

# RhoA effectors LOK/SLK activate ERM proteins to locally inhibit RhoA and define apical morphology

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1 **Abstract**

2

3 Activated Ezrin-Radixin-Moesin (ERM) proteins link the plasma membrane to the actin  
4 cytoskeleton to generate apical structures, including microvilli. Among many kinases implicated  
5 in ERM activation are the homologs LOK and SLK. CRISPR/Cas9 was used to knockout all  
6 ERM proteins or LOK/SLK in human cells. LOK/SLK knockout eliminates all ERM activating  
7 phosphorylation. The apical domain of cells lacking LOK/SLK or ERMs is strikingly similar and  
8 selectively altered, with loss of microvill, and junctional actin replaced by ectopic myosin-II  
9 containing apical stress-fiber-like structures. Constitutively active ezrin can reverse the  
10 phenotypes of either ERMs or LOK/SLK knockouts, showing that the major function of LOK/SLK  
11 is to activate ERMs. Both knockout lines have elevated active RhoA with concomitant enhanced  
12 myosin light chain phosphorylation, revealing that active ERMs are negative regulators of RhoA.  
13 As RhoA-GTP activates LOK/SLK to activate ERM proteins, the ability of active ERMs to  
14 negatively regulate RhoA-GTP represents a novel local feedback loop necessary for the proper  
15 apical morphology of epithelial cells.

16

17 **Introduction**

18

19 Essentially all eukaryotic cells are polarized, or have the potential to become polarized.  
20 This requires local regulation to define the morphology and composition of each cellular domain.  
21 A particularly well studied example is the intestinal epithelial cell with a highly ordered apical  
22 domain displaying abundant microvilli and having a protein and lipid composition distinct from  
23 the more planar basolateral membrane. While much is known about extrinsic cues that instruct  
24 the cell to polarize (Rodriguez-Boulan and Macara, 2014), much less is known about how the  
25 morphology of the individual domains is regulated. To address this, we have studied how the  
26 apical domain of epithelial cells is regulated to assemble bundles of actin filaments that support  
27 the plasma membrane of microvilli, in contrast to the flatter structure of the basolateral  
28 membrane.

29

30 The structural integrity of apical microvilli requires active ezrin/radixin/moesin (ERM)  
31 proteins (Fehon et al., 2010). These proteins exist in a cytoplasmic closed state and an active  
32 open conformation where the N-terminal FERM domain binds the plasma membrane and the C-  
33 terminal F-actin binding domain binds the underlying actin filaments (Gary and Bretscher, 1995).  
34 Activation requires phosphorylation of a conserved threonine, T567 in ezrin (Hayashi et al.,

35 1999; Matsui et al., 1998). The physiologically relevant kinase(s) responsible for ERM  
36 phosphorylation have been investigated for decades. Among the many kinases suggested are  
37 Rho kinase (Haas et al., 2007; Matsui et al., 1998; Tran Quang, 2000), PKC $\alpha$  (Ng et al., 2001);  
38 PKC $\theta$  (Pietromonaco et al., 1998), MST4 (Gloerich et al., 2012; ten Klooster et al., 2009), Nck-  
39 interacting kinase (Baumgartner et al., 2006) and LOK (Belkina et al., 2009; Viswanatha et al.,  
40 2012). More recent evidence has suggested that the related LOK and SLK are significant  
41 vertebrate kinases for ERM phosphorylation (Viswanatha et al., 2012), being the homologs of  
42 Slik that is responsible for phosphorylating the single ERM in flies, moesin (Hipfner et al., 2004).  
43 LOK and SLK belong to the germinal center-like kinase (GCK) -V subfamily of kinases  
44 (Kuramochi et al., 1997). They consist of a conserved N-terminal kinase domain, a less-  
45 conserved intermediate region and a moderately conserved C-terminal domain. The LOK C-  
46 terminal domain inhibits the kinase activity of LOK in cells, most likely through a cis-interaction,  
47 as well as targeting LOK to the apical membrane (Pelaseyed et al., 2017; Viswanatha et al.,  
48 2012). To maintain a strictly apical distribution, ezrin has to undergo  
49 phosphorylation/dephosphorylation cycles in which it is locally phosphorylated and subject to  
50 dephosphorylation by delocalized phosphatase Mypt1/PP1. As a result, constitutively active  
51 ezrin is unable to maintain apical restriction (Viswanatha et al., 2012).

52

53 The Rho family of small GTP-binding proteins are major regulators of microfilaments  
54 (Hall and Nobes, 2000). In early work Speck et al. (Speck et al., 2003) found that defects in fly  
55 moesin could be counteracted by antagonizing Rho activity, suggesting that ERM proteins might  
56 be able to regulate contractility in some manner. RhoA is capable of binding to a diverse range  
57 of effectors that influence the reorganization of actin into stress fibers and focal adhesions (Hall,  
58 1998). Among these, the effector ROCK modulates microfilament organization and function in at  
59 least two distinct ways. First, it activates myosin II by directly phosphorylating the myosin  
60 regulatory light chain (Kimura et al., 1996) and inactivating myosin light chain phosphatase  
61 (Mypt1/PP1) (Kimura et al., 1996). Second, ROCK stabilizes F-actin by phosphorylating LIM  
62 kinase to abrogate its inhibitory activity towards cofilin, an F-actin destabilizing factor (Arber et  
63 al., 1998; Maekawa et al., 2017; Yang et al., 1998). The net effect results in an increase of  
64 cortical tension that can drive cellular contraction. ERM proteins have long been suggested to  
65 be downstream targets of ROCK, first as direct targets and then as indirect targets through Rho  
66 activation of PI-4-P5K (Matsui et al., 1999).

67

68 Until very recently, the potential relationship between Rho and LOK and SLK had not  
69 been studied. A BiOLD screen for regulators and effectors of RhoA in cultured cells identified  
70 SLK (and LOK) as effectors of RhoA (Bagci et al., 2020). RhoA-GTP was shown to bind to the  
71 C-terminal domain of SLK, the corresponding domain that negatively regulates the kinase  
72 activity and targets LOK to the apical domain. Moreover, active RhoA can activate SLK in its  
73 ability to phosphorylate ezrin. Thus, a direct pathway exists from RhoA-GTP to its effector  
74 LOK/SLK to ERM protein activation.

75

76 Further uncovering the functions and exploring signaling pathways related to ERM  
77 proteins and LOK and SLK in cultured cells has been hampered by redundancy, and the  
78 possibility that they perform essential functions. Here we show that it is possible to generate  
79 cultured cells lacking all ERM proteins, and cells lacking both LOK and SLK. This has allowed  
80 us to explore the roles of these proteins, both by characterizing cells lacking them, but also by  
81 re-introduction of variants. Surprisingly, cells lacking LOK and SLK have a strikingly similar  
82 phenotype to cells lacking ERM proteins. Our results establish that LOK and SLK are the major,  
83 and possibly only, kinases that phosphorylate the conserved threonine of ERM proteins in  
84 epithelial cells. Further, rescue experiments indicate that ERM proteins are the major substrates  
85 of LOK and SLK that regulate cell morphology. Moreover, loss of either LOK/SLK or ERM  
86 proteins selectively modifies the apical actin cytoskeleton of epithelial cells by elevating RhoA  
87 signaling to redistribute actin from microvilli and junctions to generate ectopic apical stress  
88 fibers. We propose that LOK/SLK and ERM proteins function as a module that defines apical  
89 morphology of epithelial cells in a process that involves a negative feed-back loop to locally  
90 down-regulate Rho activity.

91

92

### 93 **Results**

94

#### 95 **Jeg3 cells lacking ERM proteins or LOK/SLK are viable and lack microvilli**

96

97 In earlier studies we described human cells modified by CRISPR/Cas9 to lack expression of  
98 LOK (Pelaseyed et al., 2017), but so far no cultured cells have been described that genetically  
99 lack specific ERM proteins. We set out to determine if cells lacking all ERM proteins, or lacking  
100 both LOK and SLK, are viable, and if so, what phenotypes were conferred. Our studies were  
101 performed in Jeg3 epithelial cells derived from a human choriocarcinoma as these cells exhibit

102 abundant apical microvilli (Pakkanen et al., 1987). Of the ERM proteins, Jeg3 cells express  
103 ezrin and radixin, but not moesin (Figure 1A). We first used CRISPR/Cas9 to isolate cells  
104 lacking either ezrin or radixin or SLK (Figure 1A). We then generated pairs of double knockout  
105 cells, lacking all detectable ezrin and radixin, or LOK and SLK (Figure 1A). Importantly, moesin  
106 was not expressed in the Jeg3 ezrin<sup>-/-</sup> radixin<sup>-/-</sup> cells—which can be identified as moesin is  
107 expressed in HeLa cells (Figure 1A, lane 1). Thus, the Jeg3 cells lacking all ERM proteins are  
108 viable. Likewise, Jeg3 cells lacking both LOK and SLK are viable. However, both ezrin<sup>-/-</sup> radixin<sup>-/-</sup>  
109 and LOK<sup>-/-</sup> SLK<sup>-/-</sup> cells grew slower than their wildtype counterparts (Figure S1A).

110

111 As ERM proteins and LOK and SLK have been implicated in the formation of microvilli  
112 on these cells, we examined whether single and double knockout cells retained microvilli. To  
113 assess the presence of microvilli, we could not use the traditional ERM proteins as markers, so  
114 we utilized labelled wheat germ agglutinin (WGA) that binds to plasma membrane glycoproteins  
115 and allows for the identification of cell surface structures. In wildtype cells, WGA colocalizes with  
116 ezrin in surface microvilli (Figure 1B). Consistent with earlier reports, individual reduction of  
117 ezrin or genetic loss of LOK resulted in a reduction in the number of cells with apical microvilli,  
118 whereas loss of radixin or SLK had little phenotype (Figure 1C, Figure S1B). Strikingly ezrin<sup>-/-</sup>  
119 radixin<sup>-/-</sup> and LOK<sup>-/-</sup> SLK<sup>-/-</sup> cells totally lack microvilli, with WGA staining mostly associated with  
120 membrane ruffles that form above membrane contact sites (Figure 1B, arrows and 1C). Ezrin is  
121 cytosolic in LOK<sup>-/-</sup> SLK<sup>-/-</sup> cells, consistent with phosphorylation by LOK/SLK being required to  
122 activate it. Stable expression of ezrin in ezrin<sup>-/-</sup> radixin<sup>-/-</sup> cells and LOK in LOK<sup>-/-</sup>/SLK<sup>-/-</sup> restored  
123 the presence of microvilli to the cell surface. We conclude that both an ERM protein and LOK or  
124 SLK are necessary for the presence of apical microvilli. We also examined the localization of the  
125 interactor of active ezrin, EBP50 (ERM-binding phosphoprotein of 50kD)/NHERF1 (Figure 1D).  
126 Whereas EBP50 was present in microvilli in wildtype cells, in both double knockout cell lines  
127 EBP50 was cytosolic. Both the WGA and EBP50 staining supports the conclusion that the loss  
128 of ERM or LOK/SLK disrupts the presence of microvilli from the apical surface.

129

### 130 **LOK/SLK are the major kinases for ERM phosphorylation**

131

132 ERM proteins undergo activation by C-terminal phosphorylation to exhibit their  
133 membrane-cytoskeletal linking function (Pearson et al., 2000; Turunen et al., 1994). The identity  
134 of the relevant kinase has been controversial, so we assessed the contribution of LOK and SLK  
135 to ERM phosphorylation in LOK<sup>-/-</sup> SLK<sup>-/-</sup> cells. Using an antibody that detects the relevant

136 epitope on all ERM proteins (phosphorylation of T567 in ezrin, T564 in radixin, and T558 in  
137 moesin) we found that LOK<sup>-/-</sup> SLK<sup>-/-</sup> cells appeared to be entirely devoid of all ERM  
138 phosphorylation (Figure 2A). However, because the pERM antibody produces a small  
139 background staining to unphosphorylated ezrin (Pelaseyed et al., 2017), we repeated the  
140 experiment employing phos-Tag gels in which phosphorylated ERMs migrate slower than their  
141 unphosphorylated counterparts. Again, we were unable to detect any phosphorylated ERM  
142 proteins in the absence of LOK and SLK (Figure 2B), so LOK and SLK appear to be the only  
143 significant ERM kinases in Jeg3 cells.

144

145 To explore if this is true in other cells, we also generated LOK<sup>-/-</sup> SLK<sup>-/-</sup> knockouts in HeLa  
146 cells. While these cells survived clonal isolation and lysate collection, they grew very slowly and  
147 could not be maintained. Nevertheless, analysis of cell lysates revealed that in HeLa cells as  
148 well, LOK and SLK are the major ERM kinases (Figure 2A). Further, we also tried to knock out  
149 LOK and SLK in epithelial Caco-2 cells, which proved inviable past clonal selection indicating  
150 the importance of these kinases. Likewise, we tried to isolate HeLa and Caco-2 cells lacking  
151 ezrin. Although we nursed the growth of enough cells to demonstrate loss of ezrin, they could  
152 not be passaged. These results suggest that ERM proteins are more important for viability in  
153 HeLa and Caco-2 cells than in Jeg-3 cells. It was fortuitous that we started with Jeg-3 cells as it  
154 has allowed us to use the double knock-outs to explore in more detail the phenotypes conferred  
155 by loss of these proteins, or reintroduction of variants.

