1 **TITLE: CRB3 and ARP2/3 regulate cell biomechanical properties to set epithelial**

2 **monolayers for collective movement.**

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21 **Abbreviations :**

22 ABP (apico-basal polarity), EMP (Epithelial to Mesenchymal Plasticity), CRB3 (Crumbs3), PALS1

23 (Protein Associated with Lin Seven 1), PATJ (PALS1-Associated Tight Junction), ARP2/3 (Actin

24 Related Protein 2/3 complex), KD (knockdown), PDMS (Polydimethylsiloxane)

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27    **Summary**

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29    Several cellular processes during morphogenesis, tissue healing or cancer progression involve

30    epithelial to mesenchymal plasticity that leads to collective motion (plasticity?). Even though

31    a rich variety of EMP programs exist, a major hallmark unifying them is the initial breaking of

32    symmetry that modifies the epithelial phenotype and axis of polarity. During this process, the

33    actin cytoskeleton and cellular junctions are extensively remodelled correlating with the build-

34    up of mechanical forces. As the collective migration proceeds, mechanical forces generated

35    by the actin cytoskeleton align with the direction of migration ensuring an organized and

36    efficient collective cell behaviour, but how forces are regulated during the breaking of

37    symmetry at the onset of EMP remains an unaddressed question. It is known that the polarity

38    complex CRB3/PALS1/PATJ, and in particular, CRB3 regulates the organization of the actin

39    cytoskeleton associated to the apical domain thus pointing at a potential role of CRB3 in

40    controlling mechanical forces. Whether and how CRB3 influences epithelial biomechanics

41    during the epithelial-mesenchymal plasticity remains, however, largely unexplored. Here, we

42    systematically combine mechanical and molecular analyses to show that CRB3 regulates the

43    biomechanical properties of collective epithelial cells during the initial breaking of symmetry

44    of the EMP. CRB3 interacts with ARP2/3 and controls the remodelling of actin throughout the

45    monolayer via the modulation of the Rho-/Rac-GTPase balance. Taken together, our results

46    identified CRB3, a polarity protein, as a regulator of epithelial monolayer mechanics during

47    EMP.

48

49    **Introduction**

50    Epithelia are cohesive layers of apico-basally polarized (ABP) cells that adhere and

51    communicate with each other through specialized intercellular junctions and with the

52    substrate through focal adhesions [1–3]. During development and in some diseases such as

53    cancer, epithelial tissues can perform either complete epithelial to mesenchymal transitions

54    or partial epithelial to mesenchymal transition that has been redefined as epithelial to

55    mesenchymal plasticity [4–8]. The early phase of EMP mechanically relies on the ability of cells

56    to break their symmetry switching from an apico-basal conformation to a front-rear

57    polarization.

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58    The remodeling of epithelial cells at the periphery of the monolayer requires : i) cell spreading

59    [9,10], by extending basal actin protrusion such as lamellipodia to explore the free space; and, ii)

60    cell migration, by constantly remodeling the focal adhesions that anchor the actin stress fibers

61    at the substrate [11–17]. During this initiation of migration, a well-balanced coordination of

62    protrusive and retractive forces needs to be maintained by each individual cell that compose

63    the monolayer sheet. Once engaged in the migration process, the leader cells pull via their

64    intercellular adhesions on the follower cells which in turn gradually begin to spread and

65    migrate [18,19]. Theoretical and analytical studies suggest that the leader cells might induce

66    normal strain on the follower cells and shear stress on adjacent cells, translating a local stress

67    to coordinated traction forces and cell polarization, which finally results in coordinated motion

68    [20–22]. So far, many studies have focus on the molecular mechanisms that controls the

69    mechanical forces when the cells are already migrating [1,16,21,23–28]. Despite extensive studies,

70    the molecular mechanisms that govern the breaking of symmetry of EMP together with the

71    forces underlying this process remains still elusive. In terms of biology during EMP, the

72    remodeling of the cell-cell adhesions, cell matrix adhesions and the reorganization of the actin

73    cytoskeleton correlate with the relocation of the CBR3 polarity complex proteins to the

74    migrating cell front (review in [12,29–31]). The canonical CRB (Crumbs) complex, in mammals is

75    composed of CRB3/Protein Associated with Lin Seven 1 (PALS1) and PALS1-Associated Tight

76    Junction (PATJ) and is known to regulate the actin cytoskeleton organization and intercellular

77    adhesions. CRB is the only protein to possess a transmembrane domain among the polarity

78    complexes, and it has been shown to regulate the organization of intercellular adhesions and

79    actin cytoskeleton in *Drosophila*, zebrafish and mouse embryos [29]. CRB3 has been shown to

80    play an important function in the establishment and the maintenance of cellular apico-basal

81    polarity [32–35], whereas PALS1 and PATJ have been shown to be explicitly involved in the process

82    of collective cell migration [33,36–38]. Furthermore, all these data strongly suggest a role for the

83    CRB3 polarity complex in EMP. We still ignore, however, how CRB3 protein and its partners

84    function during the breaking of symmetry of EMP [39–42].

85    Here, we systematically study how CRB3 and its partners impact the epithelial monolayer

86    architecture and mechanics during EMP. We found that CRB3 in tandem with Arp2/3 regulate

87    the breaking of symmetry during the epithelial transition. We have also shown that CRB3 is

88    required for actin remodeling through a permissive effect on Rac1 activation, thereby

3

89 promoting actin fiber remodeling and alignment along with focal adhesion maturation and

90 orientation and force alignment.

91

**Results**

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**Initial breaking of symmetry of EMP leads to actin cytoskeleton remodeling and apical polarity complex relocation**

96 We have developed a new experimental pipeline to reproducibly monitor the biomechanical

97 behavior of an epithelial monolayer transitioning from a static differentiated epithelial

98 morphology to a migratory phenotype. As a cell model system, we adopted the Caco-2 human

99 intestinal cells as they establish a robust ABP when reaching confluency [43–45] and actively

100 spread and migrate on free substrates [46–48]. We used soft lithography to fabricate thin

101 polydimethylsiloxane (PDMS) membranes with a rectangular opening, which we laid on top of

102 a soft polyacrylamide gel substrate coated with collagen-I (Fig. 1A). After 20hr of culture (time

103 t=0 hour), Caco2 cells formed a confluent polarized and symmetric epithelial monolayer and

104 display a cuboidal shape (Fig. 1A, B-E). At this stage, the actin cytoskeleton is organized in well-

105 defined structures running from the apical to the basal domain of the cells. At the apical side

106 of the cells, a dense brush border made by microvilli is observed (Fig. 1 B, red asterisk), along

107 with a lateral cortical actin belt that bridges the cellular junctions (Fig. 1C, green arrowhead).

108 At the basal side of cells, instead, stress fibers are formed (Fig. 1 D) and an actin cable is visible

109 (Fig. 1 D, blue arrowhead) that connects the free cell fronts at the monolayer's edge.

110 Classically, CRB3, PALS1 and PATJ are mainly located apically at lateral junctions (Fig.1H-J, K,-

111 M, N-0 top left graph, black arrow)[49].

112 10hr after PDMS stencil removal, the monolayer apico-basal symmetry is broken at free edges,

113 and the epithelial layer flattens (Fig. 1, E', F) and spreads (Fig. 1 C', G,). There, cells acquire a

114 migratory state and develop an elongated shape in the epithelial plane. Basally, the actin cable

115 observed at 0hr has vanished, while lamellipodia and actin arcs form (Fig. 1 D', yellow

116 arrowhead). The data show that the actin cytoskeleton is extensively remodeled during the

117 symmetry breaking, when the free edge cells initiate transition. Moreover, despite the

118 presence of well-defined junctional cortical actin (Fig. 1 C', green arrowhead), no brush border

119 could be detected, and only sparse microvilli are observed at the apical surface (Fig. 1 B', red

4

asterisk), thus suggesting a remodeling of the ABP state of free edge cells. Interestingly, actin re-organization correlates with a drastic change in the subcellular localization of the CRB3 polarity complex during EMP. Although PALS1 and PATJ remained located at the lateral junctional belt (Fig1 I' and J' green arrowhead, L', M', O, P bottom left graph, black arrow), a significant pool became redistributed at the lamellipodial membrane (white arrowhead Fig 1I', J' white arrowhead, L', M', O, P bottom left graph blue arrow, O, P right graph). In sharp contrast, junctional CRB3 was barely detected at the junction at this stage, and the protein rather accumulated in intracellular vesicles and focal accumulations at the lamellipodia edge (Fig. 1 H' white arrowheads, K', N bottom left graph blue arrow, N right graph). These data show that the remodeling of the ABP state of free edge cells triggers the recruitment of the CRB3 polarity complex during monolayer symmetry breaking, albeit with a different distribution between PALS1/PATJ and CRB3. In conclusion, apico-basal symmetry breaking at the free edge of the epithelium triggers the relocalization of the CRB3 polarity complex, which correlates with the remodeling of the actin cytoskeleton,

**CRB3 is required for the epithelial transition to collective migration.**

In order to test the function of CRB3 polarity complex in epithelial transition, we selectively silenced the expression of CRB3, PALS1 and PATJ using siRNA approach (Fig. S1) and utilized our experimental set up to quantify monolayer migration efficiency (Fig. 2A-E). Phase contrast microscopy revealed that control monolayers (siCT) and siPALS1 knock-down (KD) monolayers are able to migrate efficiently (Fig. 2A, B, E blue and purple curves), with siPALS1 migrating to further extents than the siCT cells (Fig. 2A, B, E). In contrast, depletion of PATJ or CRB3 significantly reduces the monolayer migration (Fig. 2C, D, E green and red curves). Specifically, siCRB3 monolayers exhibit the strongest phenotype with a 2-fold reduction of migration efficiency compared to controls (Fig. 2E, red curve).

