounded cell phenotype. The DV borders displayed

203   no change in the activity of RhoA signalling, indicating an anisotropic positive action of

204   p120ctn on RhoA signalling.

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206   *3) p120ctn regulates E-cad amounts and dynamics within adhesion sites.*

207   The other factor which contributes to cell shape is intercellular adhesion which is mediated

208   by E-cad in epithelial cells. In our system this intercellular adhesion is also highly anisotropic

209   with an opposite pattern to the cortical actin (Bulgakova et al., 2013). p120ctn binds to the

210   intracellular domain of E-cad, enabling it to regulate the endocytosis of E-cad (Bulgakova

211   and Brown, 2016; Iyer et al., 2019; Nanes et al., 2012; Reynolds, 2007; Sato et al., 2011).

212   First, we determined that p120ctn in *Drosophila* co-localizes with E-cad. To test this, we

213   used ubiquitously expressed p120ctn tagged with GFP (*Ubi*::p120ctn-GFP) and found that it

214   localized to both AP and DV borders with an enrichment at the DV, mimicking the

215   localization of E-cad-GFP (r = 0.868, p <0.00001, Fig. 3A-B). Note that in this system, the

216   antibody against the N-terminus of p120ctn does not work and fails to reproduce the

217   localization of the full-length GFP tagged p120ctn (Fig. S2). Given this colocalization, we

218   then examined if changes in E-cad levels were observed when altering the levels of p120ctn.

219   In cells overexpressing p120ctn we detected a reduced asymmetry of E-cad-GFP due to an

220   increase in levels specifically at the AP but not DV borders (p=0.0002 and p=0.27, Fig. 3C-

221   D, and see Fig. 2A). In contrast, the loss of p120ctn resulted in an isotropic decrease in E-

222   cad-GFP amounts at both AP and DV borders by approximately 15% (p=0.008 and p=0.035,

223   Fig. 3E-F, see also Fig. 2A).

224   Next, we examined how this increase in the E-cad amount at the AP borders

225   correlated with p120ctn localization. We overexpressed a tagged p120ctn-GFP

226   (*UAS::*p120ctn-GFP) driven by *en::*GAL4 to compare p120ctn and E-cad localization in the

227   same cell. We detected an AP:DV ratio of 2:3 for both E-cad and p120ctn (Fig. 3G-I, Fig.

228   S2). The lower than usual AP:DV ratio of E-cad was similar to that observed when untagged

229   p120ctn was overexpressed. As this E-cad distribution was identical to the distribution of

230   p120ctn itself, we concluded that additional E-cad molecules are recruited as a complex with

231   p120ctn.

232   The strength of cell-cell adhesion as well as the amount of adhesion complexes at the

233   cell surface are regulated by endocytosis, and p120ctn has been shown to inhibit and promote

234   E-cad endocytosis in mammalian and *Drosophila* cells, likely reflecting context-dependent

235   functions (Bulgakova and Brown, 2016; Ireton et al., 2002; Nanes et al., 2012; Sato et al.,

236   2011; Xiao et al., 2003). Therefore, we decided to explore whether the changes in the levels

237   of E-cad are accompanied by changes in its dynamics. We used Fluorescence Recovery After

238   Photobleaching (FRAP), which reveals the stable fraction of the protein which does not

239   exchange within the sites of cell-cell adhesion on the time-scale of experiment, and the

240   mobile fraction. E-cad-GFP mobile fraction is 70% for DV and 50% for the AP borders in

241   control, with 30% and 50% of protein being stable, respectively (Fig. 3J-K). The recovery

242   curves which describe the dynamics of E-cad-GFP mobile fraction were best-fit by a bi-

243   exponential model (Table S1, and Bulgakova et al., 2013), with the fast and slow components

244   attributed to diffusion and endocytic recycling, respectively (Bulgakova et al., 2013; Iyer et

245   al., 2019; Lippincott-Schwartz et al., 2003).

246   Measuring the dynamics of E-cad-GFP in p120ctn overexpressing cells we discovered

247   that it was less dynamic at both border types (Fig. 3J-K). The mobile fractions were

248   approximately 40% and 30% for AP and DV borders respectively, which resulted in an

249   increase of the immobile fraction at both borders to 60% and 70% relative to control

250   (p=0.0023 and p<0.0001 Table S1). This is similar to changes in the dynamics of E-cad-GFP

251   in *p120ctn* mutants, where an increase in the immobile E-cad-GFP fraction is also observed

252   (Fig. S2, and Bulgakova and Brown, 2016). Therefore, while p120ctn overexpression

253   elevates E-cad level at the AP borders, and its loss uniformly reduces it, both lead to

254   stabilization of E-cad-GFP at cell junctions. Next, we sought to determine the mechanism of

255   this stabilization and how these changes in E-cad dynamics contribute to cell shape changes

256   caused by elevated p120ctn levels.

257

258   *4) p120ctn and RhoA regulate E-cad via clathrin-mediated endocytosis*

259   To ascertain if the stabilization of E-cad-GFP by altered p120ctn levels was due to an

260   impairment of endocytosis, we examined clathrin, which is the main coat protein involved in

261   clathrin-mediated endocytosis. We used the Clathrin Light Chain (CLC) tagged with GFP

262   (*UAS*::CLC-GFP, Loerke et al., 2005; Wu et al., 2001), to monitor clathrin behaviour in the

263   plane of AJs by performing FRAP. CLC tagged with GFP incorporates functionally into

264   clathrin-coated pits without interfering with basic clathrin functions (Chang et al., 2002;

265   Gaidarov et al., 1999; Kochubey et al., 2006). CLC-GFP expressed using the *en*::GAL4

266   driver was found in spots on the plasma membrane in the plane of AJs and in the cytoplasm

267   (Fig. 4A), a localization consistent with its function (Kaksonen and Roux, 2018). p120ctn

268   overexpression reduced the recovery of the CLC-GFP, with a reduction in the mobile fraction

269   by 30% relatively to control (p<0.0001, Fig. 4B). A similar reduction in CLC-GFP mobile

270   fraction by 25% was observed in *p120ctn* mutants (p<0.0001, Fig. 4C). These reflect the

271   changes in the mobile fraction of E-cad (Fig. 3J-K), suggesting that CLC-GFP recovery is a

272   valid proxy for E-cad dynamics in these cells.

273   As p120ctn overexpression resulted in both an increase of E-cad levels and

274   anisotropic activation of RhoA signalling, we asked whether this change in the RhoA

275   signalling was responsible for the changes in E-cad, for instance by modulating its

276   endocytosis. We sought to mimic the RhoA phenotype in *p120ctn* mutants by directly

277   inhibiting the RhoA pathway using a dominant negative (Rho^{DN}) construct. Rho^{DN}, expressed

278   constitutively using *en::GAL4*, resulted in a complete loss of E-cad at the membrane by stage

279   15 of embryogenesis, therefore, we acutely induced the expression of the Rho^{DN} using

280   ubiquitously expressed temperature sensitive GAL80^{ts} and a temperature shift for 4 hours at

281   12 hours after egg laying (Pilauri et al., 2005). This acute downregulation of RhoA signalling

282   reduced the amounts of E-cad-GFP at the AP but not DV borders (p=0.0078 and p=0.37, Fig.

283   4D-E). Note that in this case E-cad asymmetry was reduced in control cells, likely due to the

284   effects of temperature shift during induction of expression. In a complementary experiment,

285   we expressed a constitutively-active Rho^{CA} to elevate RhoA signalling directly (Fig. 4F). The

286   expression of Rho^{CA} increased the amounts of E-cad-GFP at the cell surface, specifically at

287   the DV but not the AP borders (p<0.0001 and p=0.34, Fig. 4G), which is consistent with an

288   ectopic activation of RhoA signalling at the DV borders in this case. We then asked if this

289   increase in E-cad could be explained by the stabilization of E-cad-GFP at the plasma

290   membrane by elevated RhoA signalling. To this end, we measured the dynamics of clathrin

291   in the plain of AJs using FRAP as a proxy for E-cad endocytosis. Indeed, the mobile fraction

292   of CLC-GFP was reduced by 40% in comparison to control (p<0.0001, Fig. 4H-I). In cells

293   overexpressing p120ctn, increase of E-cad at the AP borders was accompanied by increased

294   activation of RhoA signalling. Overall, these data suggest that p120ctn leads to activation of

295   RhoA signalling at the AP borders, which elevates E-cad most likely by preventing its

296   endocytosis at these borders.

297

298   *5) The localization of the GTPase Arf1 at the cell plasma membrane depends on p120ctn*

299   *and RhoA, and promotes clathrin-mediated endocytosis*

300   Elevated RhoA signalling resulted in E-cad stabilization at the cell surface and inhibited

301   clathrin-mediated endocytosis, however E-cad was also stabilized when RhoA signalling was

302   downregulated in *p120ctn* mutants. Therefore, we next sought to identify molecules which

303   were responsible for this E-cad stabilization in *p120ctn* mutants. The GTPase Arf1 has been

304   reported to interact with E-cad and other AJ components (Shao et al., 2010; Toret et al.,

305   2014). Therefore, we examined if Arf1 acts downstream of p120ctn using a transgenic GFP-

306   tagged variant of Arf1 (*UAS*::Arf1-GFP, Lee and Harris, 2013) driven by *en::*GAL4.

307   *UAS*::Arf1-GFP has reduced affinity for ArfGAPs and ArfGEFs, and nucleotide exchange

308   rate (Jian et al., 2010), which allowed us to study Arf1 without hyperactivating the pathway.

309   *UAS*::Arf1-GFP localized to both the Golgi apparatus and plasma membrane (Fig. 5A,

310   Fig. S3), consistent with previous reports (Lee and Harris, 2013; Shao et al., 2010). The

311   Golgi-resident Arf1 appeared in large and distinct Arf1-GFP puncta throughout the

312   cytoplasm including in close proximity to the plasma membrane (Fig. 5A, Fig. S3). As we

313   were interested in the localization of Arf1 at the plasma membrane, we excluded the puncta

314   from our analysis (see Materials and Methods). In the control, Arf1-GFP localization at the

315   plasma membrane was uniform (p=0.36, Fig. 5C). The loss of p120ctn resulted in a uniform

316   decrease in the amount of Arf1-GFP at both the AP and DV borders (p<0.0001 and

317   p<0.0001, Fig. 5B-C), suggesting that p120ctn promotes Arf1 localization.