156

157 In transfection-based experiments, expression of LOK-GFP-Flag was able to both  
158 restore ERM phosphorylation and apical microvilli to LOK<sup>-/-</sup> SLK<sup>-/-</sup> cells (Figures 1B&C, 2D).  
159 LOK contains an N-terminal kinase domain and C-terminal domain responsible for both  
160 regulating and localizing the kinase (Pelaseyed et al., 2017). As expected, expression of a  
161 K65R kinase-inactive variant of LOK was unable to restore phosphorylation or microvilli (Figure  
162 2C,D).

163

164 **Absence of activated ERM proteins induces the formation of apical actin/myosin II  
165 bundles and alters cell-cell junctions of epithelial cells**

166

167 To explore what additional phenotypes might be associated with loss of ERM proteins  
168 and their activating kinases, we examined the structure of cell junctions and the actin  
169 distribution in the knockout cells. A characteristic feature of epithelial cells is their ability to form

170 strong cell-to-cell contact sites. Using the tight junction marker ZO-1, we observe a remarkably  
171 similar phenotype in *ezrin*<sup>-/-</sup> *radixin*<sup>-/-</sup> and *LOK*<sup>-/-</sup> *SLK*<sup>-/-</sup> cells. In both cases, the cells maintain cell-  
172 to-cell contacts, but the contact sites are uneven and occasionally have breaks in their ZO-1  
173 and greatly reduced junctional actin staining (Figure 3A, magenta arrows). In contrast, wildtype  
174 Jeg3 cells form uniform and relatively linear contacts along cell junctions co-staining with actin  
175 (Figure 3A, blue arrows). By determining the ratio between the contour length and shortest  
176 distance in ZO-1 staining from a three-cell junction to the next, we quantified the straightness of  
177 the tight junctions in cells under various conditions (Figure 3B). This measurement of tortuosity  
178 was slightly above 1.0 for wildtype cells, reflecting their almost linear nature. For either *ezrin*<sup>-/-</sup> or  
179 *LOK*<sup>-/-</sup> this rose to 1.15, and for *ezrin*<sup>-/-</sup> *radixin*<sup>-/-</sup> and *LOK*<sup>-/-</sup>/*SLK*<sup>-/-</sup> cells to about 1.2, indicating  
180 greater distortion/waviness of the interface. As with loss of microvilli, loss of just *ezrin* or *LOK* is  
181 more severe than loss of just *radixin* or *SLK*, and both double mutants exhibit the most severe  
182 phenotype. This tortuosity of the junctions was rescued by expressing *ezrin* in the *ezrin*<sup>-/-</sup> *radixin*<sup>-/-</sup>  
183 cells and *LOK* in the *LOK*<sup>-/-</sup> *SLK*<sup>-/-</sup> cells (Figure 3A).

184

185 We also noticed an additional striking effect on the actin distribution in which significantly  
186 more F-actin is seen spanning the apical domain in the double knockout cells compared to their  
187 wild type counterpart. Therefore, we examined the F-actin distribution in the basal and apical  
188 regions of the cells. ZO-1 localization is found near the apical region of both wildtype and  
189 knockout cells, so we split confocal planes into those containing ZO-1 staining and above (the  
190 apical domain), and those below ZO-1 staining (the basolateral domain). Whereas the wildtype  
191 cells display actin in microvilli and along the cell junctions, both *ezrin*<sup>-/-</sup> *radixin*<sup>-/-</sup> and *LOK*<sup>-/-</sup>/*SLK*<sup>-/-</sup>  
192 cells were noticeably different with actin bundles extending across the apical domain that  
193 correlate with the uneven contour of the tight junctions (Figure 3A). In contrast, there is no  
194 apparent difference in the basal actin network between the cells (Figure 3A), which is  
195 underscored by the similarity of focal contacts at the basal surfaces (Figure S2A). We quantified  
196 this difference by measuring the ratio of the actin intensity at the apical versus the basal side of  
197 the cells (Figure 3C). Both KO cells were found to have an increase in the density of actin found  
198 exclusively at the apical surface compared to wildtype cells (Figure 3A and C). The aberrant  
199 apical actin bundles are rescued by introduction of *ezrin* into *ezrin*<sup>-/-</sup> *radixin*<sup>-/-</sup> and of *LOK* into  
200 *LOK*<sup>-/-</sup>/*SLK*<sup>-/-</sup> cells (Figure 3 A-C).

201

202 The apical actin structures seen in *ezrin*<sup>-/-</sup> *radixin*<sup>-/-</sup> and *LOK*<sup>-/-</sup> *SLK*<sup>-/-</sup> cells contained  
203 bundles, similar in appearance to stress fibers. The expression level of the stress fiber marker  $\alpha$

204 -actinin and focal adhesion marker vinculin were unchanged in the knockout cells (Figure S3).  
205 We next explored if these bundles could have contractile properties by staining for non-muscle  
206 myosin-IIb (myo-IIb). While striated bundles of myo-IIb and actin were not completely absent in  
207 the apical region of wildtype cells, a stronger intensity and more frequent clusters of myo-IIb  
208 was observed in ezrin<sup>-/-</sup> radixin<sup>-/-</sup> cells (Figure 4A, arrows). This phenotype was also seen and  
209 enhanced in LOK<sup>-/-</sup>SLK<sup>-/-</sup> cells where the entire apical domain appeared covered in myo-IIb  
210 puncta and actin filaments. Using deconvolution microscopy, we could see characteristic  
211 myosin-II sarcomeric-like striations indicative of a contractile structure (Figure 4B). Together our  
212 results show that lack of activated ERMs, either due to loss of the proteins or their activating  
213 kinases, selectively redistributes actin in the apical domain into contractile bundles.  
214

215 **Enhanced actin assembly in the apical domain is responsible for the junctional defects of  
216 knockout cells**

217  
218 In wildtype cells, apical actin in the microvilli treadmills continuously resulting in turnover  
219 in the ~2-10 min time-frame (Loomis et al., 2003; Meenderink et al., 2019). We investigated the  
220 possibility that the abnormal actin bundles seen in knockout cells might arise in part from  
221 reduced actin turnover in the apical domain. To test this, we treated wildtype cells with the actin  
222 stabilizing drug Jasplakinolide to reduce actin turnover. Remarkably, treatment of wildtype cells  
223 with 500nM Jasplakinolide for 30 minutes induced both the formation of actin bundles in the  
224 apical domain and increased the tortuosity of the tight junctions (Figure 5A and B, lanes 1 and  
225 2) in a manner closely resembling the phenotype of ezrin<sup>-/-</sup> radixin<sup>-/-</sup> or LOK<sup>-/-</sup> SLK<sup>-/-</sup> cells (Figure  
226 3A). The addition of Jasplakinolide in all three cell lines further increased the abundance of  
227 apical F-actin networks across each cell, correlating with the increased tight junction tortuosity  
228 and breaks between neighboring cells (Figure 5A, magenta arrows and C).  
229

230 If enhanced actin assembly in the double knockout cells is responsible for the observed  
231 tortuosity of the junctions, increasing depolymerization in these cells might rescue this  
232 phenotype. Upon treatment of either of the double-knockout cells with the actin-depolymerizing  
233 drug Latrunculin B or actin plus-end-capping drug Cytochalasin D, tight junction tortuosity ratios  
234 in the knockout cells were restored to levels comparable to wildtype cells (Figure 5A, B and  
235 S5A). Interestingly, Latrunculin B treatment of cells did not completely restore the balance of F-  
236 actin between the apical and basolateral domains to wild type levels (Figure 5C). Analysis of the  
237 apical/basolateral actin distribution after these drug treatments was complicated by the finding

238 that apical actin appears to be more resistant to disassembly by Latrunculin B than its  
239 basolateral counterpart (Figure S5B). Nonetheless, in contrast to Jasplakinolide-treated cells,  
240 Latrunculin B treatment results in reorganization of actin towards junctions Figure 5A, blue  
241 arrows) and relief of junctional defects (Figure 5B). Meanwhile, stabilization by Jasplakinolide  
242 redistributes actin away from the junctions and across the cell promoting junctional defects  
243 (Figure 5A and B). In summary, our data shows ezrin<sup>-/-</sup> radixin<sup>-/-</sup> and LOK<sup>-/-</sup> SLK<sup>-/-</sup> cells become  
244 more similar to wildtype when treated with Latrunculin B, while wildtype cells become more like  
245 the knockout cells when treated with Jasplakinolide. Therefore in the absence of active ERMs,  
246 excessive actin assembly generates contractile bundles that provide forces perpendicular to  
247 junctions.

248

249 To assess how the actin redistribution might affect the mechanics of the apical surface,  
250 we utilized Atomic Force Microscopy (AFM) to measure their stiffness. Cells were allowed to  
251 grow to a confluent monolayer and then indented to a maximum force of 1nN (Figure 5D inset).  
252 The resulting force vs. distance curves were then used to identify the initial AFM probe tip  
253 contact point, allowing us to fit to a hertz equation to calculate the Young's modulus (E) stiffness  
254 parameter (Huth, Sindt, & Selhuber-Unkel, 2019). Indentations from each condition were then  
255 averaged to produce a mean curve for wild type, ezrin<sup>-/-</sup> radixin<sup>-/-</sup> and LOK<sup>-/-</sup> SLK<sup>-/-</sup> cells (Figure  
256 5D). Of the 1,091 total force indentations preformed, both LOK<sup>-/-</sup> SLK<sup>-/-</sup> (E = 260±45 kPa  
257 Median±) and ezrin<sup>-/-</sup> radixin<sup>-/-</sup> (E = 166±52 kPa) cells are significantly stiffer than WT cells (E =  
258 117±65 kPa) (Figure 5E). The finding that LOK<sup>-/-</sup>SLK<sup>-/-</sup> cells are more rigid than ezrin<sup>-/-</sup> radixin<sup>-/-</sup>  
259 cells is consistent with the enhanced levels of stress-fiber-like bundles seen in the apical  
260 domain of LOK<sup>-/-</sup>SLK<sup>-/-</sup> cells described in Figure 4. Together these results indicate that activated  
261 ERM proteins suppress the formation of apical actin bundles to affect the mechanical properties  
262 of the apical domain.

263

#### 264 **Activated ezrin Rescues both ezrin<sup>-/-</sup> radixin<sup>-/-</sup> and LOK<sup>-/-</sup>SLK<sup>-/-</sup> cells**

265

266 The phenotypic similarity of the ezrin<sup>-/-</sup> radixin<sup>-/-</sup> and LOK<sup>-/-</sup> SLK<sup>-/-</sup> cells, suggested that  
267 essentially all the phenotypes of LOK<sup>-/-</sup> SLK<sup>-/-</sup> cells are due to the lack of ERM phosphorylation.  
268 If this is the case, introduction of mutationally activated ezrin in either of the double knockout  
269 cells should rescue them in a similar manner. We therefore introduced the constitutively active  
270 phosphomimetic ezrin-T567D mutant into both ezrin<sup>-/-</sup> radixin<sup>-/-</sup> and LOK<sup>-/-</sup>/SLK<sup>-/-</sup> cells.  
271 Remarkably, phosphomimetic ezrin suppressed both equally with restoration of microvilli (Figure

272 6A). The rescue isn't perfect, as constitutively active ezrin is seen both apically and basolaterally  
273 because restriction to the apical domain requires ezrin phosphocycling (Viswanatha et al.,  
274 2012). Expression of constitutively active phosphomimetic ezrin-T567D is also able to suppress  
275 tight junction defects and the excess apical actin bundles seen in knockout cells implying that  
276 active ezrin can regulate actin at the cortex (Figure 6B-D). These results suggest that the  
277 primary role for LOK and SLK in Jeg3 cells is to phosphorylate ERMs.  
278

## 279 **Phosphorylated ERM proteins regulate myosin-II activity through RhoA Activation**

280

281 The appearance of apical stress-fiber-like cables in cells lacking activated ERMs is  
282 suggestive of local enhanced RhoA activity. Rho-associated kinase (ROCK) is a major effector  
283 of RhoA-GTP that can activate myosin-II by directly phosphorylating myosin light chain 2  
284 (MLC2), and this is counteracted by the phosphatase PP1 utilizing the Mypt1 subunit. Total non-  
285 muscle myosin-II expression showed no differences between wild type and LOK<sup>-/-</sup>SLK<sup>-/-</sup> or ezrin<sup>-/-</sup>  
286 radixin<sup>-/-</sup> cells (Figure S2B). The level of endogenous MLC2 phosphorylation was found to be  
287 very low in wildtype cells and 3-4 fold enhanced in both ezrin<sup>-/-</sup> radixin<sup>-/-</sup> and LOK<sup>-/-</sup>SLK<sup>-/-</sup> cells  
288 (Figure 7A and B). To increase the level of phosphorylation, we treated cells briefly with  
289 calyculin A that inhibits the phosphatase PP1. Treatment of wildtype cells with calyculin A  
290 enhanced the level of MLC2 phosphorylation, and greatly enhanced the level seen in both of the  
291 knockout lines. These results reveal that activated ERM proteins negatively regulate  
292 phosphorylation of MLC2 and hence myosin-II activation.  
293

294

295 MLC2 phosphorylation is either mediated by ROCK, or via Ca<sup>2+</sup>-calmodulin-dependent  
296 myosin light chain kinase. To explore which pathway is involved, we treated wildtype and double  
297 knockout cells with the specific ROCK inhibitor Y-27632. This resulted in equalization of  
298 phospho-MLC2 levels (Figure 7C), indicating that the enhanced level of phospho-MLC2 seen in  
299 knockout cells is upstream of ROCK. We therefore measured the level of endogenous RhoA-  
300 GTP by passing total cell lysates over Rhotekin-GST beads and measuring the relative levels of  
301 RhoA retained. Both ezrin<sup>-/-</sup> radixin<sup>-/-</sup> and LOK<sup>-/-</sup>SLK<sup>-/-</sup> cells were found to have about a 3-fold  
302 higher level of active RhoA than wildtype cells (Figure 7D). In addition to regulating MLC2  
303 phosphorylation, ROCK also regulates actin dynamics through the LIM kinase-cofilin pathway  
304 (Arber et al., 1998; Maekawa et al., 2017; Yang et al., 1998), which may explain in part the  
305 altered actin dynamics discussed earlier.