In addition, after 10hr, we observed a defect in the cell protrusions at the free edge of depleted monolayers (Fig. 2A-D, yellow asterisks, bottom panels). In the absence of PATJ, a milder phenotype is observed, with 45,7%± 4,2% (Fig. 2F, green undashed box plots) of leading-edge cells forming protrusions, while CRB3 depletion drastically affected the number of membrane protrusions since only 19,1%±3,2% of leading-edge cells bear protrusions (Fig. 2F, red undashed plots). Taken together, these data demonstrate a pivotal role for CRB3 in the breaking of symmetry during epithelial transition, and prompt us to further characterize

the cell protrusion defects observed in CRB3-KD monolayers. Analysis of the leading edges at 10hr showed actin filament mis-organization in the absence of CRB3 (Fig.2 G, H, middle panel insets), together with mis-localization of ARP2/3 (Fig. 2 G,H, top panel white arrowheads and inset). In fact, a thick actin cable is formed in CRB3-KD monolayer edges. In addition, ARP2/3 is not further recruited at the protrusion cortex in the mutant free edge cells. Subcellular fractionation and Western blot experiments further confirmed global decrease expression of ARP2/3 associated with the membrane in the absence of CRB3, while the total expression level of ARP2/3 is not affected (Fig.2I, J). Collectively, these results testify of the absence of lamellipodia formation in the absence of CRB3. We thus postulated that the absence of CRB3 may hamper actin remodeling at the cell membrane, preventing the branching of actin and promoting the accumulation of bundled/contractile actin at the monolayer free edge during epithelial transition.

**CRB3 promotes Rac1 activation and actin branching for proper epithelial transition to collective migration.**

One way to regulate the branched versus the bundled/contractile actin network is by controlling the balance of Rac1 and Rho small GTPases, with Rac1 activation triggering branching of actin via activation of ARP2/3, and Rho activation favoring the bundled/contractile actin [50,51]. To measure the level of activation of these small GTPases in CRB3 depleted cells, we performed a GST-pull down assay with GST fused to Rhotekin-p21Binding Domain (GST-Rhot-PBD) or PAK-p21Binding Domain (GST-PAK-PBD) to pull down activated Rho or activated Rac, respectively, in both control and siCRB3 cells (Fig. 3A-C). CRB3 depleted-cells have less activated Rac (Fig. 3 A top panel, B) while Rho activation is increased (Fig. 3 A bottom panel, C), when compared to control cells. These data show a perturbation in the balance of small GTPase activity, with Rho hyperactivation together with Rac hypoactivation in cells depleted of CRB3. This phenotype is compatible with the absence of formation of lamellipodia observed in siCRB3 at the monolayer free edges, and strongly suggest a specific defect in the remodeling of branched actin.

We further assessed whether a direct link exists between branched actin remodeling and symmetry breaking during epithelial transition. As expected from literature[52,53], inhibiting actin branching with CK666 drug treatment or using siARP2/3 transfection blocks the

183  formation of cell protrusion and prevents the global migration of the epithelial control

184  monolayer (Fig. 3D-H). These results nicely mimicked the CRB3 depletion phenotype we

185  observed in Figure 2. In addition, ARP2/3 has been shown to directly interact with CRB3 in

186  Sertoli cells[54]. We thus investigated whether CRB3 binding to ARP2/3 also takes place in our

187  system. By using affinity-precipitation (peptide pull-down) with the full-length cytoplasmic

188  domain of CRB3 (90-120 AA, CRB3 cyt) or the same cytoplasmic domain only containing the

189  FERM binding domain (90-100 AA, CRB3FERMBD) that binds to cytoskeletal associated

190  proteins such as ARP2/3 [54] (Fig. S2A), we revealed that ARP2/3 interacts with CRB3-FERM

191  binding domain construct (Fig. S2B).

192  To determine whether a functional correlation exists between CRB3 and ARP2/3 for branched

193  actin remodeling, we scrutinized actin fiber arrangement in siCT, siCRB3 and siARP2/3 cells at

194  0hr and 10hr after removing the PDMS stencil (Fig 4). Whereas CRB3 or ARP2/3 silencing did

195  not affect the initial organization of actin cytoskeleton (Fig. 4 A-C, S3), differences appear

196  between siARP2/3, siCRB3 cells and siCT cells after 10hr of PDMS removal (Fig. 4 A'-C', S3). In

197  a similar manner, both siCRB3 and siARP2/3 cells do not exhibit lamellipodia but instead still

198  display a thick actin cable at their free basal edge (Fig 4 B',C' blue arrowhead). These data

199  clearly state that the phenotypes observed result from a defect in the disassembly and

200  remodeling of the actin cytoskeleton. Further segmentation of the actin filaments and

201  quantitative analysis of their organization in the basal cell leading edge revealed that no clear

202  difference could be noted between the 3 conditions at 0hr (Fig 4D-F, G-H dashed boxes). At

203  10hr, however siARP2/3 cells developed a thick actin cable oriented orthogonally to the

204  migration direction (Fig 4 F', blue inset), as siCRB3 cells did (Fig.4E', blue inset). In addition,

205  quantification of alignment coherence and orientation index, where 0 reflects an orientation

206  of the fibers in the direction of migration and 1 reflects an orientation of the fibers

207  perpendicular to the direction of migration (see Materials and Methods), shows a reduced

208  orientation index and less coherent alignment of actin fibers in siCRB3 and siARP2/3 at 10h

209  compared to control cells (Fig.4 E', F', G, H undashed boxes). These quantifications reflect a

210  loss of the polarized state of the monolayer at the free edge in siCRB3 and siARP2/3 cells. All

211  together, these data show that CRB3 and ARP2/3 are both needed for the correct organization

212  of the actin fibers tangentially to the direction of migration. As remodeling of the actin

213  cytoskeleton is coupled to changes in cell shape, it can be thus considered as the deformability

214    potential of cells [55,56]. We therefore computed the cell area, height and use the changes in
215    cell area as a proxy to compute the strain rate of cell spreading (Fig. S3). Control cells
216    presented a high rate of deformation in spreading with a strain rate of 4,35 demonstrating
217    that Caco-2 cells strongly remodel their shape when they start to migrate at 10hr (Fig S3K). In
218    sharp contrast, a quasi-null strain rate of 0,89 and 1,16 was computed for siCRB3 and siARP2/3
219    cells respectively (Fig S3K), siCRB3 and siARP2/3 cells being taller and expanding less than
220    controls (Fig. S3A'-I', J undashed box, M). Our data shows that CRB3 together with ARP2/3 are
221    essential for the remodeling of epithelial cell shape, and in particular for cell spreading, during
222    the initial transition from epithelial to migrating cells through the remodeling of the actin
223    cytoskeleton.

224    In summary, using a variety of tools, we have shown that CRB3 is required for cells to properly
225    activate Rac1 and remodel the actin cytoskeleton in a similar way to the actin branching
226    protein ARP2/3.

227

**CRB3 and ARP2/3 regulate the remodeling of cell-matrix adhesions**

229    The remodeling of the actin cytoskeleton is required for the cell-substrate adhesion (review
230    in [57]). We therefore decided to study the focal contacts in our system, to decipher a potential
231    correlation between the organization of the actin filaments and the organization of the focal
232    adhesions. Focal adhesions were immunostained for paxillin, at 0hr and 10hr after removal of
233    the PDMS membrane in the different depletions (Fig. 5A-C, A'-C'), and image segmentation
234    and analyses of the focal contacts were performed to measure their maximal length, size
235    repartition orientation (Fig. S4, Fig.5 G, H) and spatial dispersion of focal contacts (Fig.5D-F,
236    D'-F', I). Moreover, as previous works have shown that, as cells start to migrate, their focal
237    contacts are remodeled and maturated from small nascent adhesion to larger focal adhesions
238    [58,59], we subdivided accordingly the focal adhesions into three different categories: nascent
239    <0.25μm, focal complex <0.5μm, focal adhesions >1-5μm [57]. Therefore, by looking at the ratio
240    of the different adhesion categories at 0hr and 10hr, we addressed whether the focal contacts
241    are able to be remodeled and matured from nascent to focal adhesions. At 0hr, no significant
242    difference was measured in the different conditions, indicating that focal adhesions were not
243    affected by any of the depletions when the cells are in a static epithelial state (Fig. 5A-C, G-H,
244    dashed boxplots). At 10hr, an increase in size with a higher ratio of mature focal adhesions

245  was observed in siCT cells (Fig 5G, S4G). As siCT cells spread, the length of the actin filament

246  increased and this was translated by an increase in the dispersion of the focal adhesion (Fig 5

247  D-D', I undashed blue box). At the opposite, siCRB3 and siARP2/3 cells exhibited an opposite

248  trend with smaller immature focal contacts (Fig. 5G, undashed boxplot), and a significant

249  decrease of their orientation and dispersion (Fig. 5 H-I, undashed boxplot) in comparison with

250  control cells.

251  In conclusion, defects in shape, orientation and distribution of focal adhesions in siCRB3 and

252  siARP2/3 cells showed the requirement of CRB3 together with ARP2/3 in remodeling focal

253  contacts, and may be explained by a defective remodeling and growth of the basal actin fibers.

254

255  **Loss of CRB3 or ARP2/3 perturbs the mechanics of the cellular monolayer**

256  Actin dynamics and focal adhesions are related to force generation and transmission across

257  epithelial cell monolayers [31,60]. We thus postulate that CRB3 together with ARP2/3 could be

258  involved in the generation and alignment of forces that are required for the cells to initiate

259  efficient collective motion [20–22].

260  Traction force microscopy and monolayer stress microscopy [20,21,23] were used to measure

261  forces that cells exert at the surface of the substrate. To visualize the orientation and

262  alignment of tension, we plot the vectorial fields of tension. At 0hr, before the symmetry

263  breaking, tractions exhibited a punctate pattern with higher tractions forces being exerted

264  toward the edge of the monolayer in control cells as in siCRB3 and siARP2/3 cells, with

265  depleted cells exhibiting overall higher tractions (Fig.6D-F). In a similar manner, tensions are

266  higher in siCRB3 and siARP2/3 cells compared to control cells (Fig. 6 G-I) and exhibit lower

267  anisotropy as represented by the orientation of maximal force tensor (Fig. 6J-L). After 10hr,

268  traction forces and tension increase with cells depleted for CRB3 and ARP2/3 exerting higher

269  forces in comparison with controls (Fig.6D'-F', G'-I'). Interestingly, the anisotropy of the

270  maximal force tensor increases as the vectors tend to align toward the direction of migration

271  in control cells (Fig 6J'). In contrast, in siCRB3 and siARP2/3, we still observed strong anisotropy

272  and disorganization of the vectors at 10hr (Fig. 6K', L'). To give a comprehensive understanding

273  on how the mechanics evolved in response to the different siRNAs, we computed the temporal

274  evolution of tension, tractions, and of the Shannon's entropy in the vectorial fields. This

275  analysis revealed that control cells, siCRB3 and siARP2/3 cells increased their tractions and

276  tension, albeit forces in siCRB3 and ARP2/3 being always higher than in control cells (Fig.6M,

277  N). Interestingly, cells depleted for CRB3 and ARP2/3, are not able to align their tension as the

278  control cells do, as shown by the increased entropy in siCRB3 and siARP2/3 cells (Fig.6 O).