318   Considering this role of p120ctn in Arf1 localization and the known function of Arf1

319   in intracellular trafficking, we decided to test if the reduction in Arf1 activity is responsible

320   for the CLC-GFP stabilization in *p120ctn* mutants. Therefore, we expressed a constitutively

321   active Arf1 construct (Arf1^{CA}) in *p120ctn* mutant embryos and measured the FRAP of CLC-

322   GFP. Measuring the dynamics of clathrin revealed that the mobile fraction which recovered

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323   was no longer different from the wild-type control (p=0.19, Fig. 5E-F). We concluded that

324   the expression of Arf1^{CA} rescues the clathrin dynamics in the *p120ctn* mutant. This is

325   consistent with Arf1 acting downstream of p120ctn, providing a link between the p120ctn–E-

326   cad complex and the clathrin-mediated endocytic machinery.

327   Curiously, the overexpression of p120ctn also resulted in a reduction of Arf1-GFP at

328   the AP borders (p=0.02, Fig. 5B,D). Although the reduction of Arf1 at the DV borders was

329   not significant (p=0.37), its distribution remained uniform (p=0.18). Due to evidence that

330   GTPases often regulate each other (Baschieri and Farhan, 2012; Singh et al., 2017), we asked

331   whether this reduction was a consequence of p120ctn elevating RhoA signalling. We

332   measured the membrane levels of Arf1-GFP in cells expressing RhoGEF2-RNAi or

333   *UAS*::CD8-Cherry (Fig. 5G-H). Expression of this RNAi against RhoGEF2 reduces Rok

334   amounts by 20% specifically at the AP borders (Bulgakova et al., 2013). The downregulation

335   of RhoGEF2 resulted in an increase in the amount of Arf1-GFP at both AP and DV borders

336   (p=0.049 and p=0.022, respectively, Fig. 5H), demonstrating that RhoA signalling negatively

337   regulated Arf1 localization to the plasma membrane. In a complementary experiment, we

338   measured the membrane localization of MyoII-YFP upon the upregulation of Arf1 signalling

339   using a constitutively active variant of Arf1 (Arf1^{CA}, Fig. S3). MyoII-YFP localization was

340   indistinguishable between cells expressing Arf1^{CA} and control cells (Fig. S3), suggesting that

341   RhoA signalling in the embryonic epidermis is independent of Arf1 function. Therefore, we

342   concluded that the reason Arf1 was reduced at the plasma membrane in p120ctn

343   overexpressing cells was most likely due to the elevation of RhoA signalling. The reduction

344   of Arf1 in the absence of p120ctn appears independent of RhoA and we suggest that it is

345   caused by regulation of Arf1 recruitment and/or activation by p120ctn.

346   It was surprising that RhoGEF2-RNAi led to a uniform elevation of Arf1-GFP, as it

347   affects RhoA signalling only at the AP borders (Bulgakova et al., 2013). Indeed, Arf1 was

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348   uniformly localized at the plasma membrane in all cases. FRAP of Arf1 at the plasma

349   membrane demonstrated that it recovered almost completely within 25 sec (Fig. 5I), which

350   indicated a highly dynamic exchange. Therefore, we suggest that the effect of RhoGEF2-

351   RNAi on Arf1 at the DV borders is indirect: reduced RhoA signalling results in elevated

352   recruitment of Arf1 at the AP borders, followed by rapid redistribution around the cell

353   periphery and an overall elevation of Arf1-GFP signal at the cell surface. Overall, we

354   identified two levels of p120ctn regulation of Arf1 activity and endocytosis: first, it either

355   directly or indirectly promotes Arf1 membrane recruitment, and second, it indirectly inhibits

356   this recruitment by promoting RhoA signalling activation.

357

358   *6) Adhesion dynamics regulate cells shape.*

359   So far, we have demonstrated that p120ctn regulates cortical actin via RhoA signalling, and

360   E-cad dynamics via both RhoA and Arf1. Next, we asked how this regulatory network

361   contributes to the cell shape changes caused by altered p120ctn levels. Since changes in Arf1

362   activity did not affect RhoA signalling, we first examined how inhibition and hyperactivation

363   of Arf1 alone affected cell shape. To explore this, we used Arf1 regulatory constructs to alter

364   signalling output. First, we used a dominant negative (Arf1^{DN}) expressed in the engrailed

365   segment of the epidermis (Fig. 6A). Prolonged exposure to the dominant negative Arf1^{DN}

366   construct resulted in small rounded cells (Fig. S4), with no surviving larvae, consistent with

367   previous reports (Carvajal-Gonzalez et al., 2015), likely due to gross perturbation of post-

368   Golgi protein transport causing cell death (Jian et al., 2010; Luchsinger et al., 2018).

369   Therefore, we acutely induced expression of Arf1^{DN}, using temperature sensitive GAL80^{ts}.

370   The cells which expressed the Arf1^{DN} had a reduced aspect ratio (p=0.003, Fig. 6A-B). This

371   suggests that the reduction of endocytosis, and therefore stabilization of E-cad, is sufficient to

372   reduce the cell's aspect ratio. This is further supported by the reduced aspect ratio observed

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373   in other cases in which E-cad was stabilized: namely the expression of Rho^{CA} and the

374   overexpression of p120ctn (p<0.0001, Fig. 6C-D, see Fig. 2 and 4).

375   To directly test if E-cad stabilization is sufficient to induce cell rounding, we used

376   an alternative approach to inhibit E-cad endocytosis by overexpressing a dominant-negative

377   dynamin Shibire (Shi^{DN}) construct using *en*::GAL4. Expression of this construct stabilizes E-

378   cad at the plasma membrane in a similar manner to the loss of p120ctn (Bulgakova et al.,

379   2013). Indeed, cells expressing Shi^{DN} had reduced aspect ratio in comparison to control

380   (p=0.0002, Fig. 6E-F). Overall, these results indicate that the dynamics of intercellular

381   adhesion, mediated via endocytosis of E-cad, is a key factor determining the elongation of

382   epidermal cells.

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Discussion

Epithelial cells *in vitro* are usually isotropic, and the application of external stretching or
compressing forces induce an initial anisotropy in their shape (elongation), which is quickly
resolved through cell rearrangements and divisions, or tissue three-dimensional deformation
(Duda et al., 2019; Latorre et al., 2018; Nestor-Bergmann et al., 2019). By contrast, there are
multiple examples of highly anisotropic elongated cells in whole organisms, including the
epidermal cells used in this study but also in mammalian skin (Fig. 7A, Aw et al., 2016; Box
et al., 2019). These elongated shapes are necessary for correct tissue and organism
morphogenesis (Box et al., 2019; McCleery et al., 2019). In this study, we focused on the
regulation of such elongated cell shape through the cross-talk between intercellular E-cad-
mediated adhesion and cortical actin (the findings are summarized in Table 1). We provide *in
vivo* evidence that p120ctn, a known regulator of E-cad dynamics and endocytosis
(Bulgakova and Brown, 2016; Ireton et al., 2002; Nanes et al., 2012; Sato et al., 2011),
mediates this cross-talk and regulates cell shape. It does so by promoting the activities of at
least two small GTPases with opposing effects on E-cad dynamics: RhoA, which inhibits E-
cad turnover, and Arf1, which promotes it (Fig. 7B). This is the first report of a role for Arf1
in the clathrin-mediated trafficking of E-cad from the membrane. We show a further interplay
between these GTPases with RhoA preventing the localization of Arf1 at the plasma
membrane (Fig. 7B). Finally, while p120ctn normally colocalizes with E-cad and is at higher
levels on the DV borders, we show that it regulates RhoA activity only at the AP borders,
which are under higher tension, suggesting a tension-dependent function for p120ctn.

Multiple studies have revealed that intercellular adhesions are in a state of
constant remodelling through the processes of endocytosis and recycling (recently reviewed
in Bruser and Bogdan, 2017). This recycling is important for junctional remodelling and cell
rearrangements during morphogenesis (Le Droguen et al., 2015; Levayer et al., 2011a; Ogata

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423   et al., 2007; Sumi et al., 2018). At the same time, the contribution of adhesion dynamics, in

424   particular E-cad recycling, to the shape of cells is largely overlooked, while the roles of

425   cortical actin and the levels of intercellular adhesion in shaping cells are well established

426   (Lecuit and Lenne, 2007). Here, we show that the stabilization of E-cad and thus inhibition of

427   junctional remodelling prevents the elongation of the cells in *Drosophila* embryonic

428   epidermis (Fig. 7B). This is supported by several lines of evidence: expression of Arf1^{DN},

429   Shi^{DN}, and Rho^{CA}, all of which inhibit E-cad dynamic behaviour, causing loss of elongation.

430   Moreover, the changes in cell elongation were observed only in the cases when E-cad was

431   stabilized, suggesting a central role for E-cad dynamics in cell elongation. However, it is not

432   the only contributing factor: in *p120ctn* mutant embryos cell elongation was not affected

433   despite E-cad being stabilized. As E-cad stabilization in these embryos was accompanied by

434   a reduction of RhoA signalling at the AP borders and a uniform reduction in E-cad junctional

435   levels, either one or both of these factors are likely to counteract the stabilization of E-cad

436   resulting in normal elongation. We speculate that RhoA activity and cortical actin

437   organization are likely to be responsible for compensating for E-cad stabilization (Fig. 7B).

438   The contractility of cortical actin normally acts to reduce the surface length whereas the

439   levels of adhesion acts oppositely (Lecuit and Lenne, 2007), thus reduced MyoII in the

440   *p120ctn* mutants would act to increase the length of the AP borders, whereas reduced E-cad

441   would act to reduce it. Indeed, the strongest reduction in cell elongation was observed upon

442   the expression of Rho^{CA}, which stabilized E-cad without changing its levels at the AP borders

443   and promoted actomyosin contractility. Therefore, we propose that cell elongation is the

444   product of the counteraction of E-cad dynamics and cortical contractility.

445   However, due to the tight cross-regulation of E-cad adhesion, cortical actin, and

446   endocytic machinery it is impossible to affect one without affecting the others. This makes it

447   difficult to dissect the exact contribution of each component. For example, although the

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448   expression of Arf1[DN] did not have any obvious effect on Rok localization, we cannot exclude

449   the possibility that it altered the organization of cortical actin in another way. Indeed, Arf1

450   regulates actin assembly at the Golgi by acting both upstream and downstream of Cdc42

451   (Myers and Casanova, 2008). Similarly, dynamin, which is a large GTPase, regulates actin in

452   GTPase-dependent manner in addition to driving endocytosis (Gu et al., 2010; Lee and De

453   Camilli, 2002). Furthermore, the inhibition of E-cad endocytosis using a dominant-negative

454   variant of the GTPase Rab5 resulted in an elevated level of actin in epidermal cells in

455   *Drosophila* embryos (Mateus et al., 2011). Despite this difficulty in distinguishing the direct

456   effects of endocytosis from indirect effects through the regulation of the cortex, our results

457   establish that endocytosis is an important factor in regulating cell shape.