306

306 A recent report showed that active RhoA binds directly to the C-terminal domain of SLK  
307 and this promotes its dimerization and ability to phosphorylate ERM proteins (Bagci et al.,  
308 2020). In earlier work we showed that expression of the C-terminal region of LOK (LOK\_310-  
309 968-GFP-Flag or LOK-CTD-GFP-Flag) acts as a potent dominant negative to strongly inhibit  
310 phosphorylation of ERM proteins (Viswanatha et al., 2012). To explore if this region of LOK  
311 binds to RhoA, we expressed LOK-CTD-GFP-Flag either alone or with constitutively active HA-  
312 RhoA-L30 and immunoprecipitated with Flag antibodies in WT Jeg3 cells (Figure 7E). RhoA  
313 was recovered in the Flag immunoprecipitates, indicating an interaction between the two.  
314 Therefore, both LOK and SLK are effectors of RhoA.

315  
316 Collectively these results suggest a model in which RhoA selectively regulates the apical  
317 domain of epithelial cells in a negative feedback loop involving active ERM proteins (Figure 7F).  
318 RhoA activates both the kinases LOK/SLK and ROCK to mediate phosphorylation of ERM  
319 proteins and MLC2, respectively. ROCK also negatively regulates PP1, the phosphatase that  
320 dephosphorylates both pMLC and pERMs. Phosphorylated ERMs localized exclusively in the  
321 apical domain regulate RhoA in a negative feedback loop.

322  
323 **Phosphorylated ERMs negatively regulate myosin contractility**  
324

325 If the model presented in Figure 7F is valid, a prediction is that a functional difference in  
326 contractile force production should exist between wildtype and knockout cells. It is known that  
327 cultured cells treated with calyculin A to elevate the level of myosin-II activity will ultimately  
328 contract (Ishihara et al., 1989). Therefore, we examined the contraction of spread LOK<sup>-/-</sup>SLK<sup>-/-</sup>  
329 and ezrin<sup>-/-</sup>radixin<sup>-/-</sup> cells compared with wildtype cells in the presence of 10nM Calyculin A over  
330 the course of 1 hour. Under this treatment a small fraction of WT cells showed rounding and  
331 detachment from the plate. In a dramatic difference, LOK<sup>-/-</sup>SLK<sup>-/-</sup> and ezrin<sup>-/-</sup>radixin<sup>-/-</sup> cells  
332 showed significant rounding and detachment from neighboring cells in under 30 minutes (Figure  
333 8A, Movie S1). We confirmed that the contractility is a result of enhanced myosin-II activity as  
334 the calyculin A induced contraction was prevented by inclusion of the myosin-II inhibitor  
335 blebbistatin, which showed no rounding or contraction in any cell type after 1 hour (Figure 8B,  
336 Movie S2). These results are in agreement with Figure 7A showing an increased level of MLC  
337 phosphorylation in the knockout cells in the presence of calyculin A. However, calyculin A also  
338 results in enhanced phosphorylation of ERMs (Viswanatha et al., 2012), so we wished to  
339 examine if the contractility difference induced by calyculin A was present in cells where the level

340 of active ezrin was unchanged. To achieve this, we stably expressed the active phosphomimetic  
341 ezrin-T567E in double knockout cells. Under these conditions both LOK<sup>-/-</sup> SLK<sup>-/-</sup> and ezrin<sup>-/-</sup>  
342 radixin<sup>-/-</sup> cells expressing ezrin-T567E the contraction induced by calyculin A was greatly  
343 reduced (Figure 8B, Movie S3). This implies that it is the loss of active ERM, and not the loss  
344 of LOK and SLK independent of ERM, that regulates the contractility due to enhanced RhoA-  
345 GTP.

346

347

## 348 **Discussion**

349

350 In this study we have investigated the phenotypes conferred by loss of ERM proteins, or  
351 their activators, LOK and SLK. In some cells loss of LOK and SLK, or in fact just ezrin in HeLa  
352 or Caco-2 cells, are too unhealthy to maintain, indicating that ERM proteins and their activating  
353 kinases can be almost essential. This is consistent with the situation in mice, flies and the  
354 nematode worm, where loss of ezrin or the single ERM protein (flies and worm), is lethal  
355 (Jankovics et al., 2002; Saotome et al., 2004). Additionally, the loss of SLK in mouse or the  
356 single homolog Slik in the fly is also lethal (Hipfner and Cohen, 2003). Loss of just LOK in the  
357 mouse has a more modest phenotype (Belkina et al., 2009), presumably due to partial  
358 compensation by SLK. We were fortunate to start with a cell line, Jeg-3, that can tolerate loss of  
359 either all ERM proteins, or both LOK and SLK, as this allowed us to study their phenotypes. As  
360 far as we are aware, these are the first vertebrate cells isolated genetically lacking either all  
361 ERM proteins or the kinases LOK and SLK.

362

363 Previous studies have shown that wildtype epithelial cells maintain ~50% of their ezrin in  
364 the active, phosphorylated state with cycling between active and inactive states occurring on the  
365 scale of ~2 minutes (Viswanatha et al., 2012). Proper preservation of this balanced system is  
366 critical for maintaining a polarized morphology (Viswanatha et al., 2012) and is consistent with  
367 mislocalization, overexpression, and hyper-phosphorylation of ezrin found in types of human  
368 cancers (reviewed in Clucas and Valderrama, 2015). Our finding that loss of LOK and SLK  
369 results in ablation of all detectable ezrin-T567 phosphorylation is consistent with LOK and SLK  
370 being the major, if not only, kinases that can phosphorylate the ERM regulatory threonine (T567  
371 in ezrin). This result is in agreement with earlier descriptions that Slik is solely responsible for  
372 the activation of fly moesin (Hipfner et al., 2004).

373

374        A remarkable aspect of our results is that cells lacking either all ERM proteins or LOK  
375 and SLK are phenotypically very similar. Both grow slower, have lost all apical microvilli, have  
376 greatly reduced junctional actin but also an aberrantly high level of apical F-actin and myosin,  
377 have wavy cell junctions, have a stiffer apical domain, contract abnormally in the presence of  
378 the phosphatase calyculin A, and both have elevated levels of Rho-GTP. This raises the  
379 question whether the ERM proteins are the sole substrate of LOK/SLK. An intricate multi-step  
380 mechanism is involved in phosphorylation of ezrin by LOK. In outline, it requires priming of ezrin  
381 by PIP<sub>2</sub>, then insertion of the C-terminal region of LOK between the ezrin FERM and C-terminal  
382 F-actin binding domain, which gives access for the kinase domain to bind a recognition site and  
383 ultimately phosphorylate T567 (Pelaseyed et al., 2017). This mechanism, together with the  
384 strong preference for a tyrosine two residues upstream of the targeted threonine (Belkina et al.,  
385 2009), makes phosphorylation of ERM proteins highly selective. This elaborate coincidence  
386 detection mechanism involved in LOK phosphorylation of ezrin coupled with the ability of  
387 mutationally active ezrin (ezrin-T567D) to suppress the phenotype of LOK/SLK cells strongly  
388 supports the notion that ERM proteins are the major functional substrates. Using a phospho-  
389 proteomics approach in unpublished work, we sought to identify additional LOK/SLK substrates.  
390 While we encountered many phosphopeptides whose level was elevated in wild type cells  
391 compared with LOK/SLK knockout cells, none had the appropriate LOK/SLK consensus  
392 sequence. Therefore, LOK/SLK and ERM proteins appear to work together as a functional unit.  
393 Although we were not able to identify additional LOK/SLK substrates, technical limitations may  
394 have obscured them from our analysis. In support of this possibility, the phenotypes of LOK/SLK  
395 cells enumerated above were almost always more severe than in the ERM knockout cells. Thus,  
396 the possibility remains that there is another minor substrate, functionally redundant with ERM  
397 proteins, cannot be ruled out.

398

399        While we expected to see loss of microvilli in cells lacking ERM proteins, we were  
400 surprised to encounter an extensive actin/myosin-II network in the apical domain replacing the  
401 junctional and microvillar actin. This phenotype was also seen in LOK/SLK knockout cells,  
402 implying that active ERM proteins can regulate the myosin and actin distribution in the apical  
403 domain. RhoA-GTP is known to positively regulate non-muscle myosin-II activity through  
404 ROCK1 as well as F-actin turnover through LIM kinase and cofilin. Since both actin turnover and  
405 myosin contractility are altered in the absence of pERMs, we then considered a possible over-  
406 activation of RhoA in the knockout cells. Indeed, the level of RhoA-GTP was significantly

407 elevated in both ERM and LOK/SLK knockout cells. Thus, active ERMs are negative regulators  
408 of RhoA (Figure 7F).

409

410 There have been several indications of a connection between ERM protein function and  
411 RhoA. First, ROCK1 was reported as the kinase that phosphorylates ERM proteins, in part  
412 because ROCK1 over expression resulted in formation of microvilli in cos-7 cells and its  
413 reported ability to phosphorylate radixin *in vitro* (Matsui et al., 1998; Oshiro et al., 1998). With  
414 the finding that Mypt1/PP1 is the phosphatase responsible for dephosphorylating ERM proteins  
415 (Fukata et al., 1998), and that Mypt1/PP1 is negatively regulated by ROCK1 (Velasco et al.,  
416 2002), the earlier result can be explained. In a genetic study in the fly, phenotypes resulting  
417 from loss of moesin can be rescued by also reducing the level of RhoA (Speck et al., 2003).  
418 Recent work has shown that RhoA-GTP directly dimerizes and activates SLK (Bagci et al.,  
419 2020). As phosphorylated ERM proteins act as negative regulators of RhoA-GTP, a local  
420 feedback cycle exists in which RhoA-GTP and pERMs regulate each other's activity specifically  
421 across the apical domain (Figure 7F).

422

423 RhoA is a well recognized spatial regulator of the adherens junctions between epithelial  
424 cells (reviewed in Hartsock and Nelson, 2008; Marjoram et al., 2014; McCormack et al., 2013).  
425 Active RhoA is highly enriched at junctions through the action of various regulators, including  
426 the RhoA-GEFs p114RhoGEF and ECT2, and indirectly through active myosin IIA recruitment of  
427 ROCK1 to phosphorylate and inactivate the ability of Rnd3 to recruit p190B RhoA-GAP  
428 (Ratheesh et al., 2012; Reyes et al., 2014; Terry et al., 2011). Misregulation of RhoA can affect  
429 the mechanical tension between junctions of neighboring epithelial cells (Zihni et al., 2014).  
430 Indeed, this is what we observe by knocking out active ERMs. As a consequence of removing  
431 active ERMs, we find that F-actin bundles are re-distributed away from the junctions and into  
432 actomyosin networks across the apical terminal web. This state can be mimicked in wildtype  
433 epithelial cells by introducing an actin stabilizing drug like Jasplakinolide. Conversely, the actin  
434 in knockout cells can be re-directed back towards junctions by adding actin depolymerizing drug  
435 Latrunculin B. A similar phenomenon is seen when anillin is manipulated in epithelial cells,  
436 where anillin knockdown cells produce low tensile forces and overexpression produced high  
437 tensile forces due to misregulation of medial-apical F-actin (Arnold et al., 2019). Thus, local  
438 spatial regulation of proteins like ERMs and anillin can influence the RhoA-dependent actin  
439 turnover at the apical domain and junctions of epithelial cells. As LOK is an effector of active  
440 RhoA and is located to the microvilli, and not the cell-cell junctions, a subpopulation of RhoA

441 must be specifically regulated at across the apical surface of epithelial cells. The activity of this  
442 local RhoA subpopulation is negatively regulated by phosphorylated ERM proteins, presumably  
443 through either a Rho-GEF or a Rho-GAP. In the fly RhoGAP Conundrum has been shown to  
444 bind moesin and act as a negative regulator of Rho1. However, since deletion of Conundrum  
445 has no obvious phenotype there is likely functional redundancy with another RhoGAP (Neisch et  
446 al., 2013). Our future work will be aimed at identifying the factor(s) that allow active ERMs to  
447 mediate local negative regulation of RhoA-GTP.

448 A more distant member of the ERM protein family is merlin, the product of the tumor  
449 suppressor gene (*NF2*) in which defects result in Neurofibromatosis-II (Sanson et al., 1993;  
450 Trofatter et al., 1993). Merlin has an N-terminal FERM domain and a negative regulatory C-  
451 terminal. The FERM domain of merlin can bind the C-terminal domain of ERM proteins, and vice  
452 versa (Bretscher et al., 2000). Considerable work suggests that ERM proteins and merlin act  
453 antagonistically, especially in the organization of the plasma membrane during endocytosis  
454 (Chiasson-Mackenzie et al., 2018; Hebert et al., 2012). It will be interesting to explore if active  
455 merlin can regulate RhoA activity, or if merlin-deficient cells that fail to modulate the activity of  
456 ERM proteins have a lower level of RhoA-GTP.