279  Thus, depletion of CRB3 or ARP2/3 impacted the global mechanical properties, intensity and

280  organization, of the cell monolayer.

281  To further determine whether a correlation occurs between biological and mechanical

282  phenotypes, we selected morphometric parameters (cell area, cell orientation, cell

283  elongation, focal adhesion area, focal adhesion orientation, focal adhesion elongation, actin

284  fiber orientation and coherency) and mechanical parameters (monolayer kinetics, tractions,

285  tension and entropy) and summarized our data in two matrices (Fig. 7), 0hr and 10hr, for three

286  conditions, (Fig. 7A-D). We computed two matrices in which each element contains the z-score

287  of the morphometric and mechanical properties in response to each depletion. This numerical

288  analysis demonstrates that the mechanical parameters are markedly affected at 0hr for both

289  KD cell lines, while a relevant impact is not measured in the morphometric parameters. At

290  10hr, we observed a reinforcement of the mechanical properties along the same trends

291  observed at 0hr (Fig. 7B, D), while drastic modifications can be observed for the morphometric

292  properties (Fig. 7A, C).

293

294  Collectively, these data demonstrated the reciprocity between cell behavior and mechanical

295  features, and in particular, the relationship between the remodeling of actin

296  cytoskeleton/focal adhesions, and the loaded forces the cells exert at the cell-cell interface

297  and on their underlying substrate.

298

## Discussion

300  Epithelia transition towards collective migration is associated with changes in cell polarity and

301  adhesion in epithelial tissues. The transition from a static monolayer to collective migration

302  begins with the breaking of symmetry from apical-basal to front-rear at the monolayer edge

303  and ends up with collective cell motion. These steps require a coordinated remodeling of

304  intercellular adhesions, focal contacts and actin cytoskeleton. In our study we have

305  implemented a system that allows the systematic study the transition from a static monolayer

306   where epithelial cells polarize in the ABP axis, to a more dynamic monolayer where leading

307   edge migrating cells develop instead front-to-rear polarized state.

308   Here we have identified CRB3 / ARP2/3 as mechanoregulatory module of the symmetry

309   breaking during EMP. Several studies performed on different cellular models, such as non-

310   tumorigenic human mammary epithelial cells, tumorigenic kidney-derived cells or colorectal

311   adenocarcinoma cells have led to conflicting conclusions regarding the function of CRB3

312   during collective cell motion [39–42]. In human mammary epithelial cells or tumorigenic kidney

313   cells, the loss of CRB3 expression increases cell invasion, and promotes cell scattering while

314   our study together with the work of Lioka et al[39] clearly demonstrates that loss of CRB3

315   prevents cell spreading. The discrepancies between different cell lines might be explained by

316   different factors. Firstly, the regulation of protein expression such as CRB3 depends on the

317   model system used [40,61–64]. Recently, it has been described that CRB3 gene expression differs

318   depending on the tumoral tissues or cell models [39,61–63] . As an example, CRB3 is upregulated

319   in colorectal and breast cancer whereas in glioblastoma it is downregulated. In the study of

320   Mao et al[63], the authors also described that in non-tumorigenic breast MCF10A cells, CRB3

321   expression and localization depends on the cellular density with a lower expression in

322   confluent cells when compare to sparse cells. These different expression patterns could result

323   in the formation of distinct complexes, and trigger various signaling pathways that could lead

324   to a great variety of phenotypes. These differences should be considered before raising a

325   conclusion regarding the role of CRB3 during collective cell migration. Secondly, the choice of

326   the cellular model is crucial to address properly the question of the initial step of the transition

327   from a static differentiated epithelial to a migratory epithelial monolayer, when cells

328   dramatically change their polarity and shapes as well as their actin organization. Caco2 cells

329   are highly polarized with a dense apical actin network, forming a brush border, and robust

330   cellular adhesions, mimicking i*n vivo* enterocyte organization. When cells start to spread, the

331   polarity proteins are relocalized, the apical actin network is disassembled and cellular

332   adhesions are remodeled, leading to a dramatic cell shape change. The study by Li et al, used

333   MCF10A mammary epithelial cells, which are not columnar, do not exhibit a dense apical actin

334   brush border and have weaker cellular adhesions [61,64,65]. In the case of MCF10A cells, the

335   remodeling of the actin cytoskeleton is less drastic when compared to Caco2 cells, as the cells

336   do not undergo a massive change of shape. Therefore, the results obtained with this MCF10A

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337    cell line cannot be generally transposed to understand the breaking of symmetry during EMP.

338    The Caco2 cell line used in the present study is a more suitable epithelial model to address the

339    regulation of the actin cytoskeleton during the initial cell spreading and allows us to unveil a

340    key role of CRB3 in this process.

341    Our study was, however, not limited to CRB3 but also identified new functional module for

342    EMP composed by CRB3 and ARP2/3. By performing a global quantitative analysis of several

343    biological and mechanical parameters, we were able to demonstrate with an unbiased

344    approach that CRB3 and ARP2/3 exhibit similar functions and could be part of the same

345    functional module during EMP. In line with this conclusion, our biochemical data also show

346    that CRB3 can bind to ARP2/3 independently of PALS1, confirming a heterogeneity of CRB3

347    complex composition. Using Caco2 cells, we describe ARP2/3 as a potential mechanobiological

348    effector of CRB3, and show that CRB3 expression regulates the small GTPase Rac/Rho balance.

349    CRB3 is essential for the cells to activate Rac1, and with ARP2/3 they promote the remodeling

350    of actin and the maturation focal adhesion during the breaking of symmetry of EMP. Previous

351    studies have shown a role of the CRB polarity protein complex in the regulation of the actin

352    cytoskeleton in other cellular processes [29] and here we have found a new cellular context for

353    the role of CRB3 in regulating actin organization. CRB3 is a small transmembrane protein that

354    forms a polarity complex with PALS1 and PATJ which can both recruit regulatory proteins to

355    modulate actin organization. In our study we show, however, that CRB3 can interact with

356    ARP2/3 independently of PALS1 and PATJ, via its FERM binding domain. Our data suggests that

357    to break epithelial symmetry, CRB3 might be relocated to the leading edge where, as a

358    transmembrane protein, it recruits ARP2/3, an actin binding protein that is necessary for the

359    formation of protrusions. This localization leads to cell polarization with a polarized activation

360    of the Rho/Rac balance in a rear-to-front fashion depending on the localization of CRB3 as

361    proposed by several studies [18,29,66]. During this initial cell spreading, this local activation of Rac

362    induces cytoskeletal rearrangements with a rapid actin polymerization and alignment of the

363    actin filament promoting the engagement and maturation of focal contacts [67], which in turn

364    correlates with a regulation of mechanical forces[1]. By using traction forces microscopy and

365    Shannon entropy correlation analysis, we were able to quantitatively show that CRB3 is

366    needed to fine regulate the amount and alignment of forces. These data are in line with the

367    fact that during collective motion epithelial cells increase and align their forces[20,22], however,

368  similar behavior during the breaking of symmetry initiating epithelial transition was not

369  described so far.

370

371  To our knowledge, this is the first study that clearly links a polarity protein, CRB3, to the

372  remodeling and reorganization of the actin cytoskeleton and focal adhesions to the

373  mechanical regulation leading to the breaking of symmetry during the shift between static to

374  migrating epithelium, a key process in cell, developmental biology and mechanobiology.

375

376

377  **Figure Legends**

378  **Fig. 1: F-actin is remodeled and the polarity complex CRB3 is relocalized during the onset of**

379  **EMP.** A) Scheme of the experimental set up and schematic representation of the organization

380  of the monolayer before and after migration. A PDMS membrane (in grey) is deposited on top

381  of a polyacrylamide gels. Cells are seeded within the rectangular opening and after 20hr the

382  PDMS membrane is removed. Just after removal of the PDMS membrane (0hr) the epithelial

383  cell monolayer is cuboidal and microvilli cover the apical domain, and after 10hr, cells flatten,

384  the microvilli are lost at the edge where lamellipodia form as represented in the drawing. The

385  different domains are represented together with the cellular junction and the actin

386  cytoskeleton. F-Actin organization at 0hr (B-D) and 10hr (B'-D') of migration. Red asterisks:

387  microvilli, yellow arrowhead: actin arcs, green arrowhead: lateral junctional actin, blue

388  arrowheads: thick actin cable. Lateral view of the F-actin organization at 0hr (E) and 10hr (E')

389  of migration. Scale bars= 10μm. F) Monolayer height as function of the distance from the

390  center of the cell monolayer. For the different time points several lateral views were measured

391  (0hr, n=44, grey line, 10hr, n= 44, black line). Data are presented as mean ± SEM. G)

392  Quantification of cell area at 0hr (dashed box plot, n=162) and 10hr (empty box plots, n=127)

393  of migration. Data are presented as mean ± Min Max. Localization of CRB3, PALS1, PATJ at 0hr

394  (H-J), and 10hr (H'-J') of migration. Scale Bar =10μm. White arrowheads point the leading edge

395  of the cell monolayer, green arrowheads point cell-cell adhesions, dashed lines point where

396  the intensity profiles shown in panel N-P are measured. Lateral view of the localization of

397  CRB3, PALS1 and PATJ at 0hr (K-M) and 10hr (K'-M') of migration. Scale bars= 10μm. N-P) On

398  the left panels, representative fluorescence intensity profile of CRB3 (N), PALS1 (O), PATJ (P)

399  at 0hr and 10hr. Black arrow point the junction of the cell, and blue arrow the peak detected

400  at the edges. On the right panels, quantification of the mean fluorescence intensity of CRB3

401  (N), PALS1 (O) and PATJ (P) at the edge of the monolayer at 0hr (dashed box plots) and 10hr

402 (empty box plots). For each siRNA 4 fields of views were quantified. Data are presented as
403 mean ± Min Max.