458   While the roles of RhoA and p120ctn in E-cad endocytosis are long-established

459   (Davis et al., 2003; Ellis and Mellor, 2000), the Arf1 dependent recruitment of clathrin had

460   only been shown to occur at the Golgi by recruiting the Adaptor 1 protein (Carvajal-Gonzalez

461   et al., 2015). The function of Arf1 at the plasma membrane has been described in dynamin-

462   independent endocytosis, which was presumed to be clathrin-independent (Kumari and

463   Mayor, 2008). However, the involvement of Arf1 in multiple endocytic pathways has not

464   been fully explored. We have shown that Arf1's capacity to recruit clathrin is exploited by

465   p120ctn to facilitate the endocytosis of E-cad. Whether this requires AP2, a well-documented

466   plasma membrane resident clathrin adaptor, has yet to be determined. Our findings provide a

467   mechanistic insight into the pro-endocytic activity of p120ctn which has only recently come

468   to light (Bulgakova and Brown, 2016; Sato et al., 2011) and elaborates the number of known

469   p120ctn interactors. The activities of Arf1 and RhoA are antagonistic, which was also seen in

470   previous studies on the cellularization of the early embryo (Lee and Harris, 2013).

471   In contrast to Arf1, the regulation of RhoA by p120ctn has been shown in many

472   studies, although the exact effects and mechanisms seem to be context-dependent

473 (Anastasiadis et al., 2000; Derksen and van de Ven, 2017; Lang et al., 2014; Taulet et al.,

474 2009; Yu et al., 2016; Zebda et al., 2013). We demonstrate that in the epidermal cells in

475 *Drosophila* embryos, p120ctn leads to activation of RhoA specifically at the AP but not DV

476 borders (Fig. 7B). At the same time, we show that p120ctn loss uniformly reduces

477 recruitment of Arf1 to the plasma membrane. However, we also find that Arf1 is very

478 dynamic and the changes at the DV borders, for example upon the downregulation of

479 RhoGEF2, are likely to be an indirect consequence of the effect at the AP borders (Fig. 7B).

480 It is therefore currently unclear if p120ctn performs any direct activity at the DV borders, or

481 promotes RhoA and Arf1 signalling only at the high-tension AP borders. Indeed, recent

482 evidence has suggested p120ctn has mechanosensing properties (Iyer et al., 2019). As we

483 show that the AP borders are at higher tension than the DV, our data further suggest that

484 p120ctn has the properties of a mechanotransducer. Additionally, using laser ablation we

485 demonstrated that p120ctn directly modulates tension at these AP borders, providing further

486 evidence for the novel role of p120ctn in mechanotransduction. It is yet to be determined if

487 p120ctn is directly sensing the tension though a conformational change similarly to other

488 components of cell-cell adhesion such as α-catenin and vinculin (Bays and DeMali, 2017;

489 Yao et al., 2014), or is regulated by another mechanotransducer. Our findings expand the

490 ever-increasing list of proteins involved in mechanotransduction and mechanical cell

491 responses.

492   Altogether, our findings demonstrate that cell elongation in a tissue is regulated

493 through the opposing action of cortical actin contractility and adhesion endocytosis, which

494 are however closely interconnected and regulate each other. Adhesion components modify

495 cortical actin while the latter alters adhesion endocytosis. Considering that all of the

496 molecules studied are expressed in all epithelia across evolution, we speculate that this

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497   system is likely to be more broadly applicable in development and a general feature of cell

498   biology.

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Materials and Methods

Fly stocks and genetics

Flies were raised on standard medium. The GAL4/UAS system (Brand and Perrimon, 1993) was used for all the specific spatial and temporal expression of transgenic and RNAi experiments. The GAL4 expressional driver used for all experiments was *engrailed::GAL4* (*en*::GAL4, Bloomington number 30564). The following fly stocks were used in this study (Bloomington number included where applicable): E-cad-GFP (*shg::E-cadherin-GFP*) (60584), Shg-Cherry (*shg::mCherry*, 59014), *UAS*::CLC-GFP (7109), *UAS*::Arf1-GFP (gift from T.Harris), Zipper-YFP (*Myosin II*-YFP, Kyoto Stock Center 115082), *sqh*::Rok^{K116A}-Venus (gift from J.Zallen), *UAS*::Arf1-T31N (DN) and *UAS::*Arf1-Q71L (CA) (Dottermusch-Heidel et al., 2012), *UAS*::Rho1-N19 (DN) (7328), *tubulin*::GAL80^{TS} (7017), *ubi*::p120ctn-GFP (7190), *UAS*::p120ctn-GFP (7192), and *UAS*::Shibire^{K44A} (*UAS*::Shi-DN, 5822). The p120ctn mutant genotype (p120ctn^{308}/ Δp120) used was derived from crossing two deficiency stocks: homozygously viable p120ctn^{308} females (Myster et al., 2003) with homozygously lethal Df(2R)M41A8/ CyO, *twi::*Gal4, *UAS::*GFP males (740). Thus, the p120ctn mutants examined lacked both maternal and zygotic contributions.

Embryo collection and fixation

Embryos were collected at 25°C at 3-hour time intervals and allowed to develop at 18°C for 21 hours to reach the desired developmental stage, except for the acute induction experiments, where embryos were allowed to develop for 13 hours at 18°C and shifted to 29°C for 4 hours. Then embryos were dechorionated using 50% sodium hypochlorite (bleach, Invitrogen) in water for 4 minutes, and extensively washed with deionized water prior to fixation. Fixation was performed with a 1:1 solution of 4% formaldehyde (Sigma) in PBS (Phosphate Buffered Saline) and heptane (Sigma) for 20 minutes on an orbital shaker at room

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527   temperature. Embryos were then devitellinized in 1:1 solution of methanol and heptane for 20

528   sec with vigorous agitation. Following subsequent methanol washes the fixed embryo

529   specimens were stored at -20°C in methanol until required.

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531   *Embryo live imaging*

532   Embryos were collected and dechorionated as described above. Once washed with deionized

533   water embryos were transferred to apple juice agar segments upon microscope slide. Correct

534   genotypes were selected under a fluorescent microscope (Leica) using a needle. Embryos

535   were positioned and orientated in a row consisting of 6-10 embryos per genotype. Following

536   this, embryos were transferred to pre-prepared microscope slides with Scotch tape and

537   embedded in Halocarbon oil 27 (Sigma). Embryos were left to aerate for 10 minutes prior to

538   covering with a cover slip and imaging.

539   For laser ablation, following orientation and positioning the embryos were transferred to a

540   60mm x 22mm coverslip which had been pre-prepared by applying 10 μl of Heptane glue

541   along a strip in the middle of the coverslip orientated with the long axis. The coverslip was

542   attached to a metal slide cassette (Zeiss), and the embryos were embedded in Halocarbon oil

543   27 before imaging.

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545   *Molecular cloning*

546   The p120ctn full length cDNA was obtained from Berkeley Drosophila Genome Project

547   (BDGP), supplied in a pBSSK vector. This was sub-cloned into a (pUAS-k10.attB) plasmid

548   using standard restriction digestion with NotI and BamHI (New England Biolabs) followed

549   by ligation with T4 DNA ligase (New England Biolabs) and transformation into DH5a

550   competent *E.coli* cells (Thermo Fisher Scientific). Prior to injection plasmids were test

551   digested and sequenced (Core Genomic Facility, University of Sheffield). Plasmids were

552   prepared for injection using standard miniprep extraction (Qiagen) and submitted for

553   injection (Microinjection service, Department of Genetics, University of Cambridge) into the

554   attP-86Fb stock (Bloomington stock 24749). Successful incorporation of the transgene was

555   determined by screening for (w^+) in the F1 progeny.

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557   *Immunostaining*

558   The embryos were washed three times in 1 ml of PBST (PBS with 0.05% Triton) with gentle

559   rocking. Blocking of the embryos prior to staining was done in 300 μl of a 1% NGS (Normal

560   Goat Serum) in PBST for 1 hour at room temperature with gentle rocking. For staining the

561   blocking solution was removed, 300 μl of the primary antibody: 1:100 rat anti-E-cad

562   (DCAD2, DSHB), 1:10 mouse anti-engrailed (4D9, DSHB), or 1:500 anti-Golgi (Golgin-245,

563   Calbiochem) in fresh blocking solution was added and the embryos were incubated overnight

564   at 4°C with orbital rotation. Then, embryos were washed three times with 1 ml of PBST. A

565   300 μl 1:300 dilution of the secondary antibody (goat Cy3- or Cy5-conjugated IgG,

566   Invitrogen) was added, and the embryos incubated either overnight at 4°C with orbital

567   rotation or for 2 hours at room temperature. Then embryos were washed three time with

568   PBST, following which they were incubated with 50-70 μl of Vectashield (Vector

569   Laboratories) and allowed to equilibrate for a period of 2 hours before being mounted on

570   microscope slides (Thermo).

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572   *Microscopy, data acquisition and FRAP*

573   All experiments except for laser ablation were performed using an up-right Olympus FV1000

574   confocal microscope with a 60x/1.40 NA oil immersion objective. Shi^{DN} expressing embryos

575   and the corresponding control were imaged using 100x/1.40 NA UPlanSApo objective. All

576   measurements were made on dorsolateral epidermal cells of embryos, which were near or just

577   after completion of dorsal closure, corresponding to the end of Stage 15 of embryogenesis.

578   For fixed samples 16-bit images were taken at a magnification of 0.051µm/pixel (1024x1024

579   pixel XY-image) or 0.062 µm/pixel (Shi^{DN} embryos and the corresponding control) with a

580   pixel dwell of 4µm/pixel. For each embryo, a Z-axis sectional stack through the plane of the

581   AJs was taken, which consisted of six sections with a 0.38 µm intersectional spacing. The

582   images were saved in the Olympus binary image format for further processing.