457 In summary, we have found that ERM proteins and their activators LOK/SLK function in  
458 the same pathway as a unit to build microvilli on the apical surface of epithelial cells and locally  
459 modulate the level of RhoA-GTP in a feedback inhibition cycle. The next challenge will be to  
460 understand how this feedback loop is regulated and restricted to the apical domain.

461

## 462 **Materials and Methods**

### 463 **Reagents & cDNAs**

464 Ezrin antibodies were either a mouse anti-ezrin antibody (CPCT-ezrin-1 supernatant  
465 concentrate obtained from the Developmental Studies Hybridoma) used at 1:1,250 (Western  
466 blot) or 1:100 (immunofluorescence) or a previously characterized rabbit polyclonal antibody  
467 raised against full-length human ezrin (Bretscher, 1989) and used at 1:1,000 (Western blot) or  
468 1:200 (immunofluorescence). Rabbit anti-EBP50 was also a previously characterized antibody  
469 (Reczek et al., 1997) and was used at 1:50 (immunofluorescence). Mouse anti-ZO1 (BD  
470 Biosciences, Cat #610966) was used at 1:100 (immunofluorescence). Rabbit anti-radixin (Cell

471 Signaling, Cat #C4G7) used at 1:1000 (Western blot) to blot for both radixin (80kDa) and  
472 moesin (75kDa). Rabbit anti-pERM (raised against re-combinant phosphopeptide) was used at  
473 1:1000 (Western blot). Rabbit anti-LOK used at 1:500 (western blot) and rabbit anti-SLK used at  
474 1:100 (Western blot) were purchased from Bethyl Laboratories, Inc. Mouse anti-Flag used at  
475 1:5,000 (Western blot) and mouse anti-tubulin used at 1:5,000 (Western blot) were obtained  
476 from Sigma-Aldrich. Mouse anti-GFP used at 1:100 (immunofluorescence) was obtained from  
477 Santa Cruz Biotechnology, Inc. Rabbit antibodies for anti-myosin-light-chain-2 (Cat #3672) and  
478 anti-phospho-myosin-light-chain-2 (raised against phospho-Thr18/Ser19; Cat #3674) were  
479 purchased from Cell Signaling and used at 1:500 (Western blot). Monoclonal Mouse anti-RhoA  
480 antibody (Cytoskeleton inc. Cat#ARH05) was obtained through the Cytoskeleton inc Rho A  
481 activation Assay Biochem Kit and used at 1:500. Rabbit antibody for anti non-muscle-myosin-  
482 IIB from (BioLegend, Cat. No. 909902) was used at 1:100 (Western blot and  
483 immunofluorescence). Rabbit polyclonal antibodies raised by standard procedures against  
484 Vinculin, brush boarder myosin-II and alpha-acitinin purified from chicken gizzard were used at  
485 1:2000, 1:200 or 1:200 respectively for supplemental figure 1 western blotting. For actin  
486 staining, alexa fluor 488 or 647 phalloidin (Molecular Probes) was used at 1:250. Wheat Germ  
487 Agglutinin conjugated to alexa fluor 488 was also purchased from Molecular Probes and used at  
488 1:300 to stain cell membranes.

489 Phos-tag was purchased from Wako Chemicals. Jasplakinolide (Cat #11705) and Y-  
490 27632 (Cat #100005583-5) were purchased from Cayman Chemicals. DMSO (Cat #D2650),  
491 Latrunculin B (Cat #L5288) and Cytochalasin D (Sigma Cat #C8273) were purchased from  
492 Sigma-Aldrich. Calyculin A (Cat #BML-EI92-0100) was purchased from Enzo Life Sciences and  
493 Blebbistatin (Cat #B592500) was purchased from Toronto Research Chemicals.

494 Ezrin point mutants T567E, and T567A were previously generated as described in  
495 Viswanatha et al., 2012. Sequences for LOK-GFP-Flag, LOK-CTD-GFP-Flag (or LOK\_310-968-  
496 GFP-Flag) and LOK-K65R-GFP-Flag were also previously generated in the lab (Pelaseyed et  
497 al., 2017; Viswanatha et al., 2012). To generate stable cell lines, ezrin and LOK cDNAs were  
498 subcloned into pCDH lentivector (System Biosciences). Puromycin gene in pCDH was then  
499 substituted for blasticidin using Gibson assembly. The lentivectors were then transfected with  
500 psPAX2 and pCMV-VSVG before virus collection and transduction into Jeg-3 cells. Cells were  
501 then grown under blasticidin selection at 5.0 µg/ml for 1-2 weeks prior to immunofluorescent  
502 experiments. Stable expression for ezrin was validated using either an ezrin antibody for ezrin  
503 constructs or GFP expression for LOK constructs.

504 **Cell culture**

505 Jeg-3, HeLa and Caco-2 cells (ATCC) were maintained in a 5% CO<sub>2</sub> humidified chamber  
506 at 37°C. Jeg-3 cells were maintained in MEM with 10% FBS, penicillin/streptomycin and  
507 GlutaMax (Thermo Fisher). Cells were cultured on Corning 100mm x 20mm cell culture  
508 polystyrene dishes. HeLa and Caco-2 cells were maintained in DMEM with 10% FBS and  
509 penicillin/streptomycin. Knockout cell lines were maintained with additional 2.0 µg/ml Puromycin  
510 (Sigma) selection. Transient transfections were done using either Lipofectamine 3000  
511 (Invitrogen) according to the manufacturer's instructions or polyethylenimine reagent  
512 (PolyPlus) as previously described (Viswanatha et al., 2012).

513 Single guide RNAs (sgRNAs) were designed using CRISPR analysis tools on Benchling  
514 and cloned into puromycin-resistant pLenti-CRISPRV2 (AddGene #49535) as described in  
515 Sanjana et al., 2014 and Shalem et al., 2014 (Sanjana et al., 2014; Shalem et al., 2014). The  
516 following sgRNA sequences were used: 5' - GCAATGTCCGAGTTACCA - 3' (ezrin), 5' -  
517 AGAACGAGAACGACTTGAAA - 3' (radixin), 5' - GTAAGACTCACCCAGCATGA - 3' (LOK), 5' -  
518 GCAGTACGAACACGTGAAGA - 3' (SLK). Each lentiviral construct was then transfected into  
519 293TN cells with psPAX3 and pCMV-VSVG (a gift from Jan Lammerding, WICMB/Cornell,  
520 Ithaca, NY) for 48-72 hours before virus collection. Target cells (Jeg3, HeLa or Caco-2) were  
521 then transduced with either one or two lentiviruses in order to generate a mixed population of  
522 single and double knockout cells. Cells were sorted into single cells and then expanded in  
523 puromycin selection before screening by immunofluorescence and Western blotting.

524 Growth curves of Jeg3 cells were preformed by plating cells on low evaporation lid, flat  
525 bottom 96 well plates (Corning Cat. #3595). Once plated, cells were seeded at 3,000 cells per  
526 well. Plates were then imaged once per hour for 100hrs using a 20X objective using an Incucyte  
527 Zoom v2016 (Essen BioSciences) kept in standard cell incubation chamber conditions. Raw  
528 data Images were collected and analyzed using Incucyte. Graphs were assembled and  
529 exported using Graphpad/Prism (version 8).

530 **Western blotting and immunoprecipitation**

531 Western Blot analysis of cell lysates were done using 6%-12% split SDS-PAGE gels  
532 while 6% gel was used for phos-tag experiments. Phos-tag reagent was added at a final  
533 concentration of 50uM to a standard Tris-glycine-SDS polyacrylamide gel according to the

534 manufacturer's recommendations. Gels were transferred to a PVDF membrane and blocked  
535 with 5% milk in TBS + 0.5% Tween-20. Primary antibodies were incubated with the membrane  
536 in 5% bovine serum albumin either for 1 hour at room temperature or overnight at 4°C. Bands  
537 were detected with HRP (Thermo Fisher) or infrared fluorescent secondary antibodies  
538 (Invitrogen or LI-COR Biosciences). Membranes were imaged using a scanner (Odyssey; LI-  
539 COR Biosciences).

540 For detecting myosin-light-chain and phospho-myosin-light-chain, 16% or split 7.5-17.5%  
541 SDS-PAGE gels were used and transferred to PVDF membranes with 0.2um pore size was  
542 used (Millipour Immobilon-P<sup>SQ</sup>). The membrane was then blocked with Immobilon Block-PO  
543 phosphoprotein-blocking buffer from EMD-Millipore and incubated with primary antibody  
544 solution overnight before developing with chemiluminescent reagents (Radiance Q Plus, Azure  
545 Biosystems) on a Bio-Rad ChemiDoc. Relative band intensities were calculated in ImageJ and  
546 normalized to a loading control and exported to Graphpad/Prism for statistical analysis.  
547 Calyculin A treatments for MLC Blotting were performed by incubating the drug with cells for 10  
548 minutes at 37°C prior to lysis. Lysis of cells for MLC blotting was performed with warm (70°C)  
549 Laemmli sample buffer, followed by immediate scraping and boiling.

550 To determine an interaction between LOK and constitutively active RhoA-L30, cells were  
551 transiently co-transfected with LOK-CTD-GFP-Flag and HA-RhoA-L30 (a kind gift from the  
552 Cerione lab, Cornell, Ithaca, NY). After 24 hours, cells were lysed and then solubilized in cold  
553 immunoprecipitation buffer (25 mM Tris, pH 7.4, 5% glycerol, 150 mM NaCl, 50 mM NaF, 0.1  
554 mM Na3VO4, 10 mM β-glycerol phosphate, 8.7 mg/ml paranitrophenylphosphate, 0.5% Triton  
555 X-100, 0.1 μM calyculin A, and protease inhibitor tablet [Roche]) and immunoprecipitated for 2 h  
556 using Flag M2 affinity gel (Sigma-Aldrich). Immunoprecipitates were then extensively washed in  
557 immunoprecipitation wash buffer (25 mM Tris, pH 7.4, 5% glycerol, 150 mM NaCl, 50 mM NaF,  
558 and 0.2% Triton X-100) and then eluted in 200 μg/ml 3×Flag peptide, denatured in Laemmli  
559 buffer, resolved by SDS-PAGE, transferred to polyvinylidene difluoride, and developed with  
560 HRP western detection.

## 561 **Active RhoA pull-down**

562 For GTP-RhoA pull down assay the Rho Activation Assay biochem kit (Cytoskeleton inc,  
563 Cat# BK036) was used as described in the product manual. In summary, WT Jeg-3 or KO cell  
564 lines were plated and grown for 3 days on Corning 100mm x 20mm cell culture polystyrene

565 dishes. An optimum confluence of 70-80% was used as higher confluences can result in partial  
566 loss of a monolayer and disruption of apical morphology in Jeg-3 cells. Cells were lysed using  
567 the kit lysis buffer and protease inhibitor cocktail then clarified at 10000 x g at 4°C for 1 min. The  
568 lysate was then snap frozen in liquid nitrogen as quickly as possible to reduce RhoA-GTP  
569 hydrolysis. Frozen lysates were then stored at -80°C. Protein concentrations were measured  
570 using the Bradford reagent and absorption at 595nm. Upon thawing aliquots, lysate protein  
571 concentration was then normalized to a uniform protein concentration using kit lysis buffer.  
572 Equal concentrations of lysate were then passed on 100ug of Rhotekin-RBD beads. The beads  
573 were then washed, pelleted and finally boiled with 20ul of Laemmli sample buffer. Positive and  
574 negative controls using WT-Jeg 3 lysate incubated with either GTPys or GDP for 15 minutes  
575 prior to passing over the Rhotekin-RBD bead were used to confirm the detectable range of  
576 western blot detection, to confirm Rho-A hydrolysis activity and Rhotekin bead binding capacity.  
577 Samples were run on a 7.5-17.5% split SDS-PAGE gel and blotted for RhoA; Monoclonal  
578 Mouse anti-RhoA antibody (Cytoskeleton inc. Cat#ARH05) was used at 1:500 dilution in TBST  
579 overnight.

580 **Time-lapse microscopy, immunofluorescence and image analysis**

581 Cells were treated to a final concentration of 10nM calyculin A diluted into cell media  
582 plus 10mM HEPES at the start of time-lapse imaging. Phase contrast images for timecourse  
583 movies were taken every 4 minutes for an hour using a 20x objective on a Zeiss AXIO widefield  
584 inverted microscope fitted with a 37°C temperature environmental chamber. For blebbistatin  
585 experiments, blebbistatin was added to the media to a final concentration of 25uM, 30 minutes  
586 prior to addition of calyculin A. For of calyculin A treatment timecourse movies and still frames,  
587 imaging was started immediately before addition of calyculin A and time zero was defined as the  
588 first captured frame after calyculin A addition.

589 Prior to fixation for immunofluorescence, Jeg3 cells grown on coverslips were washed  
590 with PBS and pre-stained with WGA at 1:300 for 30 minutes. For actin turnover experiments in  
591 Figure 5A, Jeg3 cells were treated with either DMSO, 500nM Jasplakinolide or 100ng/mL  
592 Latrunculin B for 30 min, prior to fixation. Otherwise, for all other immunofluorescent  
593 experiments, cells grown on glass coverslips were fixed in 3.7% formalin/PBS for 15 min at  
594 room temperature. Cells were then washed with PBS and blocked with immunofluorescence  
595 buffer (PBS + 0.5% BSA + 0.5% goat serum + 0.1% Triton X-100) for 10 min. Primary and  
596 secondary antibodies were then applied in immunofluorescence buffer containing 2% FBS.