404

405 **Fig. 2: Downregulation of the CRB3 polarity complex alters collective cell migration and the**
406 **formation of protrusions.** Representative phase contrast images showing the effect of each
407 siRNAs (control siCT (A), siPALS1 (B), siPATJ (C) and siCRB3 (D) on cell monolayer expansion at
408 0hr and 10hr of migration. Yellow asterisks: cell protrusions. Scale bar = 50 μm. E) Time
409 evolution of monolayer expansion (shown as the distance covered by the cell monolayer from
410 the initial point). Number of monolayers for siCT blue, n =7; siPALS1, purple, n =4; siPATJ,
411 green, n=4, siCRB3, red, n=3. F) Cells at the leading-edge bearing protrusions. The box plots
412 represent the ratio between the number of protrusions to the number of cells at the leading
413 edge for each siRNAs at 0hr (dashed box plots) and 10hr (empty box plots) of migration.
414 Number of cells counted for siCT, blue, n=238 0hr, n=228 10hr; siPALS1, purple, n=150 0hr,
415 n=125 10hr; siPATJ, green, n=207 0hr, n=195 10hr; siCRB3, red, n=156 0hr, n=136 10hr. Data
416 are presented as mean ± Min Max. Representative images of ARP2/3 and F-Actin
417 immunostaining in siCT (G), and siCRB3 (H) at 10hr. White arrowheads: edge of the monolayer.
418 Inset are representative zoomed region of ARP2/3 and F-actin immunostainings. Scale bars
419 10μm. I) Immunoblot analysis showing the expression of ARP2/3 5 days after transfections
420 with siCT and siCRB3. α-Tubulin was used to standardize the loading conditions between the
421 different depletions. Quantification of protein expression levels normalized to siCT cells. Data
422 are represented as mean ± SEM. For each protein, n=3 samples pooled from 3 independent
423 transfections. J) Immunblot analysis of membrane fraction purified lysate of ARP2/3 5 days
424 after transfection with siCT and siCRB3. α-Tubulin was used to standardize the loading
425 conditions between the different depletions. Quantification of protein expression levels
426 normalized to siCT cells. Data are represented as mean ± SEM. For each protein, n=3 samples
427 pooled from 3 independent transfections

428

429 **Fig. 3: Rho/Rac balance are perturbed in CRB3 depleted cells, and inhibition of branched**
430 **actin mimic siCRB3 phenotype.** A) Representative western blot images showing the effect of
431 CRB3 downregulation on the activation of Rac (top panel) and Rho (bottom panel). siCT and
432 siCRB3 lysates were pulled-down with GST-CT, GST-PAK-PBD (top panel) and GST-Rhot-PBD
433 (bottom panel) and probed to detect Rac (top panel) and Rho (bottom panel) expression. The
434 activation of Rac (B) and Rho (C) were quantified by computing the ratio between the amount
435 of Rac and Rho pull-down to the total expression detected in the siCT and siCRB3 lysates. The
436 data presented in the histograms are the mean ± SEM of 3 independent experiments for siCT
437 and 3 independent experiments for siCRB3. D-F) Representative phase contrast images
438 showing the expansion of siCT cells, siARP2/3 and siCT/CK666 treated cells at 10hr of
439 migration. Scale bar = 50 μm. G) Cells at the leading-edge bearing protrusions. The box plots
440 represent the ratio between the number of protrusions to the number of cells at the leading

441 edge for each siRNAs at 0hr (dashed box plots) and 10hr (empty box plots) of migration.

442 Number of cells counted for siCT, blue, n = 123 0hr, n =126 10hr; for siCT/CK666 treated cells,

443 orange, n =165 0hr, n =152 10hr; for siARP2/3 n=114 at 0hr, n=120 at 10hr. H) Time evolution

444 of monolayer expansion (shown as the distance covered by the cell monolayer from the initial

445 point). Number of monolayers for siCT blue, n =3; siCT/CK666, orange, n =3; siARP2/3, gray,

446 n=3.

447 **Fig. 4: Downregulation of the CRB3 complex and ARP2/3 alter the organization of the F-**

448 **actin.** A-C') Representative images showing the effect of each siRNA on the organization of

449 the F-actin at the basal domain (siCT (A, A'), siCRB3 (B, B'), siARP2/3 (C, C' (at 0hr and 10hr of

450 migration. Blue arrowheads: F-actin cable, yellow arrowheads: actin arcs, red dash line:

451 outline for the basal edge. Scale bars= 15 µm. D-F') Representation Orientation maps of actin

452 fibers (siCT (D,D'), siCRB3 (E,E') and siARP2/3 (F,F'). Scale bar in degree. Insets are

453 representative zoomed regions of actin orientation. G) Quantification of actin fibers

454 orientation area for each siRNAs at 0hr (dashed box plots) and 10hr (empty box plots) of

455 migration. For each siRNA 10 fields of views were quantified. siCT, blue; siCRB3, red; siARP2/3,

456 grey. Data are presented as mean ± Min Max. H) Quantification of actin fibers coherency for

457 each siRNAs at 0hr (dashed box plots) and 10hr (empty box plots) of migration. For each siRNA

458 10 fields of views were quantified. siCT, blue; siCRB3, red; siARP2/3, grey. Data are presented

459 as mean ± Min Max.

460

461 **Fig. 5: Downregulation of the CRB3 and ARP2/3 alters the organization of the focal contacts.**

462 Representative images of focal adhesions immunostained with paxillin (top) and paxillin/F-

463 actin (bottom) for siCT(A) , siCRB3 (B), siARP2/3 (C), at 0hr and siCT (A'), siCRB3 (B'), Arp2/3

464 (C') at 10r.. Scale bars 10µm. Representative maps of the Delaunay Triangulation of paxillin

465 for siCT (D), siCRB3 (E), siARP2/3 (F) at 0hr and siCT (D'), siCRB3 (E'), siARP2/3 (F') at 10hr.

466 Scale Bar 10µm. G) Quantification of the major length of the focal contacts is represented as

467 dashed box plots (0hr) and empty box plot (10hr). Data are represented as mean ± Min Max.

468 H) Quantification of the orientation of the focal contacts is represented as dashed box plots

469 (0hr) and empty box plot (10hr). Data are represented as mean ± Min Max. The total number

470 of focal contacts quantified for siCT, blue, n = 2530, 6 fields of view, 0hr, n= 6652, 15 fields of

471 view, 10hr; siCRB3, red, n=2230, 4 fields of view, 0hr,n=7410, 17 fields, 10hr; siARP2/3, grey,

472 n=1476, 4 fields of view, 0hr, n =5806, 13 fields of view, 10hr. Data are presented as mean ±

473 Min Max. I) Quantification of the area of the triangle obtained by a Delaunay triangulation

474 over all the focal contacts for each siRNA condition at 0hr (dashed boxplot) and at 10hr (empty

475 boxplot). The box plots represent the mean ± Min Max of all the triangles measured for each

476 siRNAs. siCT, blue, n=23232, 7 fields of view, 0hr, n= 25140, 16 fields of view, 10hr; siCRB3,

477 red, n= 12917, 4 fields of view, 0hr, n=37164, 18 fields of view, 10hr, siARP2/3, grey, n= 11737,

478 n=4, 0hr, n= 13816, 6 fields of view, 10hr .

**Fig. 6: Downregulation of the CRB3 complex and Arp2/3 alters monolayers physical properties.** Representative maps showing the effect of each siRNAs on monolayers dynamics at 0hr and 10hr of migration. Representative phase contrast images of siCT (A), siCRB3 (B), siArp2/3 (C) at 0hr and siCT (A'), siCR3 (B'), siArp2/3 (C') at 10hr . Representative traction force maps for siCT (D), siCRB3 (E), siARP2/3 (F) at 0hr and siCT (D'), siCRB3 (E'), siARP2/3 (F') at 10hr. Representative intercellular tension maps $\sigma_{xx,}$ or siCT (G), siCRB3 (H), siARP2/3 (I) at 0hr and siCT (G'), siCRB3 (H'), siARP2/3 (I') at 10hr. Representative vectorial fields of intercellular tension for siCT (J), siCRB3 (K), siARP2/3 (L) at 0hr and siCT (J'), siCRB3 (K'), siARP2/3 (L') at 10hr.. Scale bars= 100 μm. M-O) Time evolution of traction forces (M)intercellular tension (N), tension entropy (O) for the siCT (blue), siCRB3 (red), siARP2/3 (grey). Data are presented as mean ± SEM. siCT n=7 independent cell monolayers, siCRB3 n=3 independent cell monolayers, siARP2/3, n = 3 independent monolayers.

**Fig. 7: The depleted cells exhibit different mechanical properties before showing difference at the biological level.** Effect of siRNAs on biological and physical properties expressed in z-scores at 0hr (A, B) and at 10hr of migration (C, D). OI: Orientation Index, La : number of lamellipodia, EF : Elongation Factor; Disp :Dispersion, Cohe : Coherency, V : velocity, Tnr : normal Traction, Sxx : Normal component of the stress tensor in the x direction , Shr : Shear component of the stress tensor, Ent: Entropy.

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**Supplementary Figures**

**Fig. Supplementary 1: Protein expression levels after siRNA transfections.** A-B) Immunoblot analysis showing the expression of PATJ, PALS1, CRB3 and Arp2/3, 5 days after transfections with siCT, siCRB3, siPATJ, siPALS1 and siARP2/3. α-Tubulin was used to standardize the loading conditions between the different depletions. C) Quantification of protein expression levels normalized to siCT cells. Data are represented as mean ± SEM. For each protein, n=3 samples pooled from 3 independent transfections.

**Fig. Supplementary 2: CRB3 interacts with ARP2/3** A) Scheme of CRB3 full length (CRB3 FL), cytoplasmic domain of CRB3 (CRB3 cyt) and the FERM binding domain of CRB3 that are used as peptide bait for the peptide pull-down. B) siCT lysates were pulled-down with the 3

668   peptides (CRB3 FL, CRB3 cyt and CRB3 FERMBD), and probed to detect PALS1 and ARP2/3
669   expression.