583   For E-cad FRAP (adapted from Bulgakova et al., 2013) 16-bit images were taken at a

584   magnification of 0.093 µm/pixel (320x320 pixel XY-image). In each embryo, several circular

585   regions of 1 µm radius were photobleached at either DV or AP junctions resulting in one

586   bleach event per cell. Photobleaching was performed with 8 scans at 2 µs/pixel at 50-70%

587   488 nm laser power, resulting in the reduction of E-cad-GFP signal by 60–80%. A stack of 6

588   z-sections spaced by 0.38 µm was imaged just before photobleaching, and immediately after

589   photobleaching, and then at 20 s intervals, for a total of 15 minutes.

590   As rate of endocytosis depends on external factors, such as temperature [103],

591   controls were examined in parallel with experimental conditions in all experiments with

592   CLC-GFP. This dependence might also account for slight variation between datasets, e.g.

593   compare Fig. 2A and 2G. For CLC-GFP FRAP, 16-bit images were taken at a magnification

594   of 0.051µm/pixel (256x256 pixel XY-image). In each embryo a single plane was selected in

595   centre of the AJ band using Shg-Cherry for positioning. An area encompassing a transverse

596   region orthogonal to the axis of the engrailed expressing cells was selected (140x60 pixels)

597   was photobleached with 1 scan at 2 µm/pixel using 100% 488nm laser power resulting in

598   reduction of CLC-GFP signal by 70-80%. Images were taken using continuous acquisition at

599   a frame rate of 2 sec^{-1}. Prior to bleaching a sequence of 10 images was taken, and a total of

600   400 frames corresponding to 3.5 minutes were taken.

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Data processing and statistical analysis

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Membrane intensity and cell shape: Images were processed in Fiji (https://fiji.sc) by

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generating average intensity projections of the channel required for quantification. Masks

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were created by processing background-subtracted maximum intensity projections using the

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Tissue Analyzer plugin in Fiji (Aigouy et al., 2016). Quantification of the membrane

607

intensity at the AP and DV borders and cell elongation (aspect ratio) was done as described

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previously using a custom-built Matlab script (Bulgakova and Brown, 2016) found at

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(https://github.com/nbul/Intensity). In short, cells were identified as individual objects using

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the created masks, and their eccentricities were calculated. The aspect ratio was calculated

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from the eccentricity as $AR = 1/\sqrt{1 - e^2}$, where *e* is eccentricity. At the same time, the

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individual borders were identified as objects by subtracting a dilated mask of vertices from a

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dilated mask of cell outlines. The mean intensity and orientation of each border were

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calculated. The average border intensities (0-10° for the AP and 40-90° for the DV borders

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relatively to cell mean orientation) were calculated for each embryo and used as individual

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data points to compare datasets. The average cytoplasmic intensity was used for the

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background subtraction. In the case of quantification of Arf1-GFP membrane intensity, due

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to high Arf1-GFP presence inside cells both in Golgi and cytoplasm, the mean intensity of

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embryonic areas not expressing the GFP-tagged transgene were used for background

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subtraction. Statistical analysis was performed in Graphpad Prism

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(https://www.graphpad.com/scientific-software/prism/). First, the data was cleaned using

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ROUT detection of outliers in Prism followed by testing for normal distribution (D'Agostino

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& Pearson normality test). Then, the significance for parametric data was tested by either a

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two-way ANOVA or two-tailed t-test with Welch's correction.

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626   *E-cad FRAP*: images were processed by using the grouped Z-projector plugin in Fiji to

627   generate average intensity projections for each time-point. Following this the bleached ROI,

628   control ROI and background intensity were manual measured for each time point. This data

629   was processed in Microsoft Excel. First the intensity of the bleached ROI at each time point

630   was background subtracted and normalized as following: $I_n = (F_n - BG_n)/(FC_n - BG_n)$,

631   where F_n – intensity of the bleached ROI at the time point n, FC_n – intensity of the control

632   unbleached ROI of the same size at the plasma membrane at the time point n, and BG_n –

633   background intensity, measured with the same size ROI in cytoplasm at the time point n.

634   Than the relative recovery at each time point was calculated using the following formula:

635   $R_n = (I_n - I_1)/(I_0 - I_1)$, where I_n, I_1 and I_0 are normalized intensities of bleached ROI and

636   time point n, immediately after photobleaching, and before photobleaching respectively.

637   These values were input to Prism and nonlinear regression analysis was performed to test for

638   best fit model and if recoveries were significantly different between cell borders or

639   genotypes. The recovery was fit to either single exponential model in a form of $f(t) = 1 -$

640   $F_{im} - A_1 e^{-t/T_{fast}}$, or to bi-exponential model in a form of $f(t) = 1 - F_{im} - A_1 e^{-t/T_{fast}} -$

641   $A_2 e^{-t/T_{slow}}$, where F_{im} is a size of the immobile fraction, T_{fast} and T_{slow} are the half times, and

642   A_1 and A_2 are amplitudes of the fast and slow components of the recovery. An F-test was used

643   to choose the model and compare datasets.

644   *CLC-GFP FRAP:* measurements of all intensities, i.e. the bleached ROI, control ROI and the

645   background, and normalization were done using a custom-build Matlab script

646   (http://github.com/nbul/FRAP) using the same algorithm as described for E-cad FRAP.

647   Curve fitting and statistical analysis was performed in Graphpad Prism using a nonlinear

648   regression analysis as described for E-cad FRAP.

649   *Sample size:* Each experiment was performed on multiple embryos, which represent

650   biological replicates. For each embryo, 2-3 ROIs were selected for E-cad FRAP analysis, and

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651   these were treated as technical replicates and were averaged per wing. The minimum sample

652   size was estimated based on the mean values and the standard deviation of a control set of

653   embryos to allow detection of differences of 20% in the means, in a pair-wise comparison,

654   with a power of 0.8 and α 0.05 (using http://powerandsamplesize.com/, 9 embryos for

655   fluorescence intensity comparison and aspect ratio, and 5 embryos for FRAP). As standard

656   deviations were larger for some genotypes, we aimed for 15 and 6 embryos per genotype,

657   respectively. The exact numbers of embryos used in each experiment are in Table S1.

658

659   *Laser Ablation*

660   Nanoablation of single junctions was performed to provide a measure of junctional tension.

661   Embryos were imaged on a Zeiss LSM 880 microscope with an Airyscan detector, an 8-bit

662   image at 0.053 μm/pixel (512x512 pixel XY-Image) resolution with a 63x objective (1.4 NA)

663   at 5x zoom and 2x averaging was used. An illumination wavelength of 488 nm and 0.5%

664   laser power were used. Images were captured with a 0.5 μm z-spacing. Narrow rectangular

665   ROIs were drawn across the centre of single junctions and this region was ablated using a

666   pulsed TiSa laser (Chameleon), tuned to 760 nm at 45% power. Embryos were imaged

667   continuously in a z-stack consisting of 3 z-slices. The initial recoil rate of vertices at the ends

668   of ablated junctions was quantified by measuring the change in distance between the vertices

669   and dividing by the initial time step. Statistical analysis was performed in Graphpad Prism:

670   using a two-tailed t-test with Welch's correction.

671

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679

680   **Author Contribution**

681   J.G and N.A.B. designed and performed experiments and wrote the manuscript.

682

683   **Conflicts of Interest**

684   The authors declare and confirm that there is no conflict of interest for the work presented in

685   this paper.

686

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Figure legends

Figure 1. Cells of the stage 15 *Drosophila* embryonic epidermis have anisotropic cell surface adhesion, actomyosin, and cortical tension.

A-D. Overview of the stage 15 *Drosophila* embryo. (A) A cartoon sketch of a stage 15 *Drosophila* embryo, area indicted by red box is the dorsolateral epidermis region imaged in this study. (**B**) Apical view of the epidermis highlighted in the red box (A). Cells are outlined by E-cad-GFP. (**B'**) Magnified image indicated in the box of (B) displayed using rainbow pixel intensity spectrum to highlight asymmetry of distribution, scale bar is 10 µm. (**C**) A schematic of the two cell borders present in these cells: the longer Anterior-Posterior (AP, red) and the short Dorsal-Ventral (DV, blue). (**D**) Aspect ratio, which is the length of the cell in the long AP axis divided by the length in the short DV, of the cells in embryos expressing only E-cad-GFP in otherwise wild-type background. (**E**) Non-muscle Myosin II (MyoII) tagged with YFP in the same plane as E-cad (B) and displayed using rainbow pixel intensity spectrum. (**F-G**) Amounts of MyoII-YFP (F) and E-cad-GFP (G) at the AP and DV cell borders in embryos expressing only either MyoII-YFP (F) or E-cad-GFP (G) in otherwise wild-type background. (**H**) Examples of laser ablation of an AP border (upper panel) and a DV border (lower panel) in dorsolateral epidermal cells of embryos expressing E-cad-GFP to visualize cell borders, in otherwise wild type background. Image is an overlay of pre-ablation (green) and post-ablation (magenta). The arrows indicate the connected vertices of the ablated membrane used to measure initial recoil. Note, the recoil clearly seen in ablation of the AP border is absent in ablation of DV. Statistical difference between cell borders measured by two-way ANOVA. *, $P < 0.05$; ***, $P < 0.001$. 10-20 embryos per genotype were quantified.

1006 **Figure 2. p120ctn levels affect cell shape and cortical actin, resulting in concomitant**

1007 **changes in cortical tension.**

1008 (**A**) Top and middle: Apical views of epidermis expressing *UAS::*CD8-Cherry (left) and co-

1009 expressing it with *UAS::*p120ctn (right) using *en::*GAL4 with E-cad-GFP (green, top; grey

1010 middle) and *UAS::*CD8-Cherry (magenta, top). *UAS::*CD8-Cherry was used to mark cells

1011 expressing transgenes. Bottom: Control and *p120ctn$^{-/-}$* mutant epidermal cells marked by E-

1012 cad-GFP (grey). (**B**) Aspect ratio of the control (*UAS::*CD8-Cherry) and *UAS::*p120ctn

1013 expressing cells. (**C**) Aspect ratio of the wide-type control and *p120ctn$^{-/-}$* mutant cells. (**D**)

1014 The epidermis of control, *p120ctn$^{-/-}$* mutant and p120ctn overexpression (*UAS::*p120ctn)

1015 embryos visualized with anti-E-cad antibody (grey, left and middle; green, bottom),

1016 *UAS*::CD8-mCherry (magenta, bottom image, marks cells expressing *UAS::*p120ctn), and

1017 Myosin II (MyoII) tagged with YFP (rainbow pixel intensity spectrum, right, membrane

1018 indicated by arrow). (**E-F**) Amounts of MyoII-YFP in p120ctn overexpressing embryos (E)

1019 and in the *p120ctn$^{-/-}$* mutant (F). All membrane intensity data are in Table S1. Scale bars – 10

1020 μm. (**G**) Laser ablation of AP cell borders in control (top), p120ctn overexpression (middle),

1021 and *p120ctn$^{-/-}$* mutant (bottom). Images are an overlay of pre-ablation (green) and post-

1022 ablation (magenta). The small arrows indicate the connected vertices of the ablated

1023 membrane used to measure initial recoil. The large arrows represent the position of

1024 microablation, for which a small spot of discolouration can be observed. (**H**) The initial recoil

1025 velocity of the membranes in the three conditions. Statistical difference between cell borders

1026 measured by two-way ANOVA. For laser ablation statistical analysis is a two-tailed students

1027 t-test with Welch's correction. All best-fit and membrane intensity data are in Table S1. *, P

1028 < 0.05; **, P < 0.01 ****, P < 0.0001. 10-20 embryos per genotype.