597 Alexa Fluor-conjugated phalloidin, was added to the secondary. The cells were mounted in  
598 SlowFade Diamond Antifade (Thermo Fisher) and imaged using a spinning-disk (Yokogawa  
599 CSU-X1; Intelligent Imaging Innovations) Leica DMi600B microscope with spherical aberration  
600 correction device and a 100/1.46 numerical aperture Leica objective. Images were acquired with  
601 a metal-oxide semiconductor device camera (sCMOS) and image slices were assembled using  
602 SlideBook 6 software (Intelligent Imaging Innovations). Maximum or summed intensity  
603 projections were assembled in SlideBook 6 and exported to Illustrator software (Adobe). For  
604 clarity, side projections were vertically expanded using Illustrator. Deconvolution images were  
605 captured using an inverted Leica DMi8 wide-field microscope equipped a Leica 506249  
606 100X/1.49 oil objective, Leica DFC 9000 GTC camera, with Leica application suit X thunder  
607 deconvolution software.

608 The presence or absence of microvilli was scored as described previously (Garbett et  
609 al., 2010; Hanono et al., 2006; LaLonde et al., 2010; Pelaseyed et al., 2017; Sauvanet et al.,  
610 2015). More than 50 cells per replicate were stained using the WGA, ezrin and phalloidin and  
611 binned into two categories: microvilli and no microvilli. Microvilli above cell-cell junctions were  
612 ignored in the scoring.

613 For calculating tortuosity among tight junctions and relative apical and lateral actin  
614 intensities, cells were stained with a ZO-1 antibody and phalloidin and imaged on a spinning-  
615 disk microscope as described earlier. To analyze tortuosity, the ZO-1 channel was skeletonized  
616 into 1 pixel line widths using ImageJ. Between two consecutive intersection points, a straight-  
617 line length was measured and the average of two manually traced line lengths (to account for  
618 human variability) along the skeletonized line were also measured. From there, the ratio  
619 between the actual line length to the expected line length (straight length) was calculated and  
620 exported into Graphpad/Prism for statistical analysis. For comparing actin intensities between  
621 apical and lateral regions, Max-Z projections were divided between ZO-1 positive (apical) and  
622 ZO-1 negative (lateral) staining. From there, outlines of each cell were traced and measured for  
623 their relative mean intensity value in the apical stacks versus the lateral stacks. The ratio  
624 between the apical and lateral values were then calculated, plotted and analyzed for statistical  
625 significance (Welch's t-test) in GraphPad/Prism.

626 **Atomic Force Microscopy (AFM)**

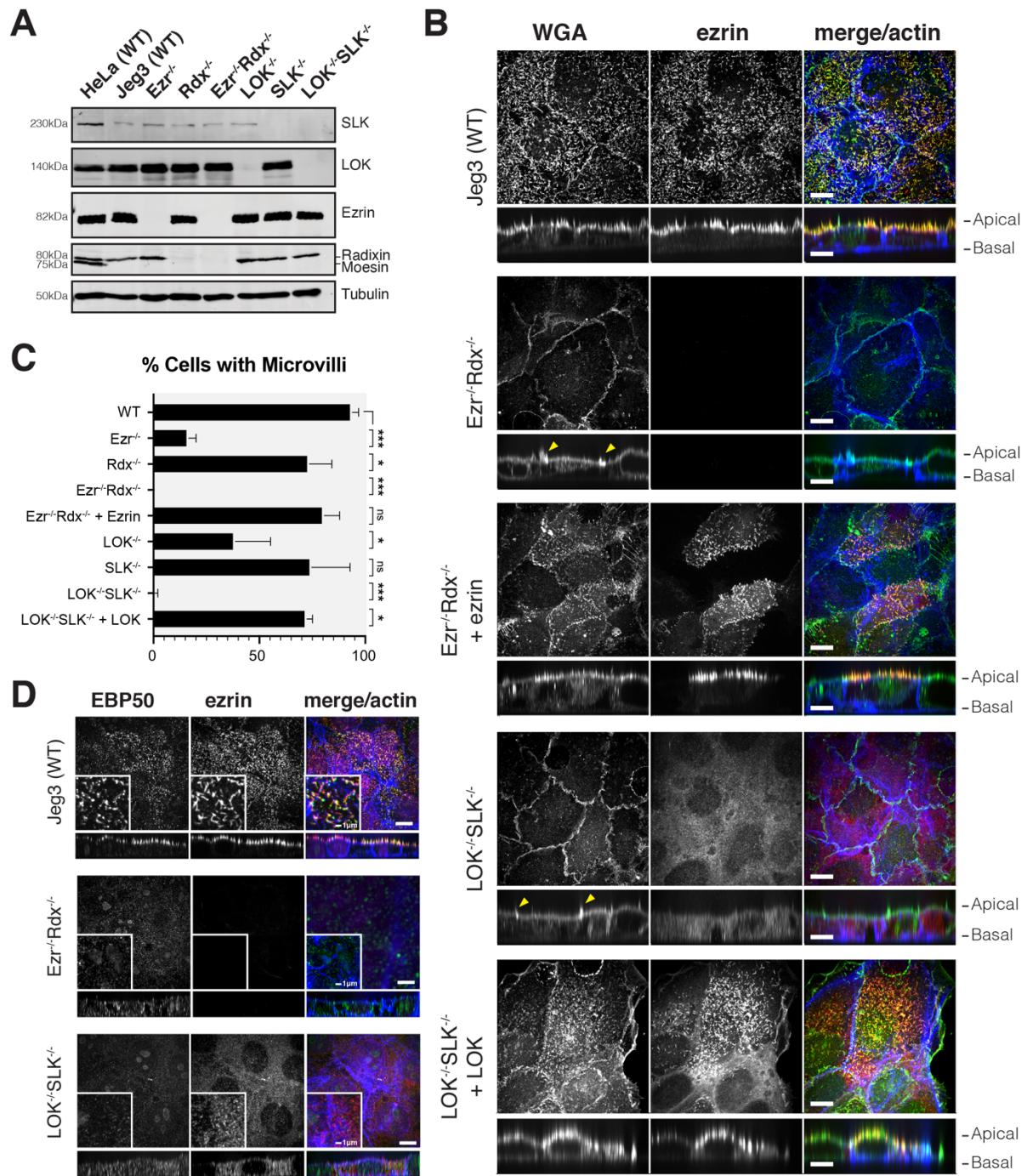
627                   Atomic force microscopy experiments were performed using a MFP-3D-BIOTM Atomic  
628 Force Microscope by Asylum Research mounted on an Olympus IX71 inverted microscope that  
629 resides on a Herzan AVI 350-S Active Vibration Isolation Table totally enclosed in an air tight  
630 BCH-45 Large Acoustic Isolation Hood. Software modules are written in Igor Pro by  
631 Wavemetrics. Cells were plated onto WillCo-Dish Glass bottom dish (size 50x7mm. glass  
632 30mm. Class #1.5, 0.17mm (Product number GWST-5030; Willco Wells)). Pyramidal shaped  
633 Probe TIP (PNP-TR-20) from Asylum Research (804.NW.PNP-TR) were used and calibrated  
634 using an in-air calculation of the spring constant. Individual cells within monolayers were  
635 selected using phase contrast live imaging with the probe tip location simultaneously illuminated  
636 onto the field of view. Probe tips were lowered onto a cell at a velocity of 1000nm/s to a trigger  
637 point of 1 nN. Individual cells were force mapped by indenting 100 times over a square 20 $\mu$ m x  
638 20 $\mu$ m area. Simultaneous force indentation and membrane retraction measurements were  
639 made by first incubating probe tips with 3.0mg/ml of concanavalin A in PBS buffer for 1.5 hours  
640 at room temperature. Deflection and z-probe position data was exported into matlab then  
641 analyzed using the open access software from Huth and colleagues (Huth et al., 2019) modified  
642 for a pyramidal shape probe tip. Force indentation traces were filtered for single force peaks  
643 then chi-value goodness fit to the Hertz model. Youngs modulus statistical analysis and  
644 graphing of AFM data was performed in Graphpad/Prism.

645

#### 646 **Acknowledgements**

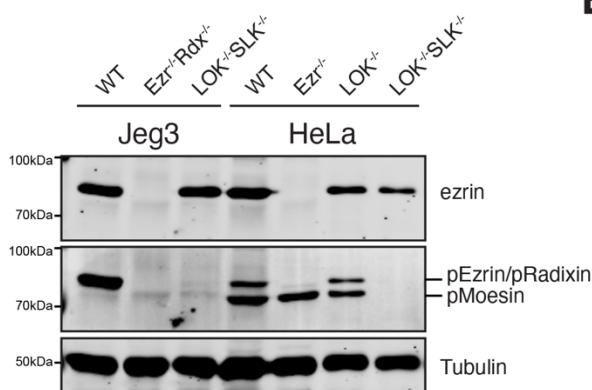
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659 GM131751 to A.B.

## Figures

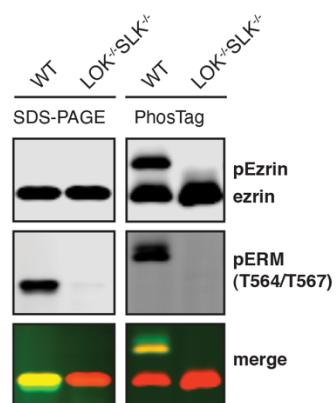


**Figure 1:** Jeg3 cells lacking ERM proteins or LOK/SLK are viable and lack microvilli. (A) Protein expression using indicated antibodies in western blots of ERM proteins and LOK and SLK in Jeg3 CRISPR cell lysates. HeLa cell lysate was used as a control for expression of moesin, which is not present in Jeg3. Tubulin expression was used as a loading control. (B) Jeg3 cell lines lacking ERM expression or LOK and SLK expression were stained with Wheat-Germ-Agglutinin (WGA), ezrin and actin. Yellow arrows indicate strong membrane ruffles. (C) Percentage of cells expressing microvilli. Bars represents mean $\pm$ SEM, N=3. Non-significant (ns) p-values are as follows: Ezr<sup>-/-</sup> Rdx<sup>-/-</sup> + ezrin = 0.0770 ; LOK<sup>-/-</sup> SLK<sup>-/-</sup> + LOK = 0.2079. (D) Staining of cells with EBP50, ezrin and actin in the indicated cells. Scale bars, 10 $\mu$ m unless otherwise noted. Vertical sections were expanded three-fold for clarity. P-values were calculated with Welch's t-test (\* p $\leq$ 0.05, \*\* p $\leq$ 0.01, \*\*\* p $\leq$ 0.001).

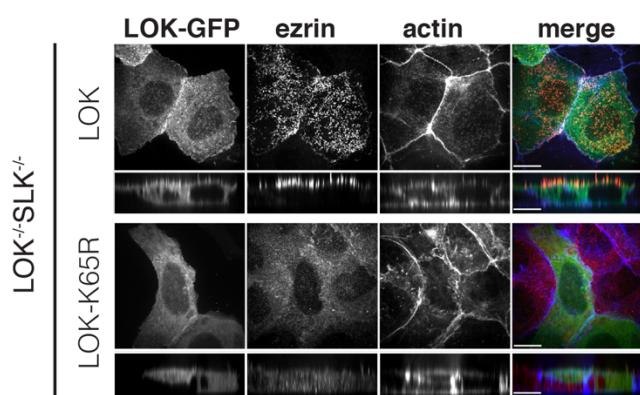
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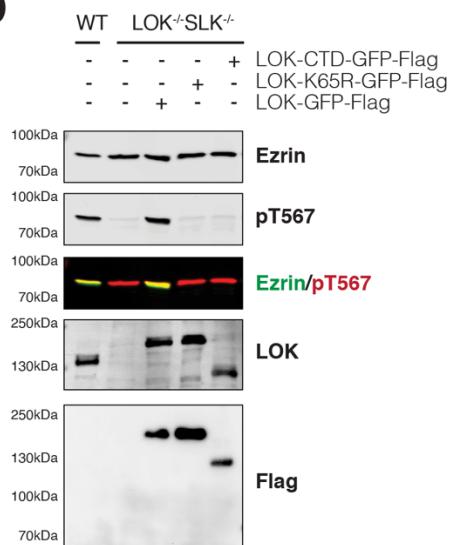
**B**