670

671   **Fig. Supplementary 3: Downregulation of the CRB3 complex and ARP2/3 alter the**
672   **organization of the F-actin.** A-I') Representative images showing the effect of each siRNA on
673   the organization of the F-actin at the apical domain: siCT (A, A'), siCRB3 (B, B'), siARP2/3 (C,
674   C'); at the lateral domain siCT (D, D'), siCRB3 (E, E'), siARP2/3 (F, F'), and in xz section siCT (G,
675   G'), siCRB3 (H, H') and siARP2/3 (I, I') at 0hr and 10hr of migration. Red dash line: outline for
676   the basal edge, red asterisk: microvilli. Scale bars= 15 μm. J-K) Quantification of cell spreading
677   (J) and deformations (K) for each siRNAs at 0hr (dashed box plots) and 10hr (empty box plots)
678   of migration. siCT, blue, n = 4 monolayers; siCRB3, red, n= 4 monolayers; siARP2/3 , gray, n =4
679   monolayers. Data are presented as mean ± SEM. L-M) Monolayer height as function of the
680   distance from the center of the cell monolayer at 0hr (L) and 10hr (M). For the different time
681   points several lateral views were measured for each siRNAs (0hr, n=44, 10hr, n= 44, siCT, blue;
682   siCRB3, red; siARP2/3 , gray. Data are presented as mean ± SEM.

683

684   **Fig. Supplementary 4: Downregulation of the CRB3 complex and ARP2/3 alter the**
685   **organization of the focal adhesions.** A-F') Representative images showing the effect of each
686   siRNA on the organization of the focal adhesion and the respective segmentation map: siCT
687   (A, A'), siCRB3 (B, B'), siARP2/3 ( C, C') at 0hr and siCT (D, D'), siCRB3 (E, E') and siARP2/3 (F,
688   F') at 10hr. G) Proportion of focal contacts as function of their sizes. The box plots represented
689   the ratio between the number of focal contacts categorized by their sizes (0.25μm: brown,
690   0.5μm: green and >1μm: blue) to the total number of focal contacts at 0hr (dashed boxplot)
691   and 10hr (empty box plot). The total number of focal contacts quantified for siCT, blue, n =
692   2530, 6 fields of view, 0hr, n= 6652, 15 fields of view, 10hr; siCRB3, red, n=2230, 4 fields of
693   view, 0hr,n=7410, 17 fields, 10hr; siARP2/3, grey, n=1476, 4 fields of view, 0hr, n =5806, 13
694   fields of view, 10hr. Data are presented as mean ± Min Max.

695

696

697   **Material and Methods**

698   **Caco2 cell line culture, transfection and drug treatment**

699   Caco2 cells were grown on DMEM-glutamax media supplemented with 20% Foetal Bovine
700   Serum, 1% of Non-Essential Amino Acid, 100U/mL Penicillin,Streptomycin.

701   siRNA transfections were performed by electroporation using Amaxa technology. 100pmoles
702   of a pool of 3 siRNAs and $1.5 \; 10^6$ freshly trypsinized Caco2 cells were transfected using the
703   B24 program following the manufacturer recommendations. Cells were then seeded on a 6-

704  well plates. 4 days after transfections, cells were trypsinized, 50000 cells were seeded on soft
705  polyacrylamide gels and the remaining cells were processed by western blot.

706  The inhibition of branched actin was performed by adding 67µM, directly in the cell medium,
707  of CK666 drug directly after the removal of the PDMS membrane.

**Preparation of soft polyacrylamide gels**

709  Polyacrylamide gels with a Young's modulus of 12 kPa were prepared as described previously
710  (Bazellieres et al 2015). A solution of 19% acrylamide, 8% bis-acrylamide, 0.5% ammonium
711  persulfate, 0.1% tetramethylethylenediamine, 0.64% of 200nm diameter red fluorescent
712  carboxylate-modified beads and 2mg/mL NH-acrylate was prepared and allowed to
713  polymerize at RT for 1hour. After polymerization, gels were incubated with 0.1mg/mL of
714  collagen I overnight at 4°C.

**Fabrication of PDMS membrane**

716  Polydimethylsiloxane (PDMS) membranes were fabricated according to procedures described
717  previously (Bazellieres et al 2015). SU8-50 masters containing rectangles of 300×2,500 µm
718  were obtained using conventional photolithography. Uncured PDMS was spin-coated on the
719  masters to a thickness lower than the height of the SU8 rectangular feature and cured
720  overnight at 60°C. A thick border of PDMS was left at the edges of the membranes for handling
721  purposes. PDMS was then peeled off from the master and kept in ethanol at 4°C until use.

**Epithelial cell monolayer patterning**

723  To pattern the cells on top of the polyacrylamide gels, a PDMS membrane was deposited on
724  top of the polyacrylamide gel and 50000 cells were seeded within the rectangle defined by
725  the PDMS stencil. Cells were allowed to adhere and differentiated on the gel for 24 hours. 15
726  minutes before time lapse imaging, the PDMS membrane was carefully removed allowing the
727  cells to migrate toward the freely available substrate.

**Time lapse imaging and monolayer expansion quantification**

729  Multidimensional acquisitions were performed on an automated inverted microscope (Zeiss
730  AxioObserver, 10× lens) equipped with thermal, CO2, and humidity control, using Zen
731  software. Images were obtained every 10 minutes during 600 minutes. 6 independent
732  monolayers were imaged in parallel using a motorized XY stage.

733  Based on the phase contrast images, a segmented imaged of the edges of the monolayer was
734  created at each time frame. The Euclidean distance between the two edges, at each pixel, for
735  each siRNA was analyzed using a custom written MatLab codes based on the bwdist function.

**Immunoprecipitation and western blotting**

737  For protein expression level confluent caco2 cells were lysed in lysis buffer (50 mM Tris HCL
738  pH 7.4, 150 mM NaCl, 0.5% NP40) with protease inhibitor cocktail containing 1 µg/ml antipain,

739　1 µg/ml pepstatin, 15 µg/ml benzamidine, and 1 µg/ml leupeptin. Lysates were cleared by
740　centrifugation at 20000g for 30min at 4°C. Protein expression levels were measured using
741　Western Blot. Cell lysates were then mixed with Laemmli 1X and heated at 95°C for 5 minutes.
742　Next, cell lysates were loaded to NuPAGE 4-12% Bis-Tris gel (ThermoFisher Scientific,
743　Courtaboeuf, France) for electrophoresis. Proteins were then transferred to a nitrocellulose
744　membrane (Whatman, GE Healthcare Life Sciences), which was blocked with 5% dry-milk-Tris
745　Buffer saline, 0.2% Tween, and incubated with primary antibodies (overnight at 4°C) followed
746　by the horseradish peroxidase coupled secondary antibodies (1h, room temperature). Bands
747　were revealed using chemi-luminescence reagent plus (Perkin Elmer) and visualized by MyECL
748　imager (ThermoFisher Scientific, Courtaboeuf, France). The intensity of the bands was
749　quantified using ImageJ software. Tubulin was used as an endogenous control for
750　normalization. Protein concentrations are reported relative to the control.

751　A phase separation method was used to quantify the amount of ARP2/3 that is associated at
752　the cell membrane in siCT and siCRB3 conditions. Confluent caco2 cells were lysed in lysis
753　buffer (50 mM Tris HCL pH 7.4, 150 mM NaCl, 2% Triton X-114) with protease inhibitor cocktail
754　containing 1 µg/ml antipain, 1 µg/ml pepstatin, 15 µg/ml benzamidine, and 1 µg/ml leupeptin.
755　Lysates were cleared by centrifugation at 20000g for 30min at 4°C, and the supernatant was
756　brought up to 37°C for 5min allowing to collect the phase that contains the membrane bound
757　proteins [68]. After 4 washes with lysis buffer without Triton-X114, bound proteins were
758　processed by western blotting.

759　CRB3 peptide pull-down assays: 150 µL pelleted streptavidin beads (streptavidin agarose
760　resin, ThermoFisher Scientific, Courtaboeuf, France) were coated with 2mg of a biotinylated
761　peptide mimicking the cytoplasmic part of CRB3A (amino-acids 90 to 120) or with the
762　cytoplasmic part containing the FERM binding domain (amino-acids 90 to 100) called CRB3
763　FERMBD, all synthetized by CovaLab (Cambridge, UK). The cell lysates were centrifuged at 20
764　000g during 30 min and the supernatants were incubated with 20 µl of streptavidin beads
765　CRB3cyt or CRB3 FERMBD at 4°C overnight. After 4 washes with lysis buffer, bound proteins
766　were processed by western blotting.

767　**Quantification of activated Rac and Rho**

768　Caco2 cells siCT and siCRB3 were lysed in lysis buffer (50mM Tris pH 7.4, 500mM NaCl, 10mM
769　$MgCl_2$, 1% Triton X-100, 0.1% SDS, 0.5% Sodium Deoxycolate) with protease inhibitor cocktail
770　containing 1 µg/ml antipain, 1 µg/ml pepstatin, 15 µg/ml benzamidine, and 1 µg/ml leupeptin,
771　for 5min on ice. Cell lysates were centrifugated and the supernatants were incubated with the
772　GST fused to Rhotekin-p21Binding Domain (GST-Rhot-PBD) or PAK-p21Binding Domain (GST-
773　PAK-PBD) at 4°C for 30 minutes. The constructs were kindly provided by Michael Sebbagh,
774　CRCM, Marseille. After 3 washes with lysis buffer, bounds proteins were processed by western
775　blotting.

776　**Immunofluorescence**

777  Caco2 cells were washed with PBS, fixed with 3% paraformaldehyde for 10 minutes and
778  permeabilized in 0.5% triton X-100 for 5 minutes. Cells were blocked in 10% FBS for 1 hour at
779  room temperature before being incubated for 4 hours, room temperature, with primary
780  antibodies. After incubation with the appropriate fluorescence-conjugated secondary
781  antibodies for 1hr at room temperature, cells were washed and mounted in DABCO/Mowiol
782  mounting media. Images were acquired with a Zeiss 510 meta confocal microscope, using a
783  63× Objective with 1.4 NA lens.