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Figure 3. p120ctn regulates both the levels and dynamics of E-cad.

(**A**) Apical view of epidermal cells expressing a *Ubi::*p120ctn-GFP transgene, cell borders visualised by transgene itself (grey, left) and stained with E-cad antibody (grey, right). (**B-B'**) Quantification of the localization of the *Ubi::*p120ctn-GFP transgene in the epidermal cells. (B) Pearson's correlation of the signal intensities between E-cad and *Ubi::*p120ctn-GFP. (B') The localization of the *Ubi::*p120ctn-GFP transgene on the two cell borders. (**C-D**) Effect on E-cad of overexpressing p120ctn using *UAS*::p120ctn construct. Apical views (C) and the membrane E-cad levels (D) in cells expressing E-cad-GFP (C, grey, top; green bottom) and *UAS*::CD8-mCherry (C, control, magenta, left) or *UAS*::p120ctn co-expressing a single copy of *UAS*::CD8-mCherry (C, magenta, right). (**E-F**) Apical views (E) and the membrane E-cad levels (F) in epidermis of control and *p120ctn*^{-/-} mutant cells both expressing endogenous E-cad-GFP. (**G-I**) Localization of E-cad and p120ctn in embryos expressing a *UAS*::p120ctn-GFP. (G) *UAS*::p120ctn-GFP expressed in engrailed compartment cells using the *engrailed*::GAL4 driver. (G) The same cells stained for E-cad to compare with internal control cells adjacent to engrailed stripe. (H-I) The localization of *UAS*::p120ctn-GFP (H) and E-cad (I) in the *UAS*::p120ctn-GFP expressing cells between the two borders. (**J-K**) Dynamics of E-cad-GFP measured by FRAP in control and *UAS::*p120ctn cells at the AP (J) and DV (K) borders. Average recovery curves (mean ± s.e.m.) and the best-fit curves (solid lines) are shown. All best-fit and membrane intensity data are in Table S1. Statistical difference between cell borders was measured by two-way ANOVA. For comparison of the difference between cell borders a two-tailed students t-test with Welch's correction. Scale bars – 10 µm. *, $p < 0.05$, **, $p < 0.01$ ***, $p < 0.001$; ****, $p < 0.0001$. 10-20 embryos per genotype were used for quantifications; 8-10 embryos – for FRAP.

42

1056 **Figure 4. p120ctn and RhoA change the dynamics of the clathrin mediated endocytosis.**

1057 (**A-C**). The localization and dynamics of the clathrin light chain (*UAS*::CLC-GFP) in control,

1058 embryos overexpressing p120ctn (*UAS*::p120ctn), and *p120ctn*$^{-/-}$ mutant embryos. (A)

1059 Distinct puncta (spots, magenta in left and black in right images, arrows) are observed at the

1060 membrane and in the cytoplasm. Cell outlines are visualized by anti-E-cad antibody (green,

1061 left). FRAP of *UAS*::CLC-GFP using a rectangular region in the plain of AJs in embryos

1062 overexpressing p120ctn (*UAS*::p120ctn) (B) and *p120ctn*$^{-/-}$ mutant (C). Average recovery

1063 curves (mean ± s.e.m.) and the best-fit curves (solid lines) are shown. (**D-G**). Localization of

1064 E-cad in the epidermis following downregulation (the induction of Rho^{DN} expression for 4

1065 hours, membrane indicated by arrow, D-E) or upregulation (expression of Rho^{CA}, F-G) of

1066 RhoA signalling. Top images in **F** are the corresponding control expressing *UAS*::CD8-

1067 Cherry alone. Cell borders are marked by E-cad-GFP (D and F, rainbow spectrum, right;

1068 green, left). Cells expressing transgenes are marked with an antibody for Engrailed (D) or by

1069 *UAS::*CD8-Cherry (F). Levels of the E-cad-GFP at the membrane of the Rho^{DN} (E) or Rho^{CA}

1070 (G) expressing cells in comparison to internal control (E) or external controls (G). (**H-I**).

1071 Localization of clathrin (*UAS*::CLC-GFP, grey, right; magenta, left) in cells co-expressing

1072 Rho^{CA} (bottom) and corresponding control co-expressing *UAS*::CD8-Cherry (top). Cell

1073 borders are visualized by anti-E-cad antibody (green, left, arrow indicates membrane

1074 localized puncta). (**I**). FRAP of *UAS*::CLC-GFP in Rho^{CA} expressing cells. Average recovery

1075 curves (mean ± s.e.m.) and the best-fit curves (solid lines) are shown. All best-fit and

1076 membrane intensity data are in Table S1. Scale bars – 10 μm. Statistical analysis is a two-

1077 tailed students t-test with Welch's correction. *, $p < 0.05$; **, $p < 0.01$ ****, $p < 0.0001$. 10-

1078 20 embryos per genotype were used for quantifications; 8-10 embryos – for FRAP.

1079

1080   **Figure 5. Arf1 signalling is downstream of p120ctn and promotes clathrin mediated**

1081   **endocytosis, but is inhibited by RhoA signalling**

1082   (**A**) Apical views of the epidermis expressing *UAS*::Arf1-GFP (Arf1, magenta, left; grey,

1083   middle and right) with *en*::Gal4. Cell outlines are visualized with anti-E-cad antibody (green,

1084   left). The large UAS::Arf1-GFP puncta in the cytoplasm (large arrow in A) mark the Golgi.

1085   The presence of the secondary Arf1-GFP population at the cell surface is revealed with a

1086   single plane section in the centre of the AJ (grey, right, small arrow), and is less prominent in

1087   an average projection spanning the whole depth of the AJ (middle). (**B**) Apical views of the

1088   epidermis expressing *UAS*::Arf1-GFP in cells overexpressing p120ctn (*UAS*::p120ctn) and

1089   *p120ctn$^{-/-}$* mutant. (**C-D**) The amount of *UAS*::Arf1-GFP at the plasma membrane between a

1090   control and *p120ctn$^{-/-}$* mutant (C), and between a control and p120ctn overexpressing

1091   embryos (D). (**E**) Localization of *UAS*::CLC-GFP (grey, right; magenta, left) in control,

1092   expressing *UAS*::CLC-GFP alone (top), and in embryos expressing a constitutively active

1093   Arf1 (Arf1^{CA}) in a *p120ctn$^{-/-}$* mutant genetic background (bottom). Cell borders were

1094   visualized with anti-E-cad antibody (green, left). (**F**) FRAP of *UAS*::CLC-GFP in the plane of

1095   AJs in the Arf1^{CA}; *p120ctn$^{-/-}$* embryos. Average recovery curves (mean ± s.e.m.) and the best-

1096   fit curves (solid lines) are shown. (**G-H**). The apical view (G) and amount (H) of *UAS::*Arf1-

1097   GFP (grey, right; magenta, left) in control and cells expressing RhoGEF2 RNAi. (**I**).

1098   Dynamics of Arf1-GFP in control cells using FRAP. Statistical analysis between cell borders

1099   measured by two-way ANOVA. *, $p < 0.05$; ***, $p < 0.001$. All best-fit and membrane

1100   intensity data are in Table S1. 10-20 embryos per genotype were used for quantifications; 8-

1101   10 embryos – for FRAP.

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Figure 6. Inhibition of the dynamics of surface adhesion leads to reduced cell elongation.

(**A-B**). Apical view (A) and cell aspect ratio (B) following downregulation of Arf1 (the induction of Arf1^{DN} expression for 4 hours). (**C-D**) Apical view (C) and cell aspect ratio (D) following upregulation of RhoA signalling (Rho^{CA}). (**E-F**) Apical view (E) and cell aspect ratio (F) following inhibition of endocytosis by expression of the dominant-negative variant of dynamin (Shibire^{DN}). Cell outlines are visualized by E-cad-GFP (A and C) or anti-E-cad antibody (E) in green (left) or grey (right). Cells expressing constructs are labelled with *UAS*::CD8-mCherry (A,C,E, magenta, left). ***, $p < 0.001$, and ****, $p < 0.0001$. All best-fit and membrane intensity data are in Table S1. 10-20 embryos per genotype were used for quantifications

Figure 7. Model of cell shape regulation in anisotropic elongated cells in epidermis

(**A**). Schematic illustration of cellular anisotropy in embryonic epidermis of stage 15 *Drosophila* embryos. E-cad and p120ctn (blue) are enriched at the DV borders, whereas MyoII and Rok (green) at the AP borders, while Arf1 (magenta) is uniformly associated with the plasma membrane. The long AP borders are under tension, while the short DV borders are under compressive force (**B**). Model of cell shape regulation by cortical actin and E-cad dynamics at both cell borders. High cortical tension and actomyosin contractility at the long AP borders inhibit cell elongation, whereas adhesion dynamics, i.e. E-cad endocytosis, promote it. Actomyosin is activated downstream of RhoA signalling, while Arf1 GTPase promotes clathrin-mediated endocytosis. Furthermore, RhoA additionally inhibits this endocytosis by either directly or indirectly preventing Arf1 localization at the plasma membrane (dashed lines). Finally, both RhoA and Arf1 are activated downstream of p120ctn at the long AP borders. In contrast, at the short DV borders, where tension is low, RhoA

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1129   seems to be low resulting in low MyoII and Rok levels and is thus not involved in cell

1130   elongation. It is also not regulated by p120ctn at these borders, despite p120ctn being

1131   normally enriched there. Arf1 promotes E-cad endocytosis at the DV borders. Arf1 is highly

1132   diffusive and its levels at the DV borders are most likely to be influenced by recruitment to

1133   the plasma membrane at the AP borders via diffusion (magenta arrow). Finally, it is unclear

1134   whether Arf1 or other molecules are regulated downstream of p120ctn at the DV borders,

1135   neither if E-cad dynamics via endocytosis contributes to cell shape and elongation (question

1136   marks).

