Biomimetic niches reveal the minimal cues to trigger apical lumen formation in single hepatocytes.

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Abstract

Symmetry breaking of protein distribution and cytoskeleton organization is an essential aspect for development of apico-basal polarity. In embryonic cells this process is largely cell autonomous, while differentiated epithelial cells collectively polarize during epithelium formation. Here, we demonstrate that the *de novo* polarization of mature hepatocytes does not require the synchronized development of apical poles on neighboring cells. It proves inducible at the single cell level by the mere contact with Extra-cellular matrix and immobilized cadherin defining a polarizing axis. The creation of these single cell liver hemi-canaliculi allows unprecedented imaging resolution and control and over the lumenogenesis process. We show that the density and localization of cadherins along the initial cell-cell contact act as a key triggers of the reorganization from lateral to apical actin cortex. The minimal cues necessary to trigger the polarization of hepatocytes enable them to develop asymmetric lumens with ectopic epithelial cell originating from kidney, breast, or colon.

Introduction

The development of apico-basal polarity in epithelial cells requires signaling from the extracellular matrix as well as from cell-cell contact. The matrix/cell junction direction provides an axis of external cues along which epithelial cells break their symmetry (*1-3*), organize their acto-myosin cortex (*4*), and direct the vectorial transport of proteins (*5, 6*). Mechanical constrains and tensions also play a role (*7-9*). The segregation of membrane proteins between apical, basal, and lateral poles is ensured by the fencing activity of tight junctions as well as by the antagonist pathways of the polarity complexes (Par1/Par2, Par3/Par6/aPKC, Crumbs and Scribble complexes). The situation differs in the early embryo when single cells can polarize in the absence of external cues. In *C.elegans* zygote, Par 1 and Par 3 complexes segregate based on antagonist kinase activity (*10, 11*) and acto-myosin cortical flow (*12*). In mouse embryo, blastocysts develop apical poles (*13, 14*) in the absence of extracellular matrix interactions in a fully cell autonomous manner (*15, 16*). In single intestinal epithelial cells LS174T cells, activation of LKB1 by inducible

33 STRAD stimulates the autonomous development of an ectopic brush border and the
34 localization of polarity complexes (*17*). The question then arises whether *de novo*
35 establishment of epithelial polarity requires solely a pre-existing set of external cues
36 triggering a single cell response, or requires the concomitant development of
37 polarity in the neighboring cells resulting in a collective response. Conventional
38 epithelial models (tissue, cell monolayer, or cysts) inherently fail to address this
39 question. Considering an individual cell polarizing in these multi-cellular contexts,
40 the concomitant reorganization of cell-cell contacts of the neighboring cells can act
41 as a polarization cue as well as a response to its polarization establishment.
42 This study shows that single primary hepatocytes develop independent *bona fide*
43 secretory apical poles when grown in synthetic microenvironments. In this context,
44 we demonstrate for the first time that the spatial assembly of junctional complexes
45 and polarity markers apical lumen development is inducible at the single cell level. It
46 depends mainly on actin cortex rearrangements triggered by the biophysical
47 properties of the cadherin-mediated adherens junction along the initial lateral cell-
48 cell contact and not on the subsequent response of the neighboring cells.
49
50 **Results:**
51 **Time sequence of single hepatocyte polarity development.**
52 Mature hepatocytes develop intercellular secretory apical lumen called bile
53 canaliculi. They consist of small, elongated tubules (2µm in diameter) extending
54 between two adjacent cells and sealed by tight junctions. Bile salt transporters (eg:
55 Bile salt export pump BSEP) and ion pumps (eg: Multidrug resistance-associated
56 protein MRP2) accumulate at the apical membrane to secrete bile into the lumen.
57 We devised microniches with an asymmetric distribution of extra-cellular matrix
58 (matrigel, collagen, laminin or fibronectin) and cell-cell contact proteins (E or N
59 cadherins) to test the minimal cues that a cell senses to trigger the development of
60 an apical lumen. We plated single hepatocytes on substrates with 700 μm^2
61 biofunctionalized patterns (cue 1) and covered with a feeding medium or an ECM
62 hydrogel (cue2). The asymmetry of the cues defined a vertical polarizing axis (**Figure
63 1a**). **Supplementary Figure 1** summarizes the several combinations of cues we tried.
64 Amongst all, ECM overlay (Matrigel, collagen or laminin) and cadherin patterns (E or

65 N cadherins interchangeably) (**Figure 1a**) created a functional polarizing axis for
66 primary rat hepatocytes (**Supplementary Figure 2**). It uniquely led to the
67 development of actin rings at the interface between the cells and the substrates
68 (**Figure 1b, Supplementary Figure 1a**). Concomitantly, the transporter MRP2
69 localized in the inner part of the actin ring. Similar structures developed with primary
70 mouse hepatocytes (**Supplementary figure 1b).** The development of the actin ring
71 did not depend on the cadherins type (E or N), or on the substrate rigidity (10 kPa to
72 70 GPa), or on the nature of the matrix overlay (Matrigel, laminin, collagen)
73 (**Supplementary Figure 1 b,c,d)**

74 We focused our study on E-cad patterns and 6% matrigel overlay. We
75 established the time sequence of the development of the actin-ring and the
76 concomitant recruitment of polarity associated proteins. We identified five phases
77 based on the different prevailing acto-myosin structures imaged by Structured
78 Illumination Microscopy (**Figure1b, c, d**).

79 In phase 1, 3h pre-matrigel induction, cells first adhered to the E-cad patterns.
80 During this phase an acto-myosin ring accumulated at the cell edge. Cadherins and
81 ZO1 co-localized with the ring. Other markers showed no specific localization.