**C**

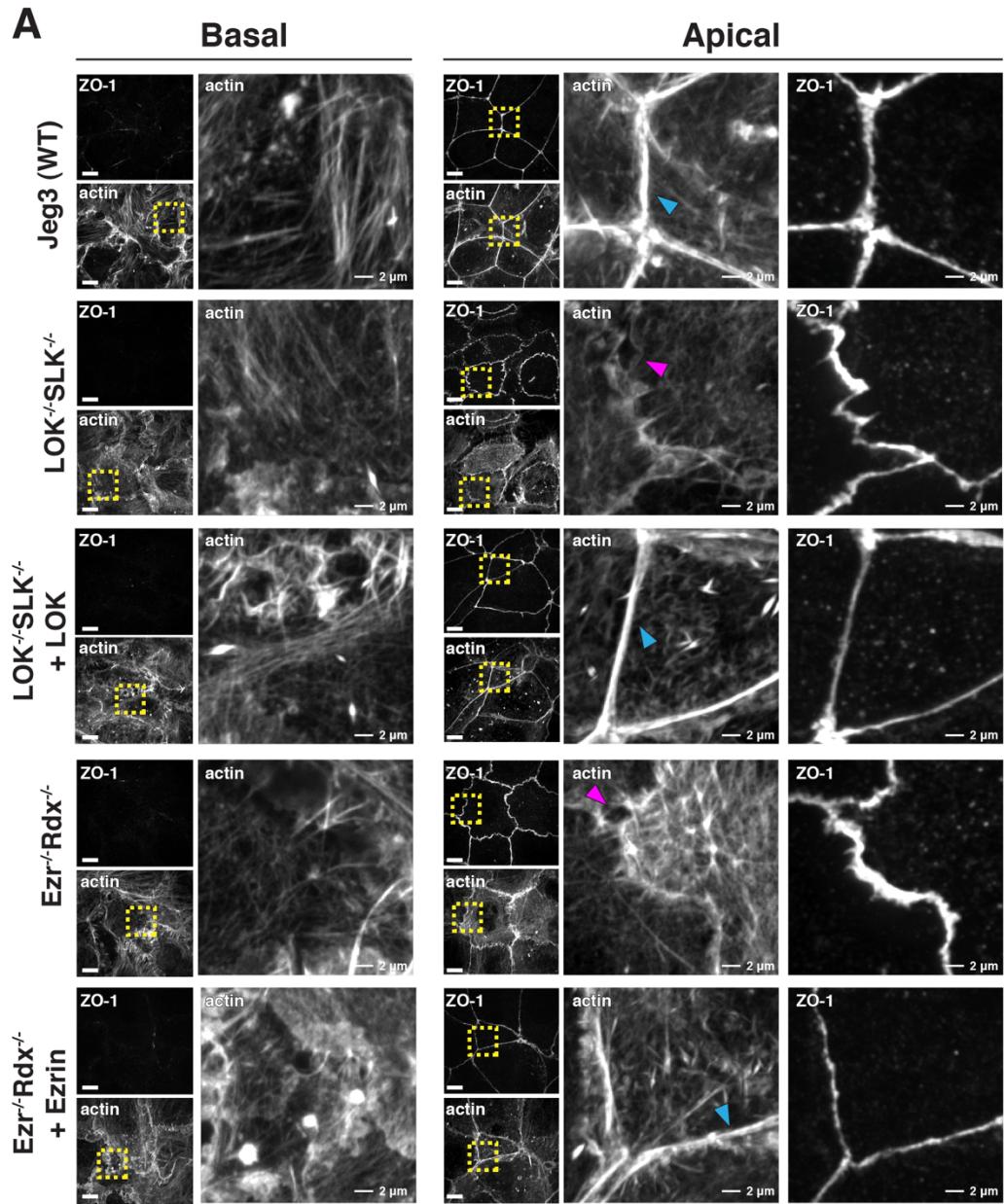


**D**

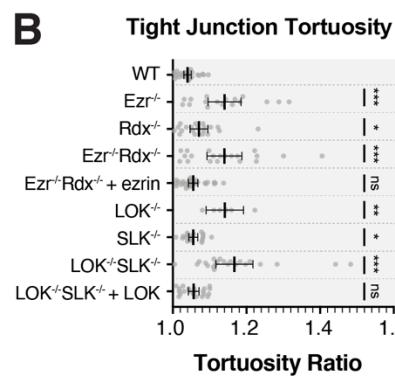


**Figure 2:** LOK/SLK are the major ERM kinases. (A) Ezrin and phospho-ERM levels in Jeg3 or HeLa knockout cells. Tubulin is used as a loading control. (B) Extracts of wildtype or LOK<sup>-/-</sup>SLK<sup>-/-</sup> cells were resolved by either 6% SDS-PAGE gel or 6% Mn-Phos-Tag SDS-PAGE gel and blotted for ezrin and phosphoERM. Approximately half of endogenous ezrin is phosphorylated in wildtype cells as seen by the shift in band size. No phospho-shift is detected in LOK<sup>-/-</sup>SLK<sup>-/-</sup> cells. (C) LOK<sup>-/-</sup>SLK<sup>-/-</sup> cells transfected with either LOK-GFP-flag or K65R-LOK-GFP-flag and then co-stained with ezrin and actin. K65R-LOK is unable to rescue apical ezrin localization. (D) Extracts of cells transfected with wildtype LOK or LOK mutants were collected and blotted for endogenous ezrin or phosphoERM. Lysates were also blotted for flag or LOK to check expression of the constructs relative to wildtype LOK. Scale bars 10 $\mu$ m.

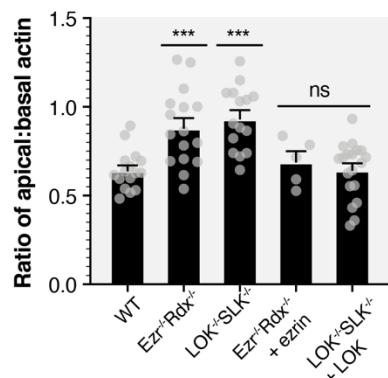
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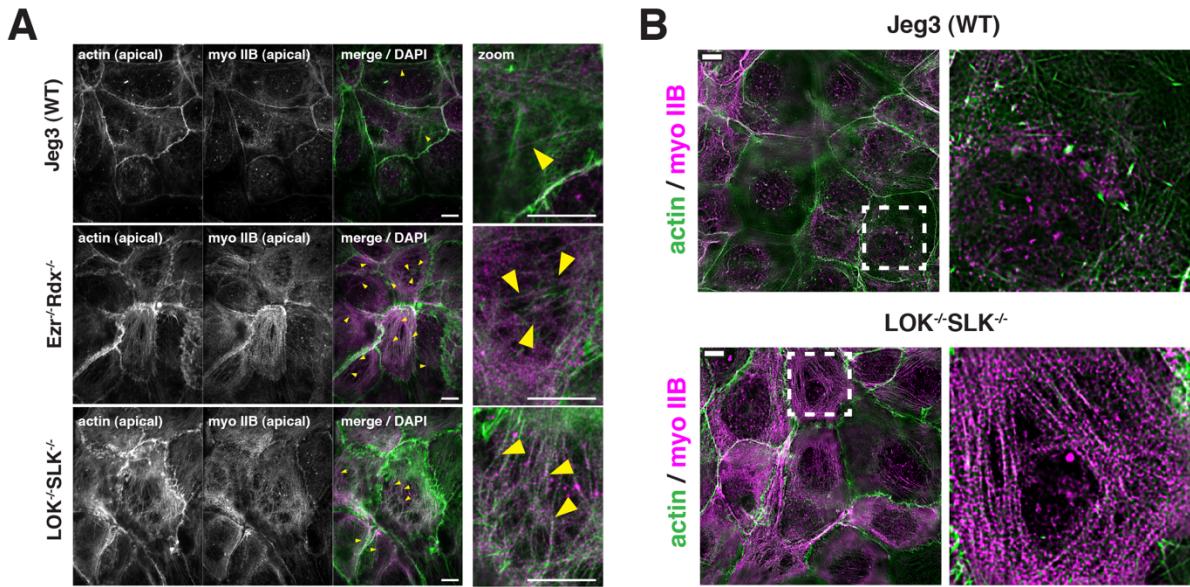
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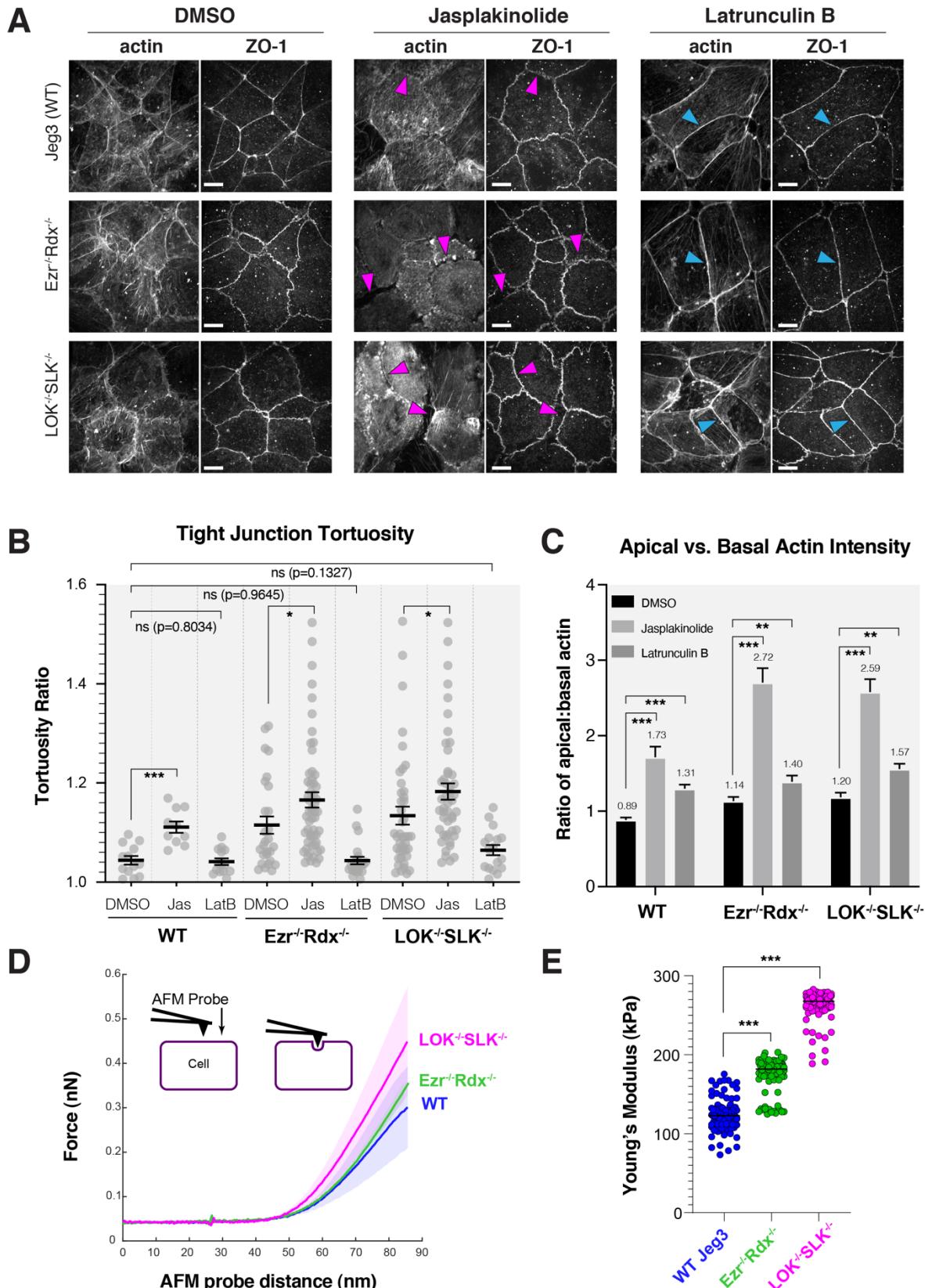
**C Apical vs. Basal Actin Intensity**