1137

1138   **Table 1. Summary of cell shape and protein distributions in the genotypes examined.**

1139   Genotypes of the embryos presented in this study on the with corresponding changes

1140   observed in the aspect ratio of the cells; and the levels of representative cell adhesion,

1141   cytoskeletal, and signalling pathway components at both the AP and DV cell borders. OE –

1142   overexpression.

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Supplementary Information

Table S1. Numerical values for each experiment presented in paper.

Figure S1. Rok-Venus is effected is the same manner as MyoII by changes the levels of p120ctn.

A. Apical view of the epidermis of control, *p120ctn*$^{-/-}$ mutant, and p120ctn overexpression (*UAS::*p120ctn) embryos visualized with anti-E-cad antibody (**A**, grey in the top images, green in the top right image), *UAS*::CD8-mCherry (**A**, magenta in the right image, marks cells expressing *UAS::*p120ctn) and Rho-Kinase (Rok) tagged with Venus (**A,** lower panel rainbow pixel intensity spectrum, membrane indicated by arrow). **B-C.** The amount of Rok-Venus in the *p120ctn*$^{-/-}$ mutant (**B**) and p120ctn overexpressing embryos (**C**). Scale bars – 10 μm. Statistical analysis between cell borders measured by two-way ANOVA. **, $p < 0.01$. 15-20 embryos per genotype were used for quantifications.

Figure S2. p120ctn antibody staining fails to replicate localization of GFP tagged variant and genetic ablation results in stabilization of endogenously tagged E-cad-GFP.

A. Apical view of epidermal cells of the *ubi::*p120ctn-GFP expressing embryos. The localization of the p120ctn-GFP (grey, left panel) compared to the localisation of E-cad stained with an antibody (grey, middle panel) the staining pattern of the anti-p120ctn antibody in the same cells (grey, right panel). **B.** The FRAP curves of endogenously tagged E-cad-GFP in *p120ctn*$^{-/-}$ mutant embryos. AP borders (left graph) and DV borders (right graph) are shown separately. All best-fit and membrane intensity data are in Table S1. Scale bars – 10 μm. 8-10 embryos used for quantification in FRAP.

1169 **Figure S3. Arf1-GFP cytoplasmic puncta are representative of the Golgi apparatus and**

1170 **hyperactivation of Arf1 signalling has no effect on MyoII-YFP.**

1171 **A-E.** Apical view of the dorsolateral epidermis of *UAS::*Arf1-GFP expressing control

1172 embryo with cells borders visualized with anti-E-cad antibody (black in **A**, white in **E**), Golgi

1173 marked by immunostaining of Trans-Golgi (black in **B**, magenta in D and E), and Arf1-GFP

1174 in the *engrailed* expressing cells (black in **C,** green in **D** and **E**). The same region is shown in

1175 main text (see Fig. 5). (**F-G**) Localization of MyoII-YFP (rainbow in left image) in the cells

1176 expressing a constitutively active Arf1 (Arf1^{CA}) with *en*::GAL4 marked by *UAS::*CD8-

1177 Cherry (magenta in right image). Cell borders are visualized with anti-E-cad antibody

1178 staining (green in right image). **G.** The amount of MyoII-YFP at the plasma membranes of

1179 the Arf1^{CA} cells and the adjacent internal control cells. Scale bar is 10 μm. Statistical analysis

1180 between cell borders measured by two-way ANOVA. 15-20 embryos were used for

1181 quantification.

1182

1183 **Figure S4. Arf1^{DN} induces cell morphology changes leading to cell death.**

1184 **A-B.** Apical view of the dorsolateral epidermis of Arf1^{DN} transgene expressing embryos, co-

1185 expressing *UAS::*CD8-cherry in the engrailed stripes to mark the cells (magenta in **A**). The

1186 cell borders are marked by Shg-GFP (green in A, black in B). Scale bar is 10 μm. **C.** Cell

1187 area of the internal control and Arf1^{DN} expressing cells, normalized to control. **, $p < 0.01$.

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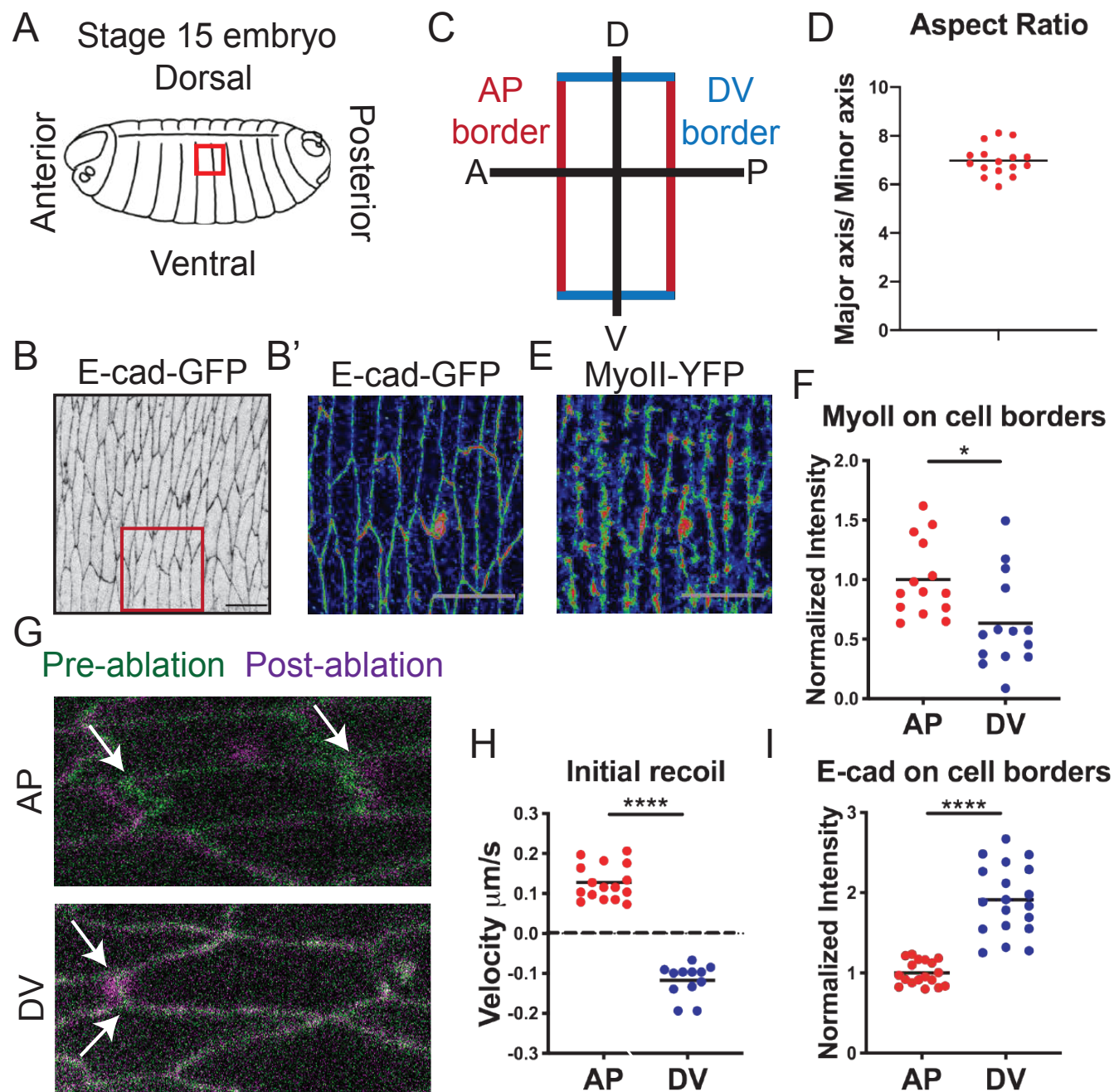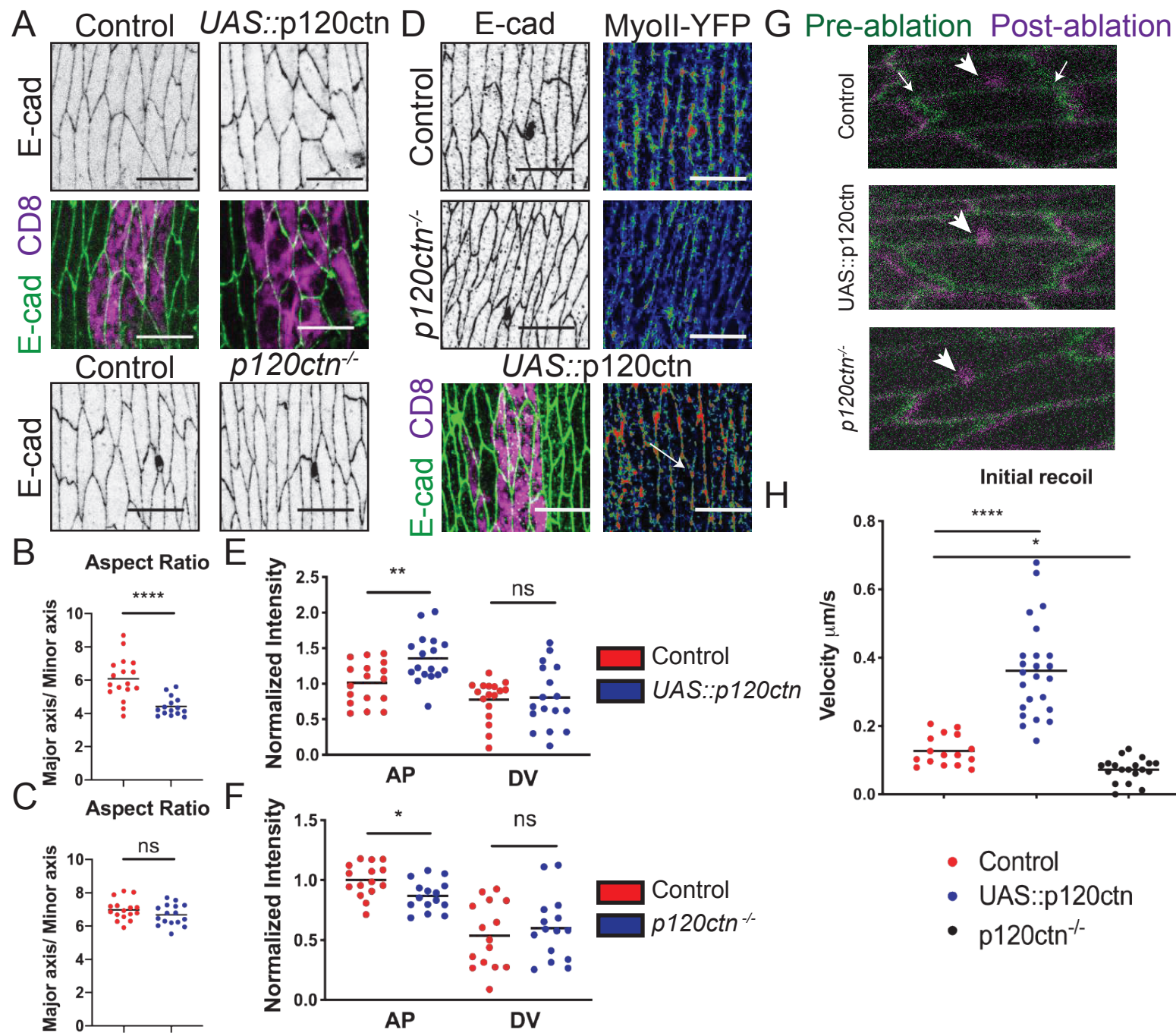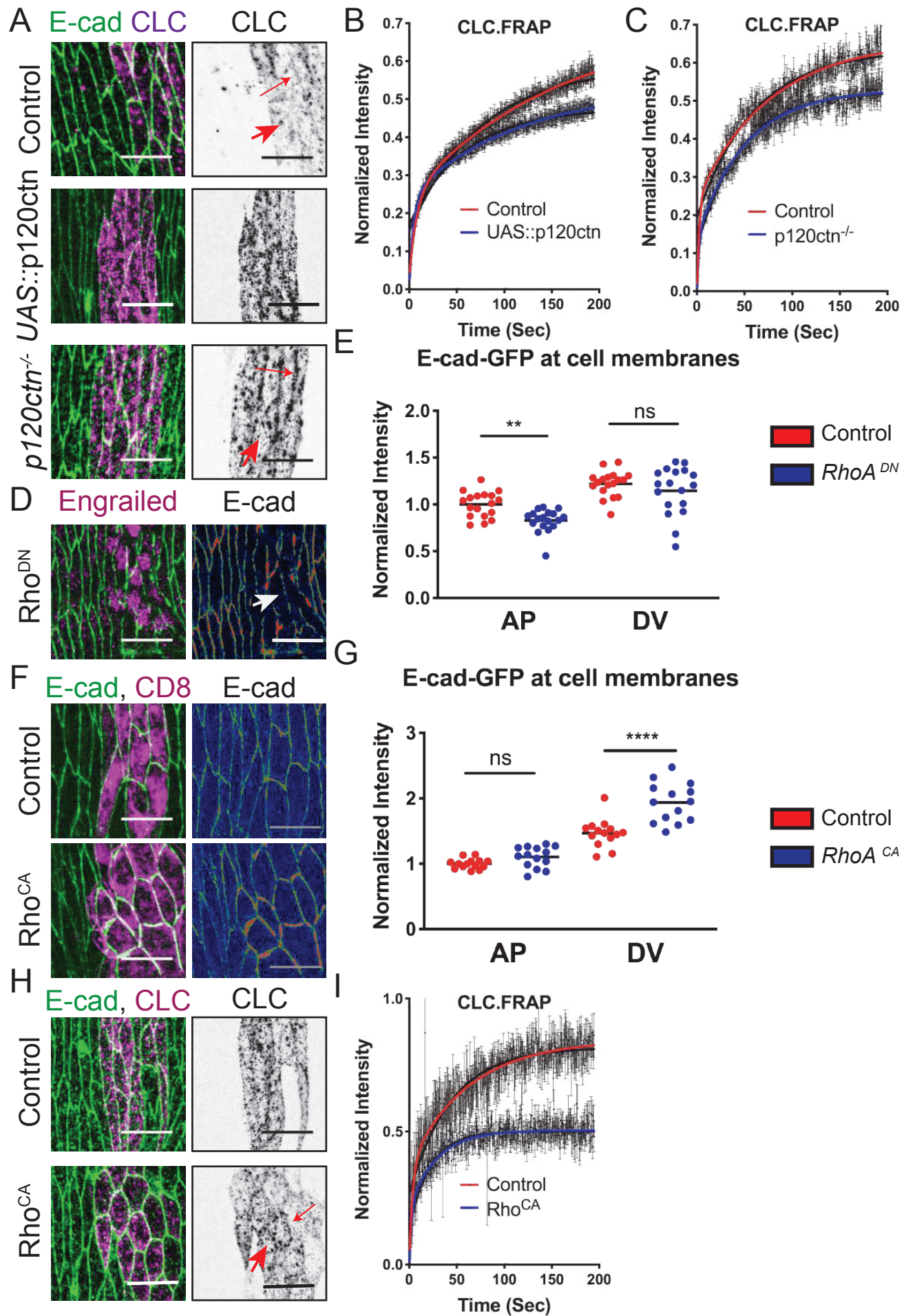Fig. 1