784

785

## Quantification of actin properties

787  Images were taken using a Zeiss 510 Meta confocal microscope (Zeiss) and were analyzed as
788  following. Actin orientation and coherency were measured using the OrientationJ plugin, the
789  vector field to compute the different parameters was extracted using a grid size of 30 pixels
790  and an α value of 2 pixels. The orientation was defined as the orientation index such as OI =cos
791  (Θ), Θ defining the angle between the axis of the actin fiber and the direction of migration.

792

## Quantification of cell and focal adhesion properties

794  Images were taken using a Zeiss 510 Meta confocal microscope (Zeiss) and were analyzed as
795  following. Cell contours were detected using cortical actin fluorescent signals, monolayer
796  heights were detected using Z confocal section using actin staining and focal contact were
797  detected using paxillin fluorescent signals in the immunostaining images. Using custom
798  written Matlab codes based on the regionprops function, all the cell contours, monolayer
799  heights and focal contacts within an image were automatically segmented and localized.

800  Based on the segmented images obtained for the cell contours and focal contacts, an ellipse
801  was fitted on each feature and different parameters were extracted such as ellipse area,
802  length of the major and minor axis. The ellipse orientation was defined as the orientation
803  index such as OI =cos (Θ), Θ defining the angle between the major axis and the direction of
804  migration.

805  Based on the segmented images obtained for the monolayer height, the Euclidean distance
806  between the two edges, at each pixel, for each siRNA was analyzed using a custom written
807  MatLab codes based on the bwdist function.

## Quantification of focal adhesion dispersion

809  The centroid of each focal contact was automatically determined from the segmented images
810  obtained previously with custom written Matlab scripts. XY position of all centroids were used
811  to build triangles between the nearest neighbors with the Delaunay Triangulation Matlab

812   script. Once the triangulation was obtained, the areas of the triangles were calculated in
813   Matlab.

**Traction Force Microscopy**

815   Traction forces were computed using Fourier Transform Traction Microscopy with finite gel
816   thickness. Briefly, as cells migrate, they exert force on the underlying the substrate. Gel
817   deformations are observed by imaging the fluorescent beads embedded within the gels. Gel
818   displacements between any experimental time point and a reference image obtained after
819   cell trypsinization were computed using particle imaging velocimetry software. To reduce
820   systematic biases in subpixel resolution and peak-locking effects, we implemented an iterative
821   process (up to four iterations) based on a continuous window shift technique (Serra-Picamal
822   et al., 2012). Traction vectors $T_{i,j}(t)$ within the field of interest are obtained from displacement
823   vectors $u_{i,j}(t)$ for all time points $t = 1,…, n$ and locations $(i,j)$ of the $M × N$ gel interface matrix.

**Monolayer Stress Microscopy**

825   Maps of inter- and intracellular tension within the monolayer were computed using
826   monolayer stress microscopy. In a 2D approximation, monolayer stress is fully captured by a
827   tensor possessing two independent normal components ($\sigma_{xx}$ and $\sigma_{yy}$) and two identical shear
828   components ($\sigma_{xy}$ and $\sigma_{yx}$). At every pixel of the monolayer, these four components of the
829   stress tensor define two particular directions of the plane, one in which the normal stress is
830   maximum and one in which it is minimum. These directions, which are mutually orthogonal,
831   are called principal stress orientations, and the stress values in each principal orientation are
832   called maximum ($\sigma_{11}$) and minimum ($\sigma_{22}$) stress components. The average normal stress is
833   defined as $\sigma_n = (\sigma_{11}+\sigma_{22})/2$. The spatial resolution and force precision of MSM are formally
834   set by those in the original traction maps.

835

**Shannon Entropy Analysis**

837   To apply Shannon's entropy, we partition the range of the angle represented as the angle
838   between two dimensional vectors, into 30° angle bins histogram. With this histogram, the
839   probability of angles in each bin can be calculated, and the information content can be
840   computed using Shannon's entropy as

841    $H(X) = − \sum_{x_i \in X} p(x_i)\log_2 p(x_i)$

842

843   The Shannon's entropy allows to measure and thus compare the amount of variation in angle
844   between the vectors in siCT, siCrb3 and siARP2/3 conditions. When the angles between the
845   vectors are different (less aligned) the number of information is high (1), whereas an
846   alignment of the vectors tends to have a more deterministic number of information (0).

**Computation of z-scores**

848 The z-score is defined as the signed number of standard deviations an observed quantity
849 deviates from the mean of that quantity. In our study the the z-score of a quantity x (a physical
850 or biological property) in response to a siRNA perturbation is defined as:

851
$$Z = \frac{\bar{x} - \bar{x}_c}{\sigma_c}$$

852 Where $\bar{x}$ is the mean of x under the siRNA perturbation, $\bar{x}_c$ is the mean of x under control
853 conditions, and $\bar{\sigma}_c$ is the standard deviation of x under control conditions.

854

855 **siRNAs, Antibodies and reagents**

856 The siRNA sequences used were: siCRB3 (5'-GCAAAUACAGACCACUUCU-3', 5'-
857 CUGCUAUCAUCGUGGUCUU-3', 5'-GUGCGGAAGCUUCGGGAGA-3', 5'-
858 GCUUAAUAGCAGGGAAGAA-3', Dharmacon (On-Target plus Smart Pool)), siCT (5'-
859 CGUACGCGGAAUACUUCGAtt-3', Ambion), siPALS1 (5'-UUCCUUAUGAUGAACUGGCtt-3') and
860 siPATJ (5'-CCAGAUACUCACACUUCAGtt-3', Ambion), siARP2/3 (5'-
861 GGAUUCCAUUGUGCAUCAAtt-3', 5'-GGGAUGAUGAGACCAUGUAtt-3', 5'-
862 AAAUCCUAAUGGAGACAAAtt-3', Ambion)