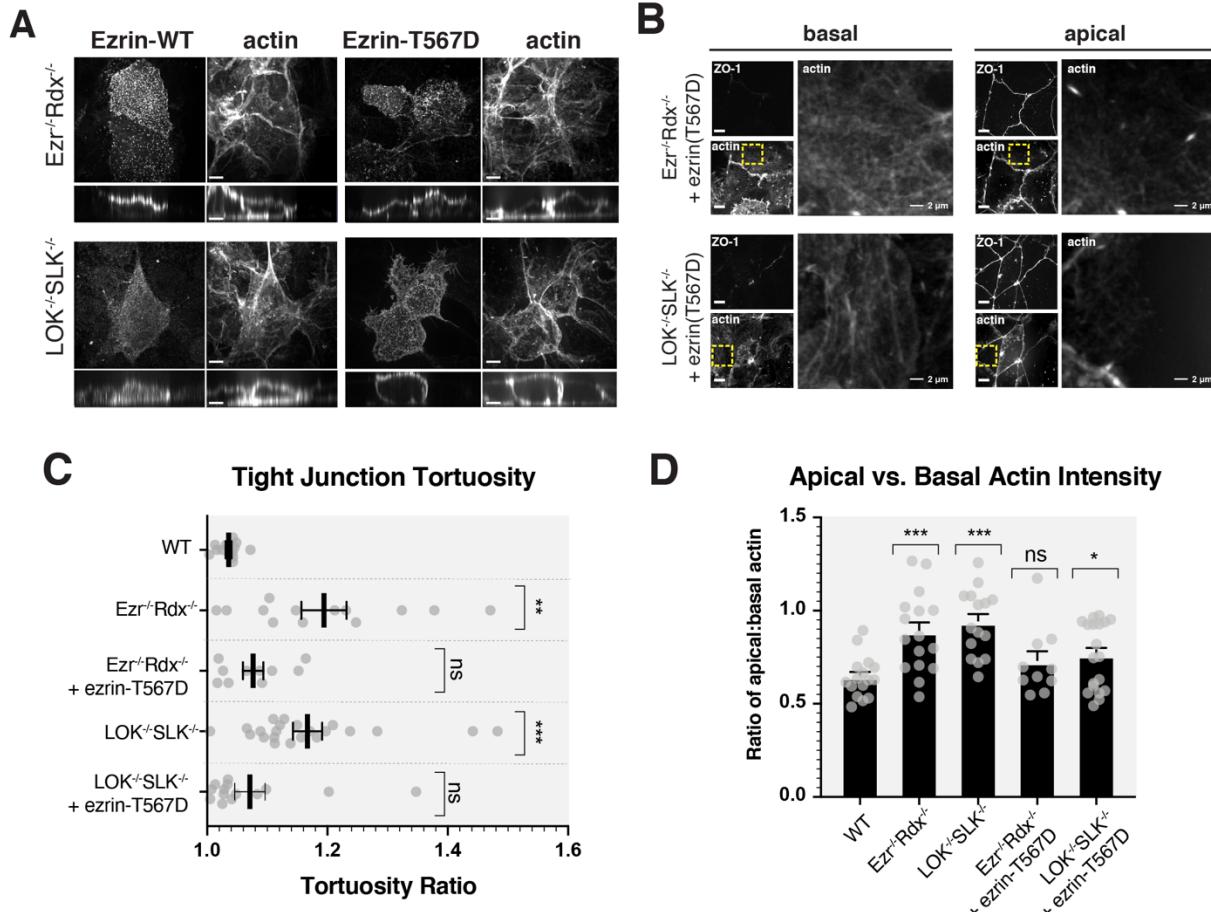
**Figure 3:** Absence of activated ERM proteins induces the formation of apical actin bundles, alters cell-cell junctions, and compromises the apical rigidity of epithelial cells. (A) Comparison of ZO-1 and actin between basolateral and apical confocal slices. Apical Z-slices were determined by the presence of ZO-1. Blue arrows indicate actin present at cell junctions. Magenta arrows point to actin breaks between neighboring cells. Scale 10 $\mu$ m unless otherwise noted. (B) Quantification of tight junction tortuosity using ZO-1 staining. Each point represents the tortuosity from one tight junction intersection to the next intersection. Center lines represent mean $\pm$ SEM. Non-significant (ns) p-values are as follows: Ezr<sup>-/-</sup> Rdx<sup>-/-</sup> + ezrin = 0.0578 ; LOK<sup>-/-</sup> SLK<sup>-/-</sup> + LOK = 0.0573. (C) The ratios of relative mean actin intensity values per cell between apical and basolateral cross-sections. Bars represent mean $\pm$ SEM. Non-significant p-values are as follows: Ezr<sup>-/-</sup> Rdx<sup>-/-</sup> + ezrin = 0.4188 ; LOK<sup>-/-</sup> SLK<sup>-/-</sup> + LOK = 0.9555. P-values were calculated with Welch's t-test (\* p $\leq$ 0.05, \*\* p $\leq$ 0.01, \*\*\* p $\leq$ 0.001).



**Figure 4:** A dense actomyosin network forms at the apical surface of ERM and LOK/SLK knockout cells (A) Maximum projection of immunofluorescence images of the apical confocal Z-slices (2.8 $\mu$ m or 10 confocal slices) indicating actin and non-muscle myosin IIB localization. Areas with contractile fibers are indicated by yellow arrows. (B) Similar apical max projection from deconvolution imaging of the same samples as in (A) showing numerous non-muscle myosin puncta along the apical actin bundles in LOK/SLK knockout cells. Scale bars 10 $\mu$ m.

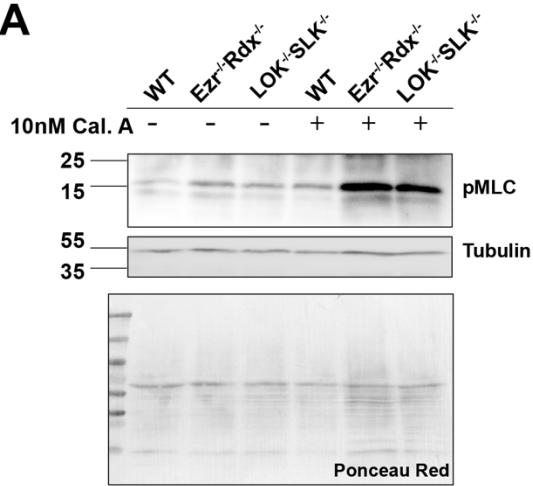


**Figure 5:** Increased actin polymerization in ERM or LOK/SLK knockout cells leads to junctional defects and mechanical stiffening of the cell. (A) Jeg3 cells treated with DMSO, 500nM Jasplakinolide or 100ng/mL Latrunculin B for 30 min before immunostaining with ZO-1 and actin. Magenta arrows point to actin gaps at junctions while blue arrows represent actin present at cell junctions. Scale bars, 10  $\mu$ m. (B) Quantification of tortuosity between tight junction markers as described in Figure 3. Jasplakinolide treatment increases tortuosity values, while Latrunculin B rescues tortuosity to wildtype levels.  $N \geq 10$  cells per condition. Lines represent mean $\pm$ SEM. P-values were calculated with Welch's t-test (\*  $p \leq 0.05$ , \*\*  $p \leq 0.01$ , \*\*\*  $p \leq 0.001$ ). (C) Comparison of actin levels between apical and basolateral regions after treatment with drugs as visualized in panel A.  $N \geq 21$  cells per condition. Bars show mean $\pm$ SEM. (D) Averaged force indentation curves for WT Jeg3 (blue), ezrin<sup>-/-</sup>radixin<sup>-/-</sup> (green) and LOK<sup>-/-</sup> SLK<sup>-/-</sup> (magenta); semi-transparent area around each line represents the SEM of the data. A steeper curve indicates a stiffer cell. (E) Young's modulus stiffness parameters; black line indicates each condition's mean value (WT  $N=356$ , ezrin<sup>-/-</sup>radixin<sup>-/-</sup>  $N=304$ , LOK<sup>-/-</sup> SLK<sup>-/-</sup>  $N=431$ ). Both KO conditions were significantly stiffer than WT cells (\*\* $p < 0.0001$ , K/S Test).

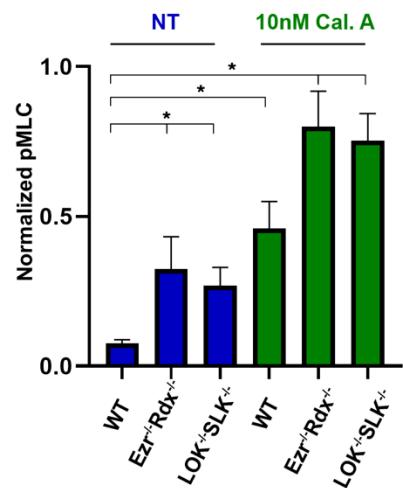


**Figure 6:** Constitutively active ezrin rescues apical actin distribution in knockout cells. (A) Jeg3 knockout cells expressing wildtype ezrin or T567 phosphomimetic were stained for ezrin and actin. Maximum intensity projections and vertical cross-sections are shown. (B) Comparison of ZO-1 and actin between basolateral and apical confocal slices between Jeg-3 knockout cells expressing phosphomimetic ezrin-T567D. Apical Z-slices were determined by the presence of ZO-1. Yellow boxes indicate the region of the image that was expanded on the right. (C) Quantification of tight junction tortuosity using ZO-1 staining.  $N \geq 10$  cells per condition. Center lines show mean $\pm$ SEM. Non-significant (ns) p-values are as follows:  $Ezr^{-/-} Rdx^{-/-} + ezrin-T567D = 0.0623$ ;  $LOK^{-/-} SLK^{-/-} + ezrin-T567D = 0.2445$  (D) The ratios of relative mean actin intensity values per cell between apical and basolateral cross-sections.  $N \geq 21$  cells per condition. Non-significant p-value for  $Ezr^{-/-} Rdx^{-/-} + ezrin-T567D = 0.4188$ . Bars show mean $\pm$ SEM. P-values were calculated against wildtype with Welch's t-test (\*  $p \leq 0.05$ , \*\*  $p \leq 0.01$ , \*\*\*  $p \leq 0.001$ ). Scale bars, 10 $\mu$ m; unless otherwise noted.

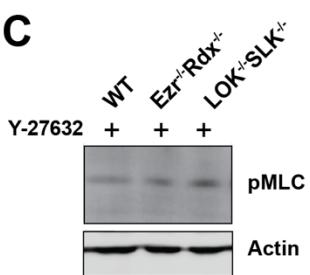
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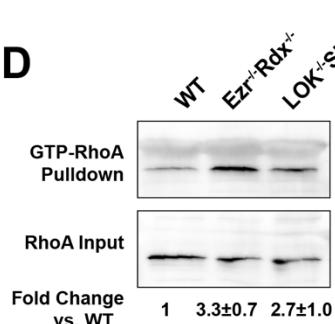
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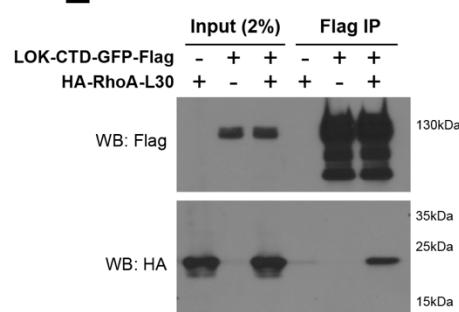
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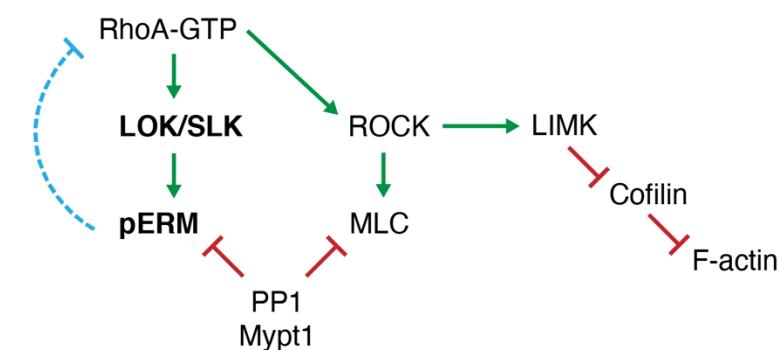
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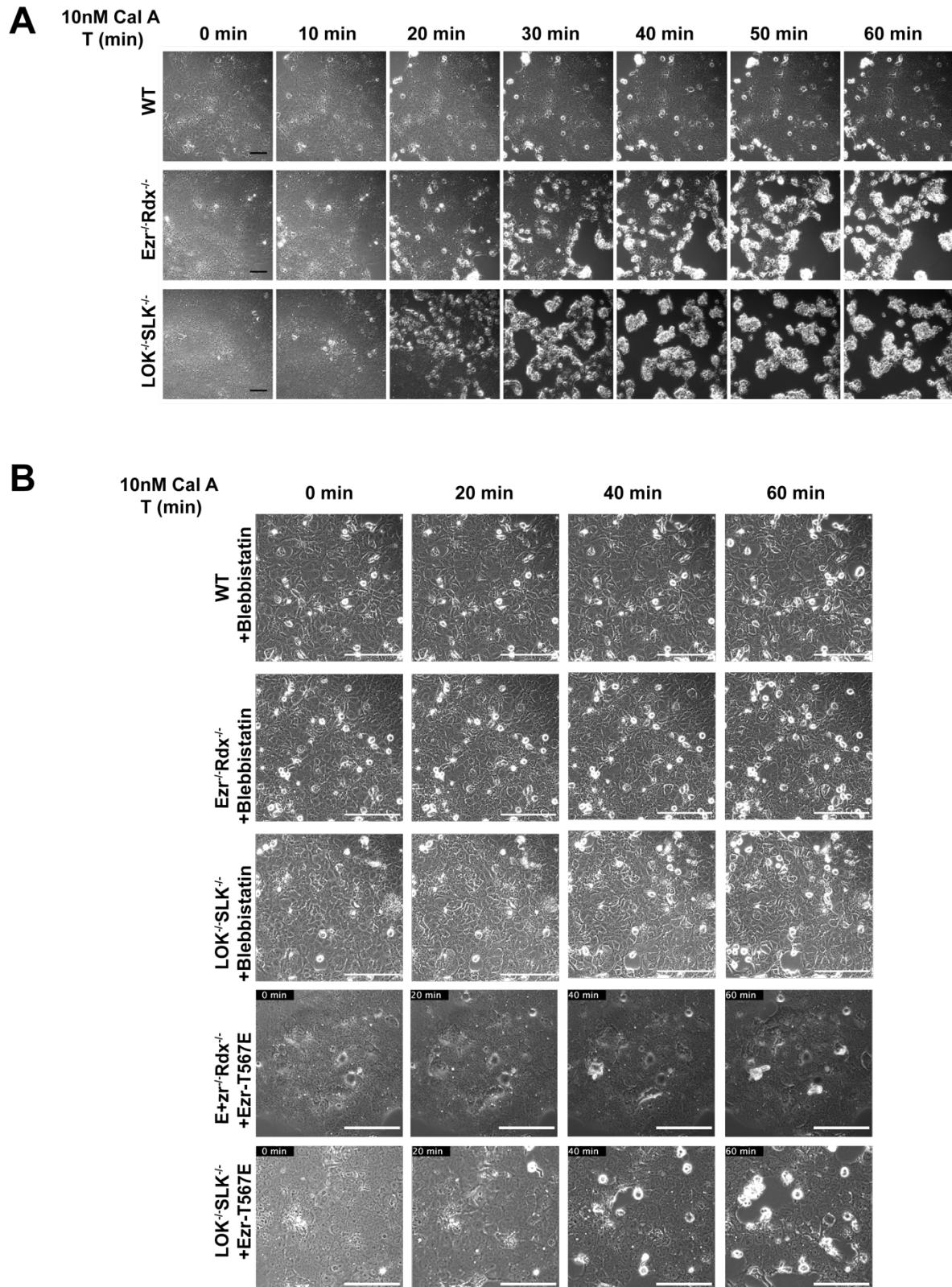
**E**