Fig.2

Fig. 3

Fig. 4
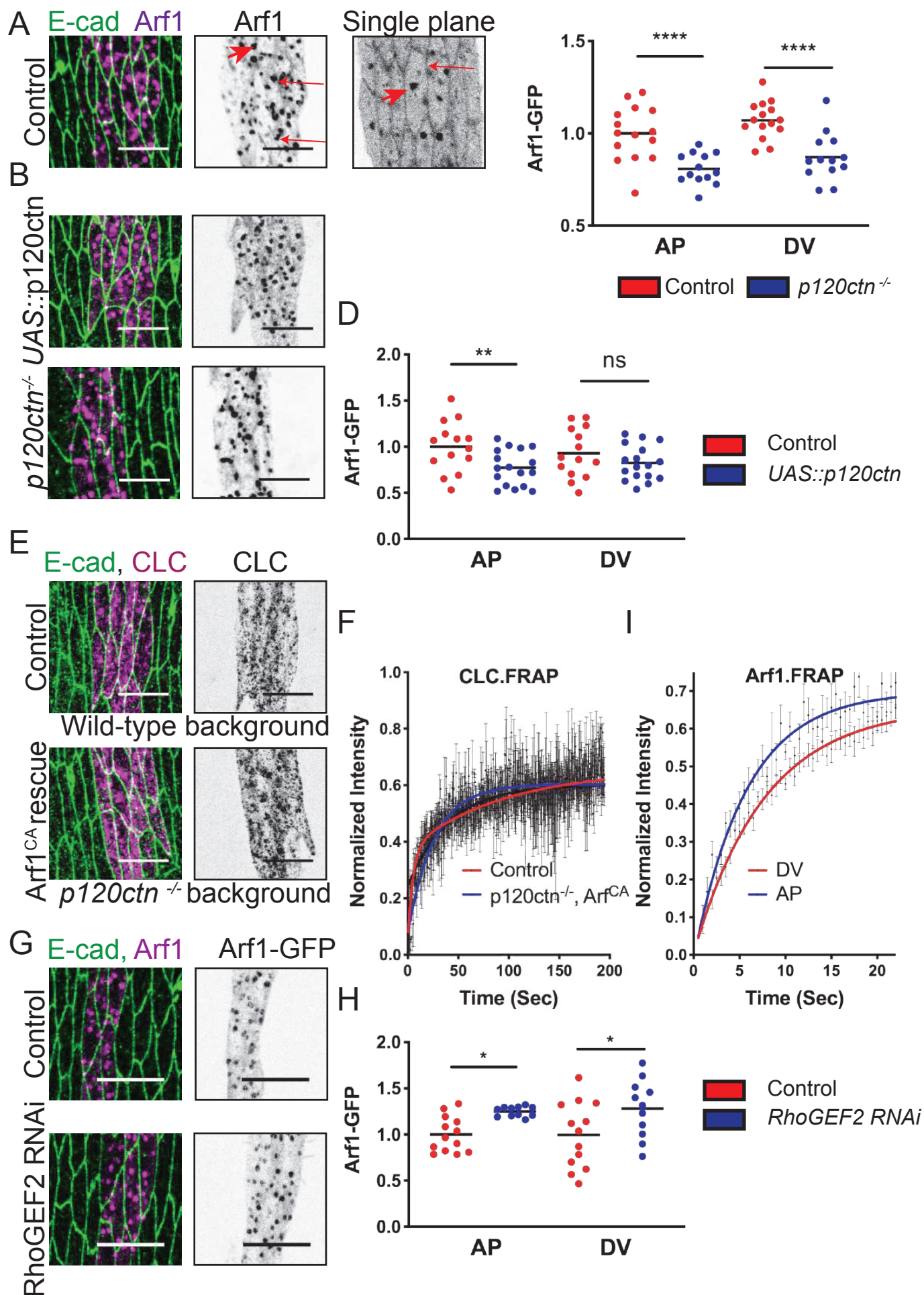
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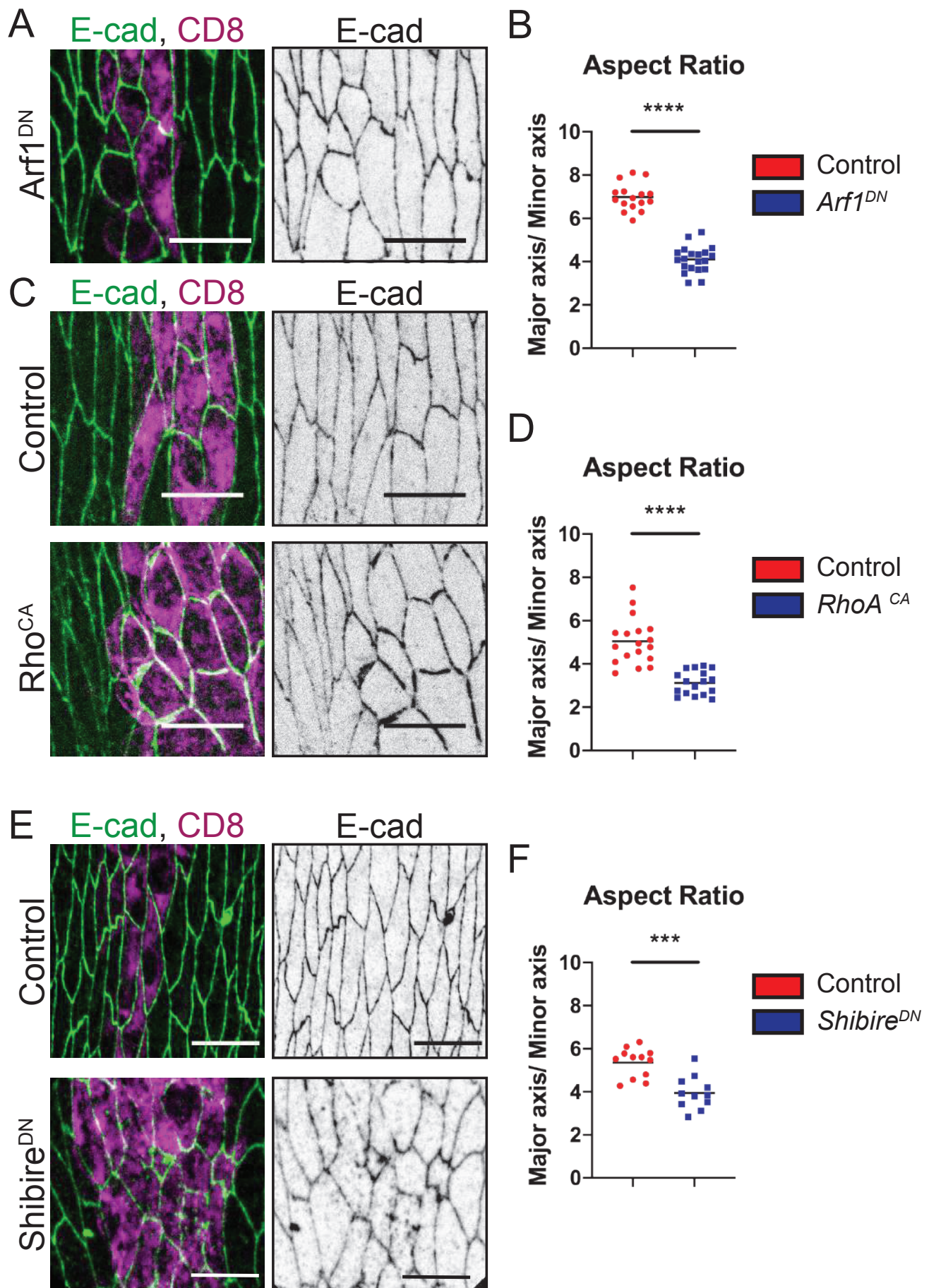
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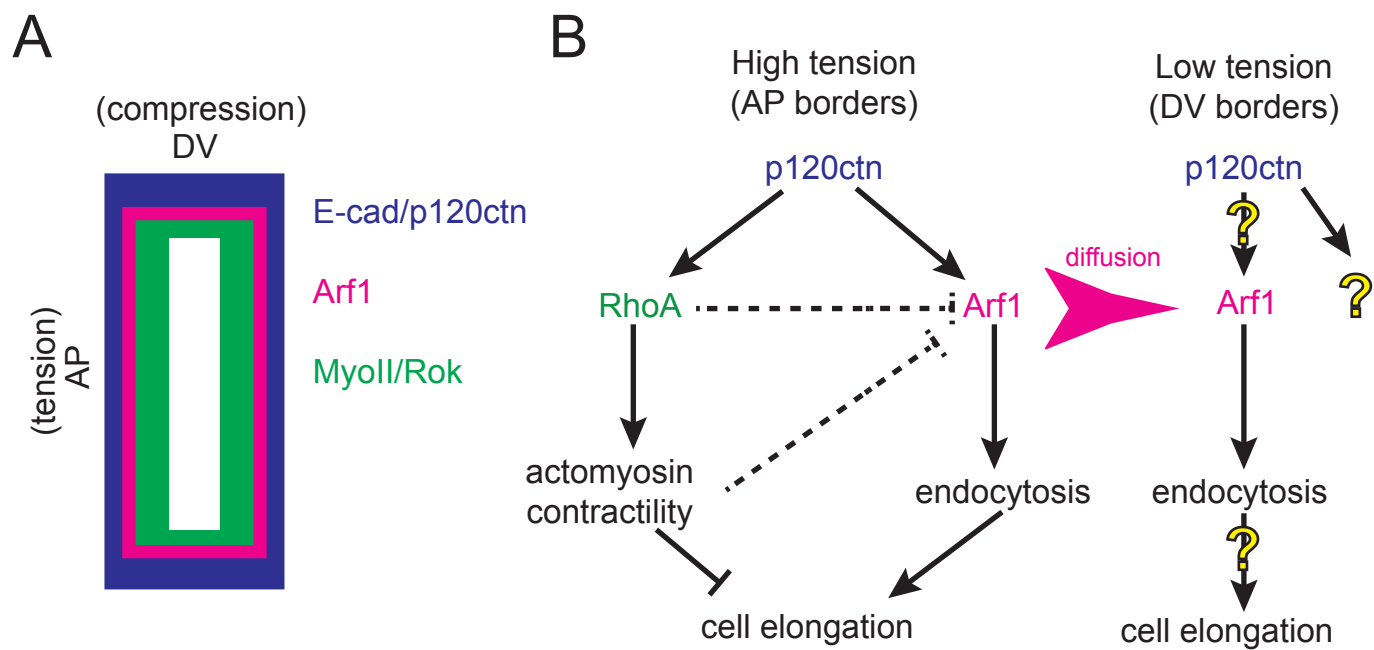
Fig. 7

Genotype	Aspect ratio	Amount at AP			Amount at DV		
		E-cad AP	MyoII AP	Arf1 AP	E-cad DV	MyoII DV	Arf1 DV
p120ctn OE	down	up	up	down	ns	ns	down
$p120ctn^{-/-}$	ns	down	down	down	down	ns	down
Rho^{DN}	ns	down	down	up	ns	down	up
Rho^{CA}	down	ns	up	down	up	up	down
$Arf1^{DN}$	down	ns	ns	down	ns	ns	down
$Arf1^{CA}$	ns	ns	ns	up	ns	ns	up
RhoGEF2RNAi	ns	down	down	up	down	ns	up