863 The following primary antibodies were used : rabbit anti CRB3 D2 (Lemmers et al 2004), rat
864 anti CRB3 1E6 (MABT1366 Merck), mouse anti-paxillin (BD transduction 612405), rabbit anti-
865 PATJ Ina2 (Lemmers et al, 2002), chicken anti-PALS1 SN47II (gift from Jan Wijnholds,
866 Kantardzhieva et el, 2005), mouse anti-PALS1 (MPP5, Abnova H00064398), rabbit anti-p34-
867 ARPC2 (07227I, Sigma Aldrich), rabbit anti-pEzrin (Abcam ab47293), mouse anti-Rac (BD
868 transduction, Clone 102), mouse anti-RhoA (SantaCruz 26C2, sc 418). The secondary
869 antibodies used were : Alexa Fluor 488 anti-rat (Invitrogen, A21208), Alexa Fluor 488 anti
870 mouse (Invitrogen, A21202 ), Alexa Fluor 647 mouse (Jackson Immuno Research 715-605-151)
871 Alexa Fluor 488 anti-rabbit (Invitrogen, A21206), and HRP anti-rat (Jackson Immuno Research
872 712 035 153), HRP anti-mouse (Jackson Immuno Research ,715 035 151), HRP anti-rabbit
873 (Jackson Immuno Research 111 035 003), HRP anti-chicken (Jackson Immuno Research 703
874 035 155). The following probe was used for actin labeling: Phalloidin 647 (Cell Signalling
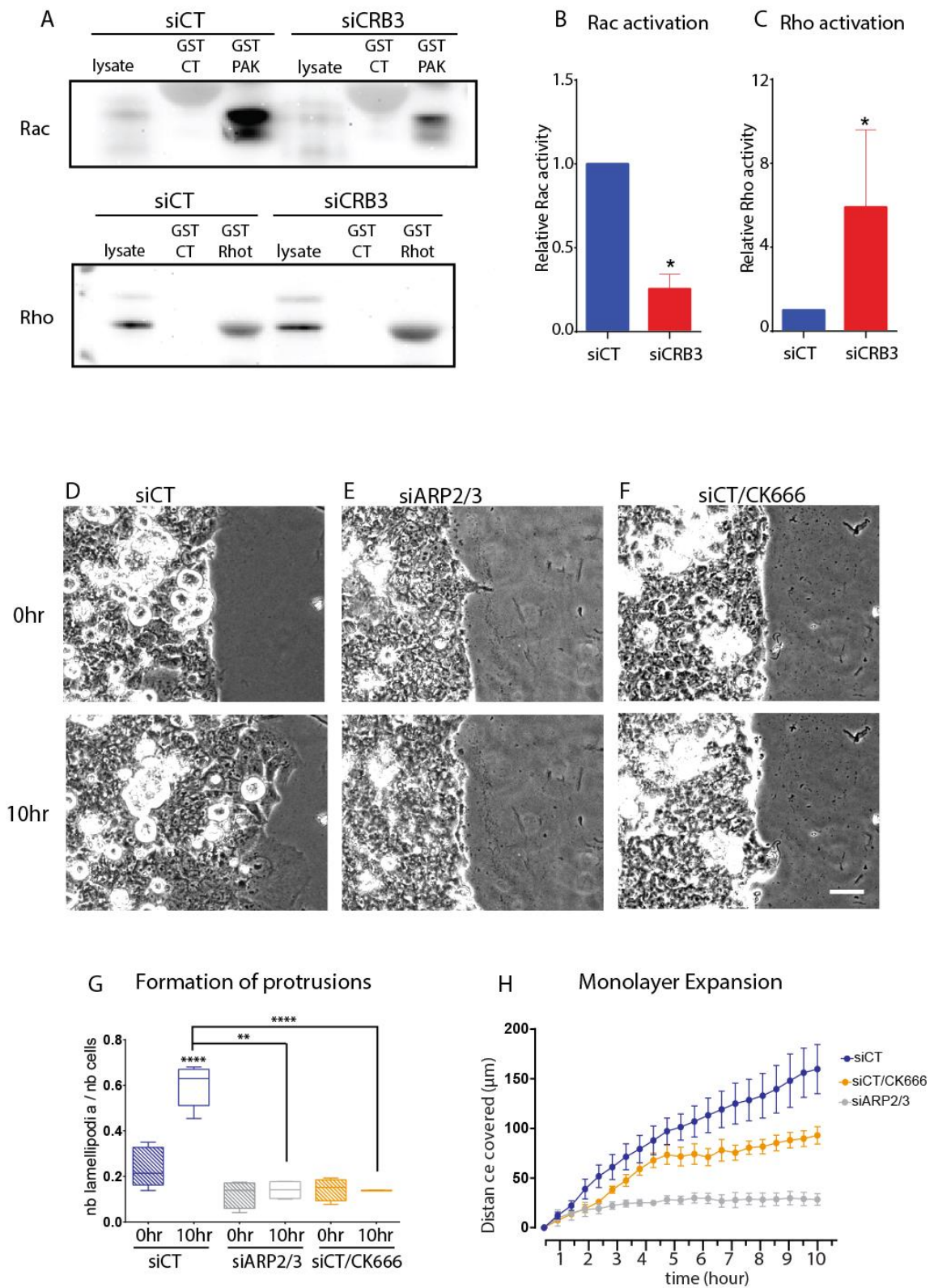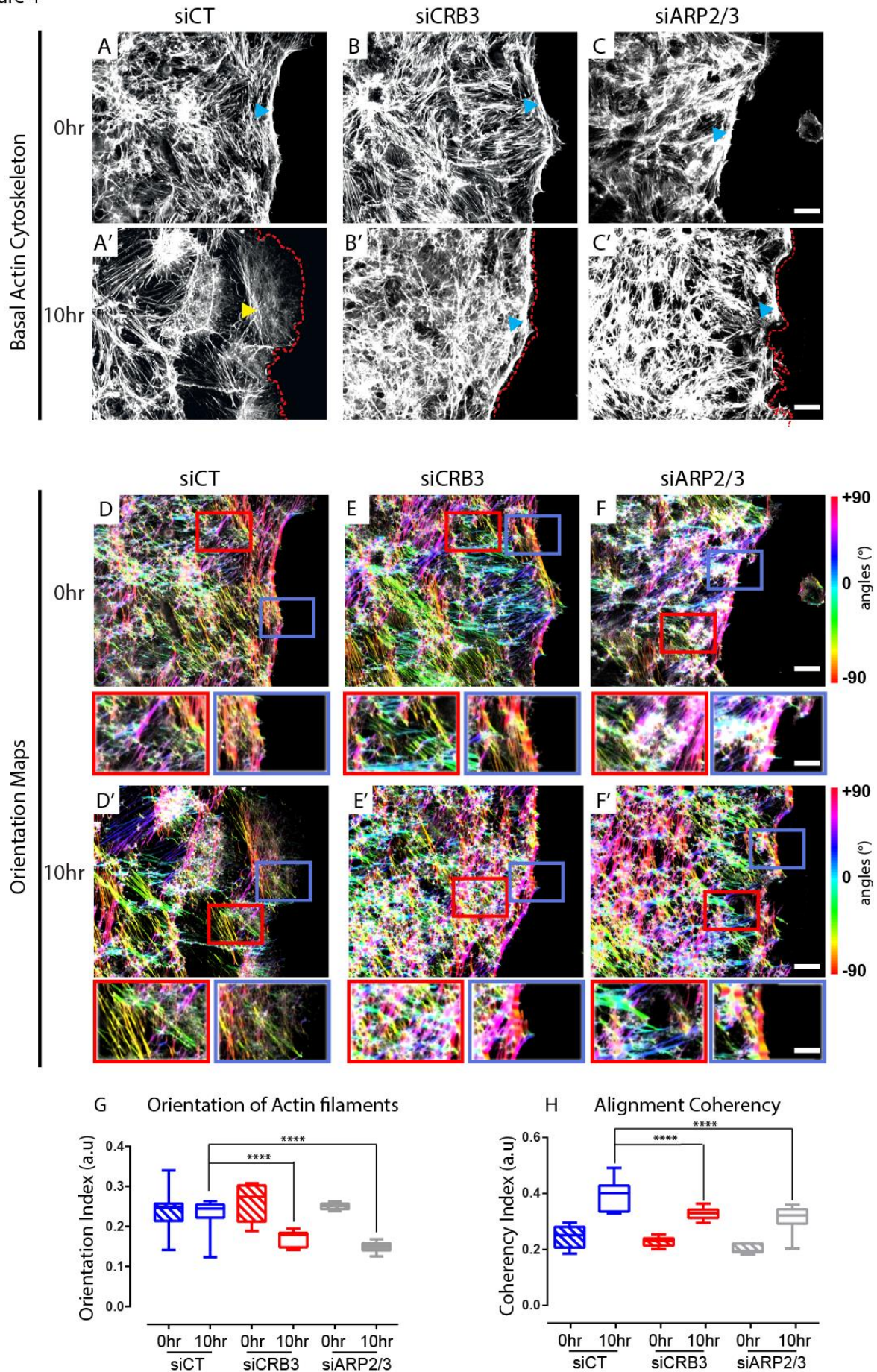875 technology, 8940S).
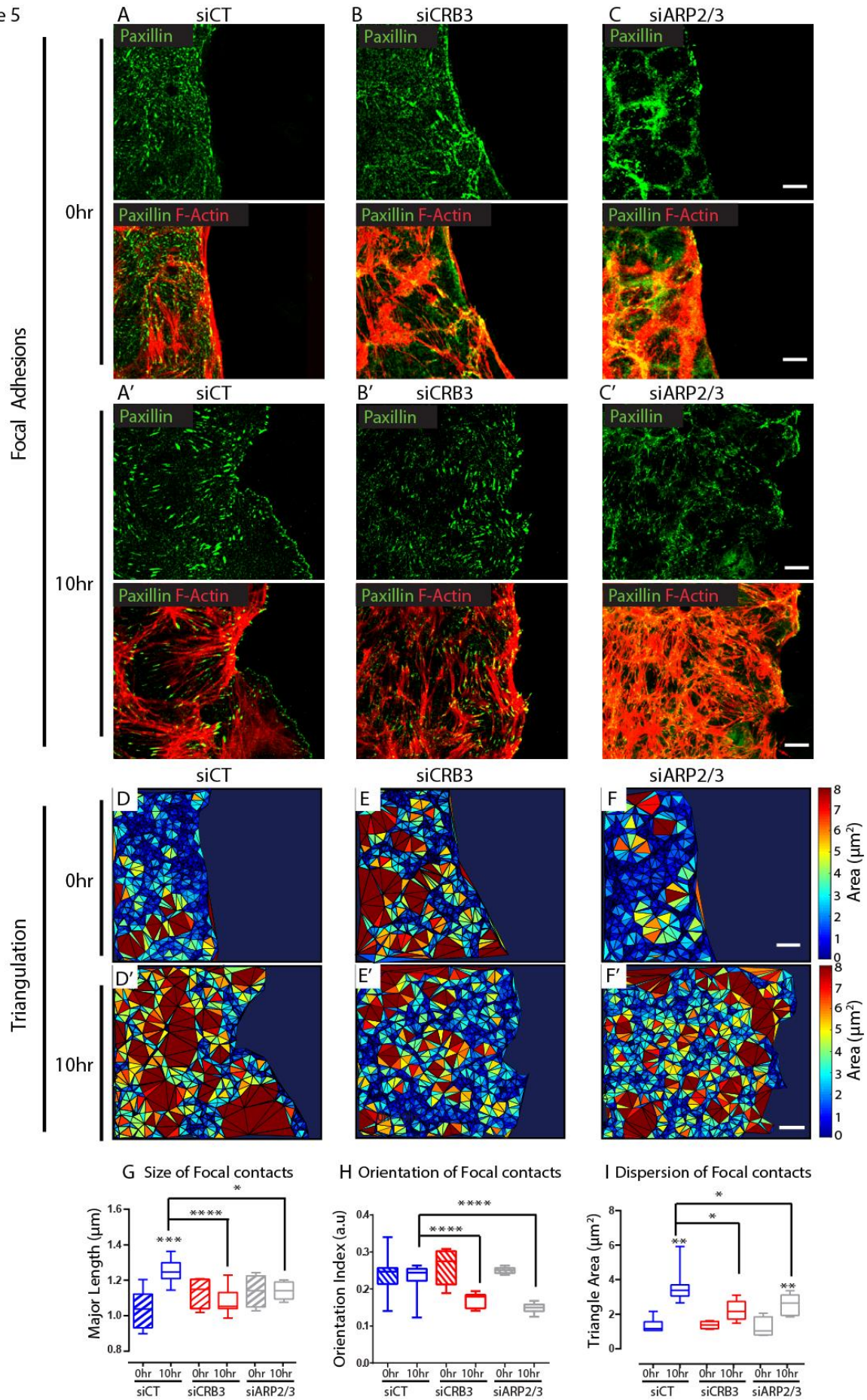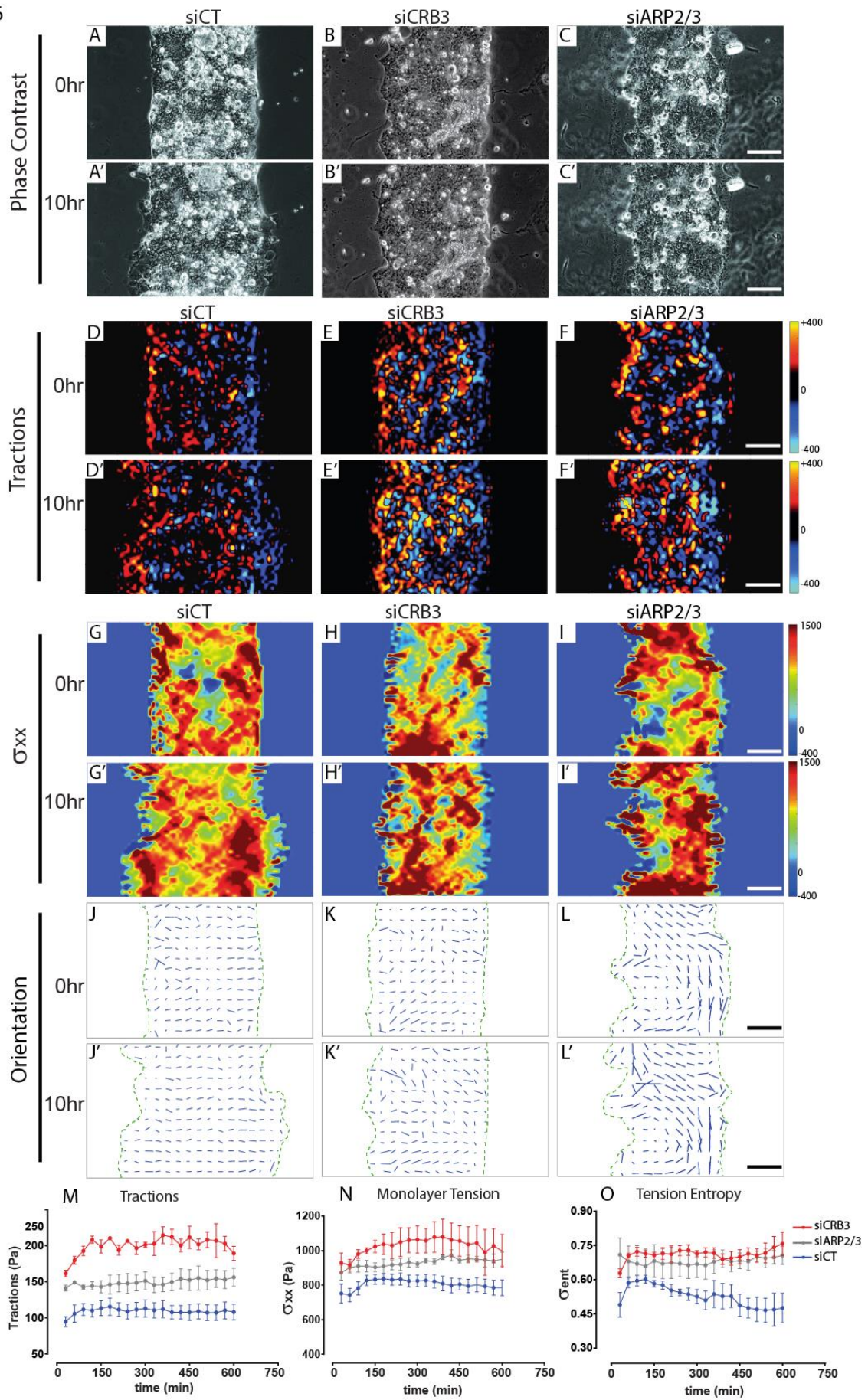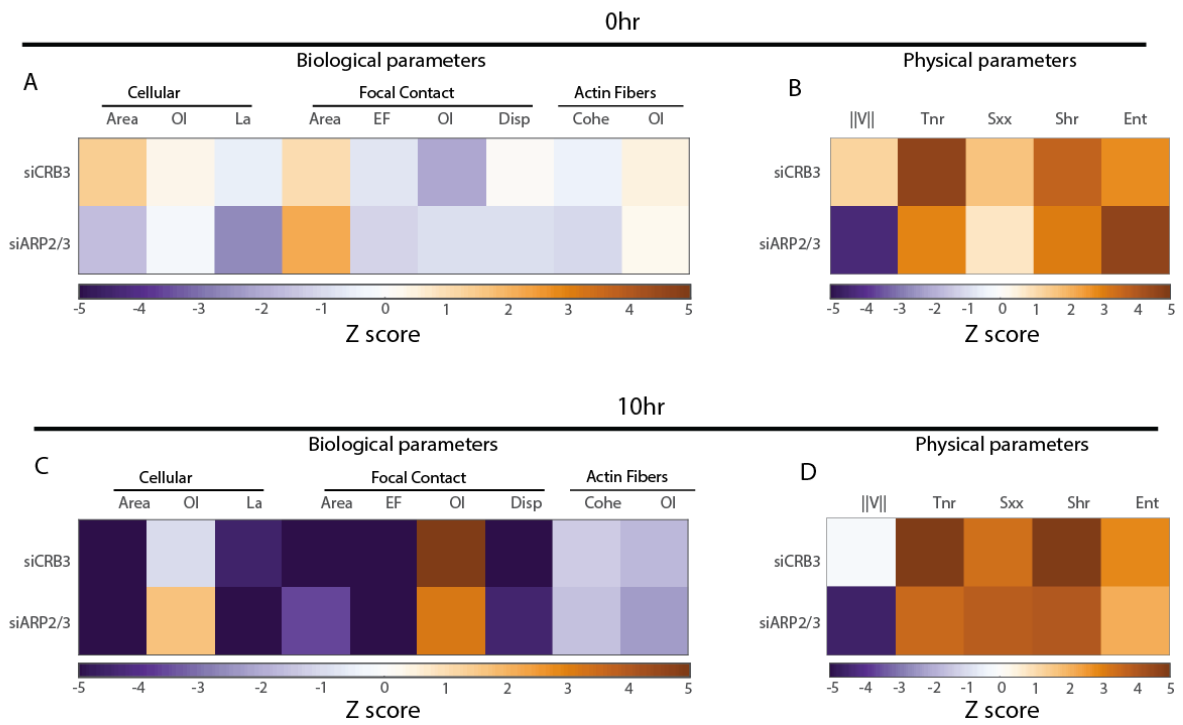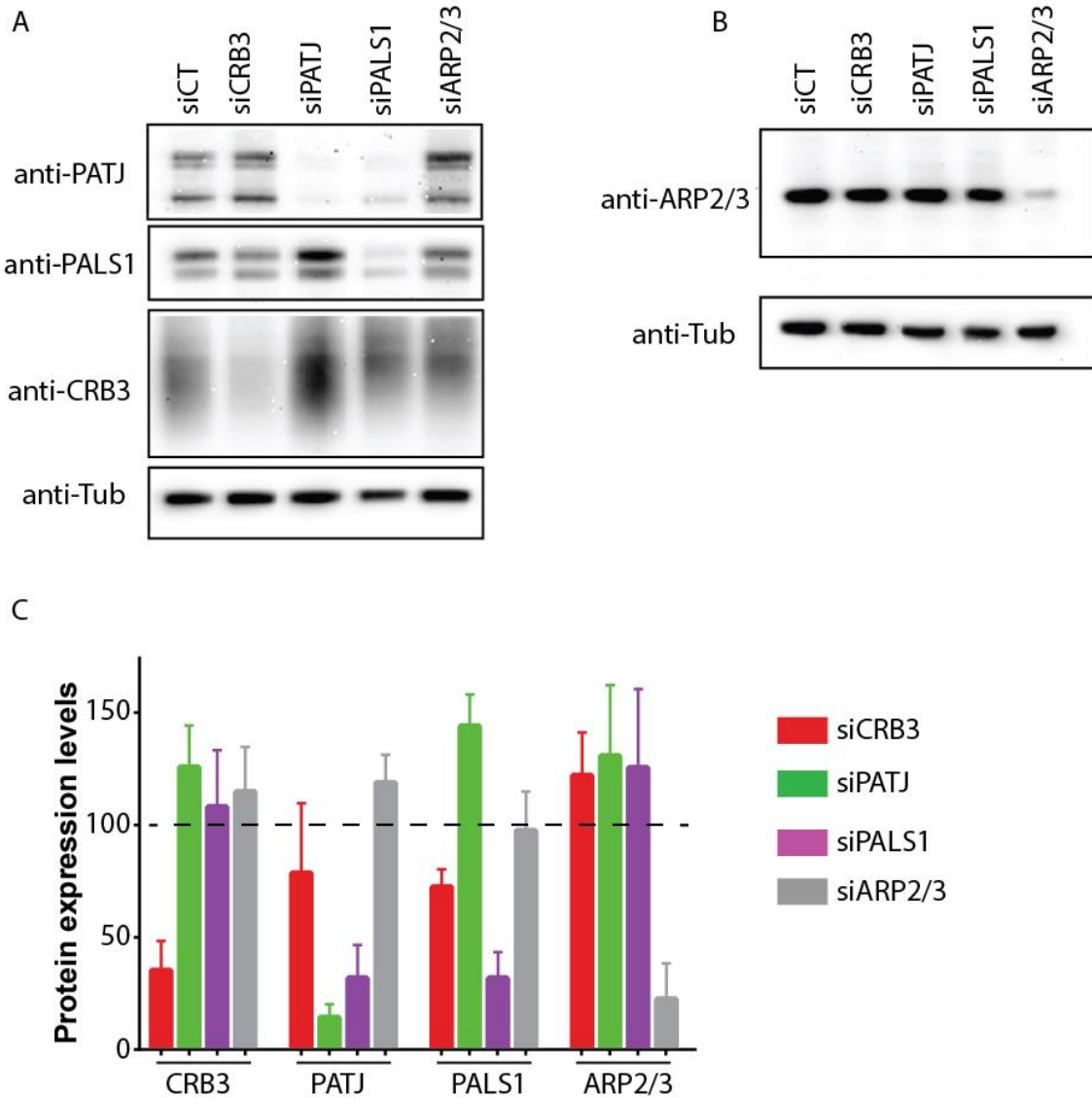
876