**F**

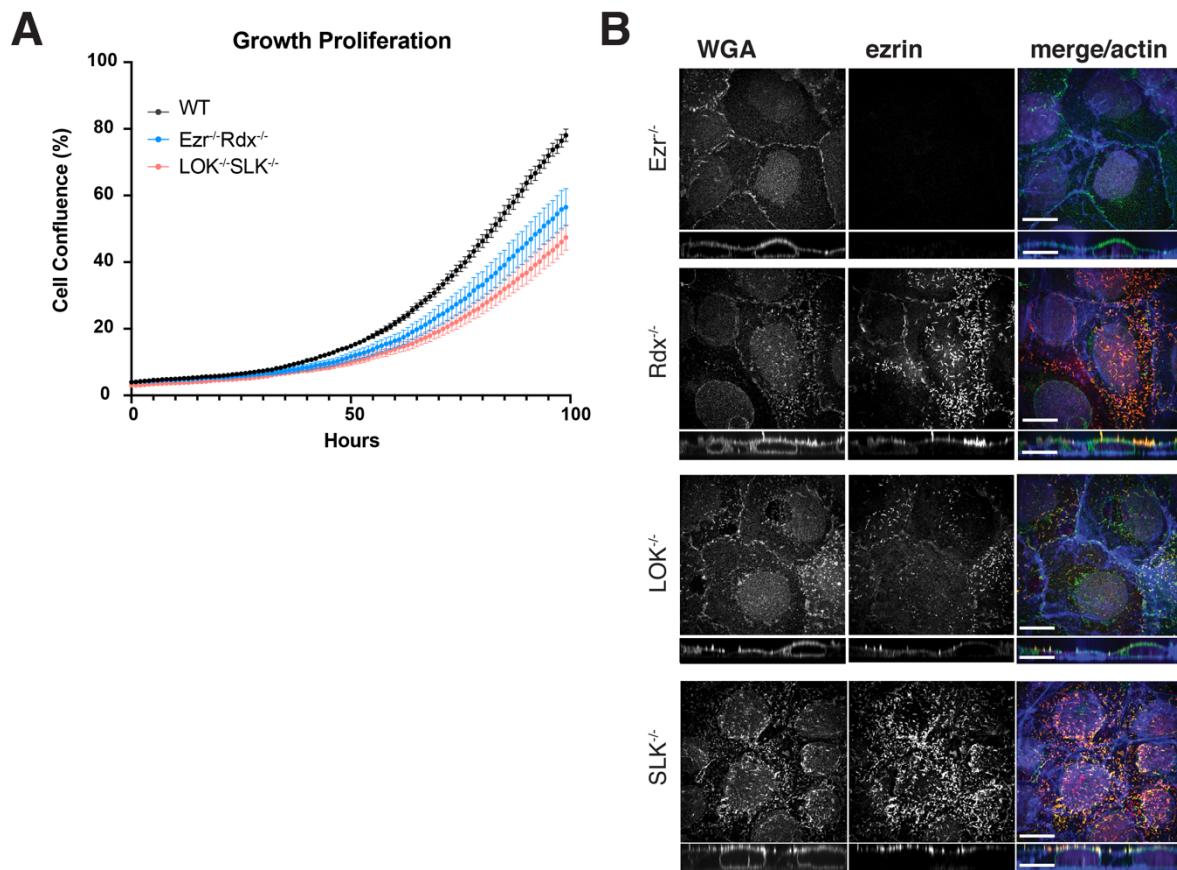


**Figure 7:** Phosphorylated ERMs negatively regulate myosin activation through RhoA. (A) Phospho-myosin light chain (pMLC) (T18/S19) western blot with tubulin and total protein staining to indicate equal loading. (B) Quantification of pMLC staining normalized to loading control (bars show mean±SEM; control:N = 4; 10 min of 10nM CalA: N = 3). (C) Western blot of pMLC with actin loading control in Jeg3 cells first treated with ROCK inhibitor Y-27632 showing reversal of the pMLC hyperphosphorylation phenotype in KO cells. (D) Representative Western blot of active RhoA-GTP pull down results. Upper row: Rho-A blot of the Rhotekin beads following pulldown, Lower row: blot of Rho-A of 4% of input lysate. Normalized fold change of the RhoA pull down band intensity compared to WT (mean±SEM; N = 5). (E) LOK-CTD-GFP-Flag pulldown of cells expressing constitutively active RhoA (RhoA-L30) indicating that C-term LOK binds active RhoA. (F) Model of Rho A signaling negatively regulated by phospho-ERMs (dotted blue line).

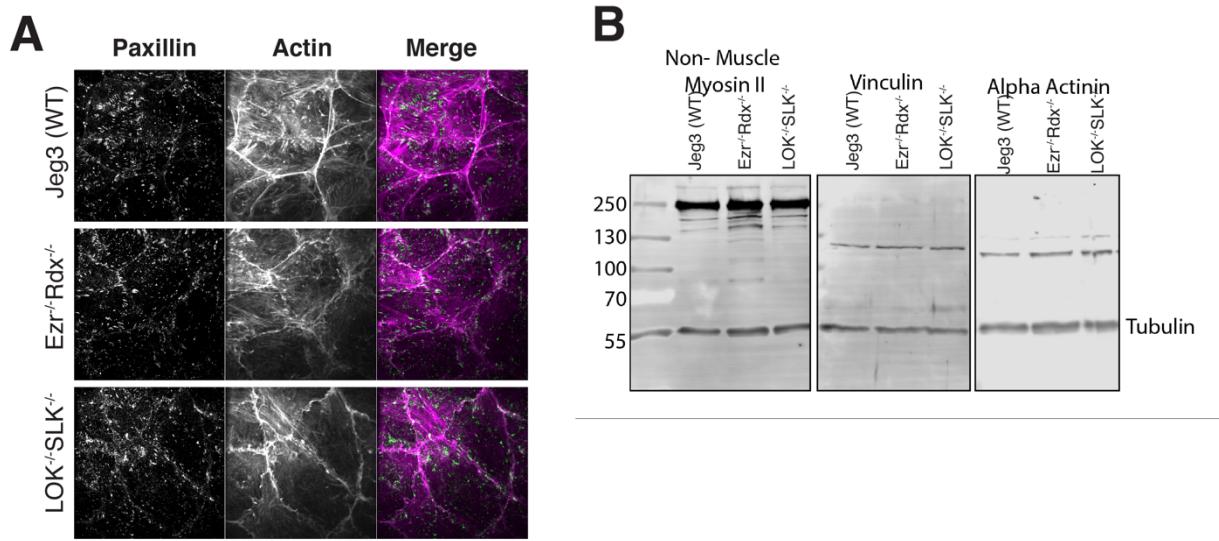


**Figure 8:** Phosphorylated ERMs negatively regulate myosin contractility. (A) Representative phase contrast still images from movies (S1) showing the effect of 10nM calyculin A on WT or KO Cells. (B) Same as (A) except for 30 min treatment with 25uM blebbistatin (Movie S2), or transfection to express phosphomimetic ezrin-T567E (Movie S3), prior to calyculin A treatment. Scales 100μm.

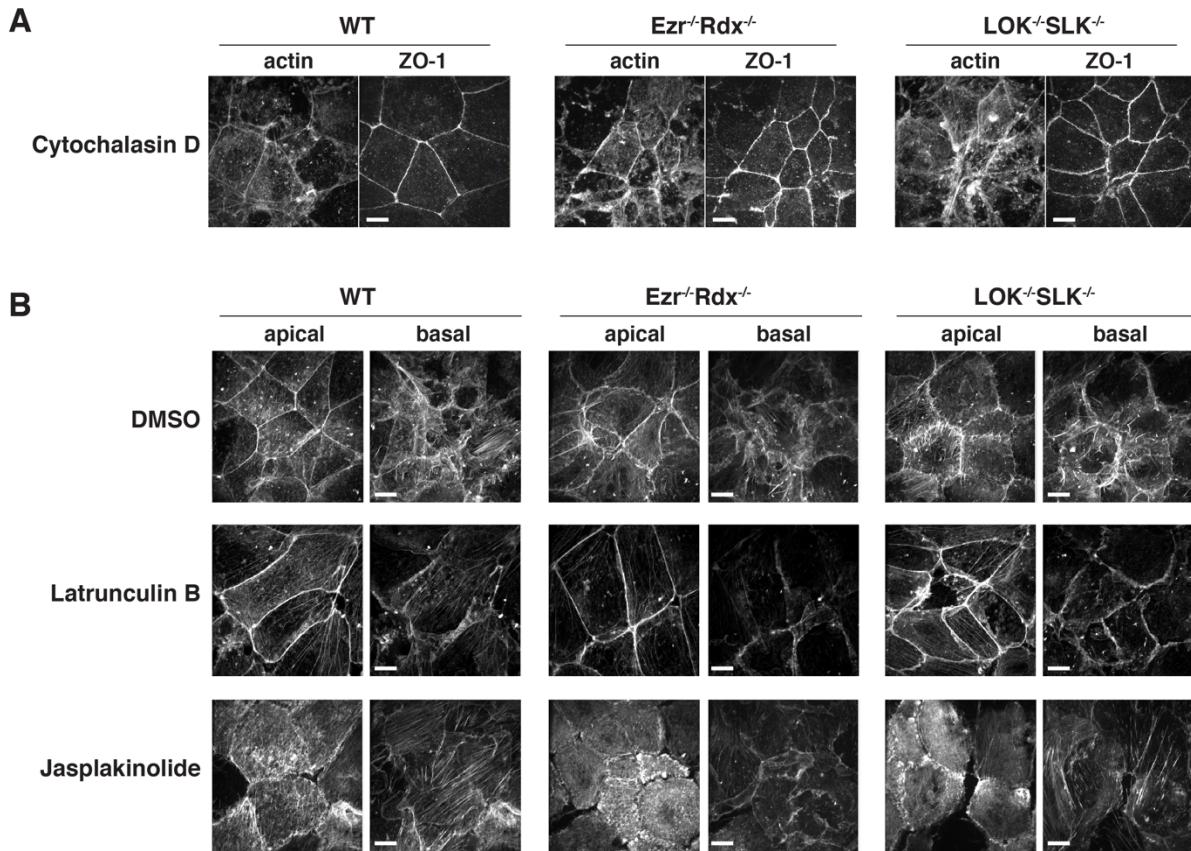
## SUPPLEMENTAL MATERIALS



**Figure S1:** Single KO phenotypes in Jeg3 cells. (A) Growth curve WT (grey) vs. Ezr<sup>-/-</sup>Rdx<sup>-/-</sup> (Blue) and LOK<sup>-/-</sup>SLK<sup>-/-</sup> cells (orange). N=15 wells of cells shown WT ; N=15 Ezr<sup>-/-</sup>Rdx<sup>-/-</sup>; N=15 LOK<sup>-/-</sup>SLK<sup>-/-</sup>. 3,000 cells were seeded per well on 96-well plates and imaged every hour using Incucyte Live-cell analysis system. Each point represents cell confluence, measured by Incucyte over time per hour, from 0 to 100 hours. Error bars = SEM (B) Representative immunofluorescent staining of microvilli in single KO cells used for microvilli quantification and assessment of partial defects.



**Figure S2:** Focal contacts in WT Jeg3 vs LOK<sup>-/-</sup>/SLK<sup>-/-</sup>, Ezr<sup>-/-</sup>/Rdx<sup>-/-</sup> cells. (A) Immunofluorescent staining of Paxillin focal adhesion marker and actin shows similar organization. (B) Western blotting of Non-Muscle Myosin II, Vinculin and alpha-actinin and tubulin in Jeg3 WT and double knockout cells.



**Figure S5:** Effects of actin polymerizing drugs on Jeg-3 epithelial cells. (A) Jeg3 cells treated with 1 $\mu$ g/mL of Cytochalasin D for 30 min prior to fixation and staining with ZO-1 and actin. (B) Actin staining of apical and basolateral slices from maximum projection images in Figure 5A. Notably, Latrunculin B treatment favors disassembly of basolateral actin over apical actin, while maintaining a strong presence of junctional actin. Scale bar 10 $\mu$ m.

**Movie S1:** Time course movie of Jeg3 WT, Ezr<sup>-/-</sup>Rdx<sup>-/-</sup> and LOK<sup>-/-</sup>SLK<sup>-/-</sup> cells treated with 10nM calyculin A over 1 hour.

**Movie S2:** Time course movie of Jeg3 WT, and Ezr<sup>-/-</sup>Rdx<sup>-/-</sup> LOK<sup>-/-</sup>SLK<sup>-/-</sup> cells treated with 10nM calyculin A and 25uM blebbistatin over 1 hour. Blebbistatin treatment was added 30 minutes prior to movie start.

**Movie S3:** Time course movie of Ezr<sup>-/-</sup>Rdx<sup>-/-</sup> and LOK<sup>-/-</sup>SLK<sup>-/-</sup> cells transfected with phosphomimetic ezrin (Ezrin-T567E) treated with 10nM calyculin A over 1 hour.

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