$p120ctn^{-/-}$; $Arf1^{CA}$	down			up			up
$Shibire^{DN}$	down	up			up		

Table 1. Summary of cell shape and protein distributions in the genotypes examined.

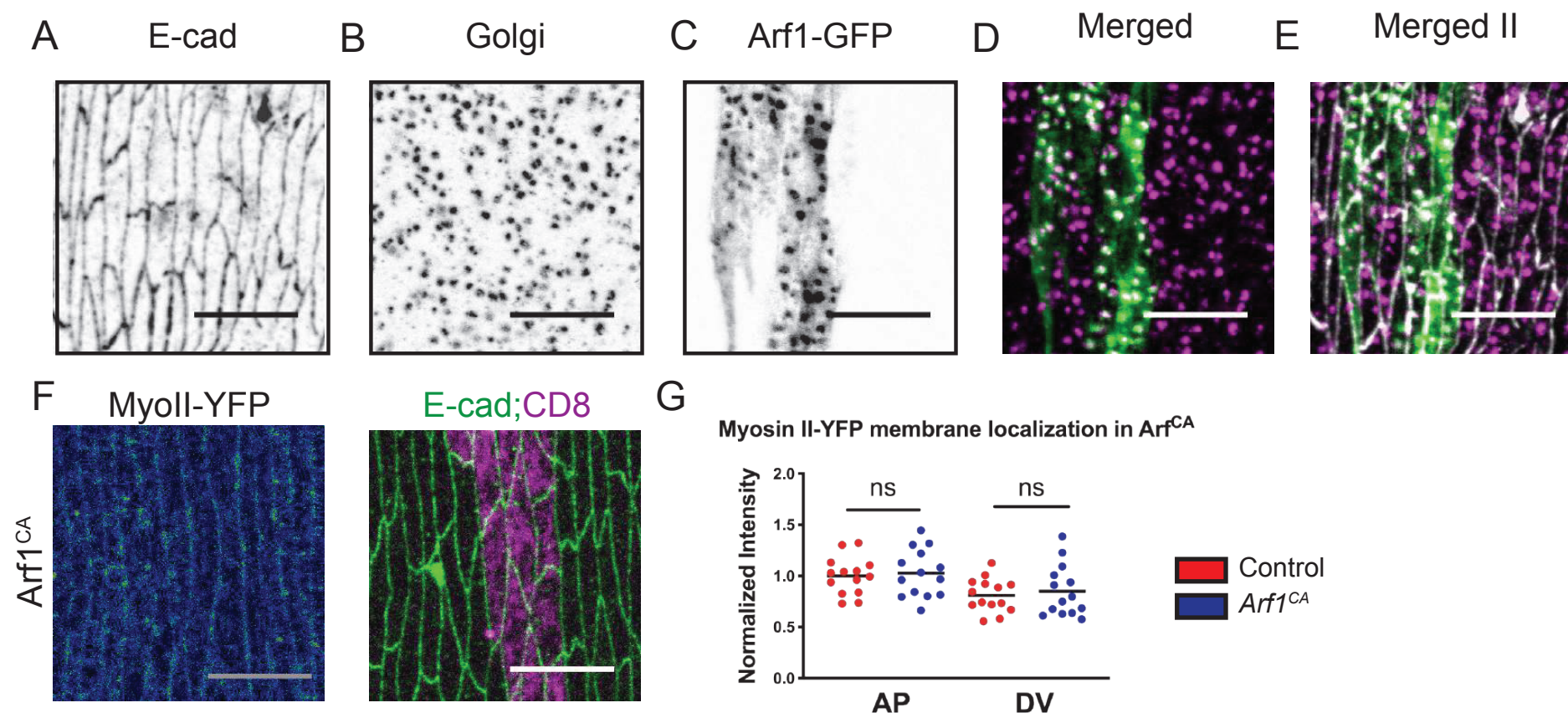**A**

Control   *p120ctn*^{-/-}   *UAS*::p120ctn

Rok-venus

B

Rok membrane localization

Normalized Intensity

AP   DV

**   ns

■ Control
■ *p120ctn*^{-/-}

C

Rok membrane localization

Normalized Intensity

AP   DV

**   ns

■ Control
■ *UAS*::p120ctn

Fig. S1

Fig. S2

| A E-cad | B Golgi | C Arf1-GFP | D Merged | E Merged II |

| F | MyoII-YFP | E-cad;CD8 | G Myosin II-YFP membrane localization in Arf^{CA} |

Fig. S3

A E-cad; CD8

B E-cad

C Cell Area

Fig. S4