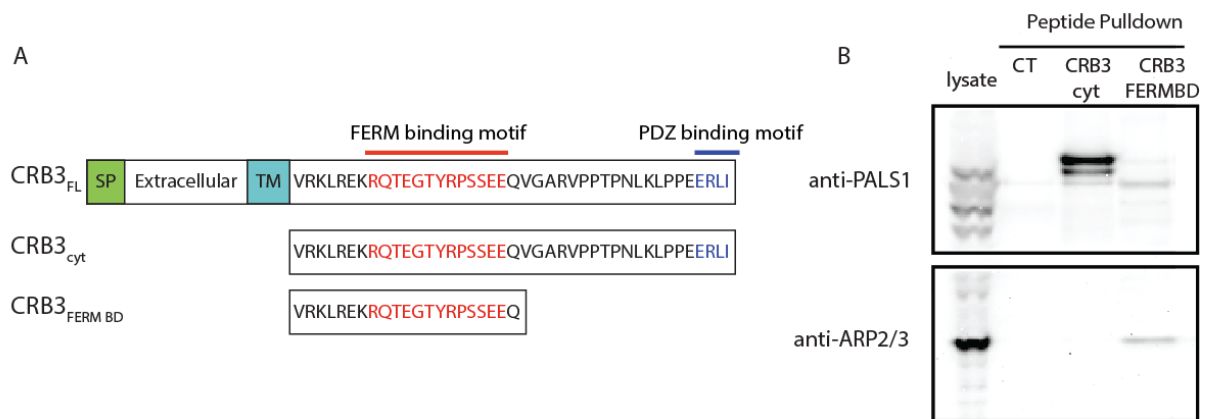890

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7

Figure S1

Figure S2

Figure S3

Figure S4