82 In phase 2, 0h to 4h post induction*,* disorganized actin fibrils developed at the center
83 of the cell contact with the substrate. Myosin IIA and ZO1 were recruited along
84 these fibers. Par3 accumulated diffusively in the central region.

85 In phase 3, 4h to 7h post induction, a diffuse acto-myosin patch developed in the
86 center of the cells (50% cases, N=56). ZO1 and ZO2 accumulated at the outer edge of
87 the patch into a discontinuous ring. Par3 and MRP2 localized diffusively over the
88 patch. Cadherin attachment to the substrate remained homogenous across the
89 whole contact area.

90 In phase 4, 7h to 14h post induction, the acto-myosin patch densified into a
91 disorganized central region surrounded by radial fibers. Myosin was largely recruited
92 on the disorganized patch (**Supplementary Figure 3**). Cadherin detached from the
93 underlying substrate beneath the central patch region. Polarity markers (ZO1-2,
94 Par3) were densely recruited around the interface between the patch and the radial
95 fibers. MRP2 accumulated within the central patch region.

96 In phase 5, 14h post induction, hepatocytes developed the ring phenotype described
97 previously (**Supplementary Figure 1a**) with 85% occurrence rate (N=72). Note that
98 ZO1/2 were fully excluded from the edge of the cell. The transition from Phase 4 to
99 Phase 5 was suppressed by inhibition of actomyosin contractility or bile secretion
100 (**Supplementary Figure 4**), strongly suggesting that the transition from actin patch to
101 actin ring was mechanically driven by acto-myosin contractility and osmotic
102 gradients. This phase constituted the steady morphology without structural
103 alteration over more than 48 hours.
104 We then compared this sequence to the development of real lumens between two
105 cells imaged by 3D Structured Illumination Microscopy imaging (**Supplementary**
106 **Figure 5**). It revealed large similarities including the gradual accumulation of a dense
107 actin cortex (300 nm thick) at the center of the contact and the gradual relocalization
108 of ZO1 and Par 3 from the contact edge to the lumen edge. It also resembles pre-
109 apical actin patches (PAP) and Apical Membrane Initiation Sites (AMIS) (*4, 18*) in
110 previously described in MDCK doublets. However, in our case the actin structure
111 formed *in vivo* is far more localized at the membrane than what is reported for PAP
112 of MDCK (*4*).
113
114 **Structural and functional characterization of hemi-lumens**
115 We then detailed of the spatial and functional self-organization of the membrane
116 domains delineated by the actin ring. **Figure 2**a shows the positions of cellular
117 cadherin, ZO1/ZO2, Par-3, Claudin-3, Myosin IIA, MRP2 relative to the actin ring. The
118 circular shape of the ring enabled the computation of the average localization map
119 (**Figure 2b and Supplementary Figure 6a**) for each protein (**Material and Methods**).
120 From this map we found that myosin II microfilaments accumulated strongly along
121 the ring. Structured illumination microscopy (**Supplementary Figure 3**) revealed their
122 radial orientation across the ortho-radial actin fibers constituting the ring, strongly
123 suggesting that the ring is highly contractile. ZO1/ZO2 and Par3 accumulated
124 precisely at the edge of the actin ring (**Figure 2a,b**). However, transmembrane tight
125 junction proteins (occludin, claudin1 and claudin3 did not show any spatial
126 accumulation at any time (24h to 48h) (**Figure 2a,b and Supplementary Figure 7**).
127 Whereas cellular cadherins (**Material and Methods**) were depleted from the central

128 region of the ring, they accumulated 1.5 ± 0.5 µm away from the outer edge of the
129 ZO1-ZO2-Par3 ring and was diffusively present along the rest of the contact with the
130 substrate. The distribution of the recombinant cadherin on the patterns, however,
131 remained homogenous (**Supplementary Figure 2a**). Our experiment strongly
132 suggested that claudins and occludin localization required trans-binding with their
133 counterparts on neighboring cells, whereas the segregation of ZOs and Par3 from
134 adherens junctions did not require the complete development of tight junctions or a
135 concerted response of the neighboring cells. Note however that the concentric rings
136 of actin and ZO/Par 3 extend laterally over 2 ± 0.5 µm. In canaliculi formed between
137 2 cells, the peri-canalicular acto-myosin ring was smaller than 2 microns, and ZOs
138 and Par3 localized around a 200-300 nm away from the actin belts (*19*).
139 3D imaging (**Figure 2c**) revealed also that the Golgi overlapped precisely (95%
140 overlap N=40, **Material and Methods**) the actin ring region. It extended vertically to
141 reach the nucleus independently of the position of the nucleus in the cells. Similarly,
142 the network of microtubules was highly concentrated and structured above the actin
143 ring. Its 3D reconstruction (**Figure 2d**, **Supplementary Figure 6c**) unambiguously
144 indicated the polarized distribution inside the cells towards the E-cad substrate.
145 Beyond structural proteins we then tested the localization of the functional apical
146 (MRP2, BSEP, DDP-IV) and baso-lateral (NTCP, Na+/K+ ATPase) transporters and
147 channels (**Figure 2e, Supplementary Figure 6b**). Baso-lateral markers all segregated
148 away from the central region of the ring and localized dominantly at the interface
149 with the matrix (**Supplementary Figure 6b).** Sodium pumps also showed a systematic
150 enrichment at the edge of the actin ring, overlapping the ZO belt. Apical markers
151 mostly localized within the actin ring.: DPP-IV preferentially localized on actin rich
152 region, BSEP and MRP2 diffusely accumulated at the apical membrane and in apical
153 vesicles (**Figure 2c**). We concluded that the adhesion to ECM and inert immobilized
154 cadherin is sufficient to induce *bona fide* basal, lateral and apical domains in primary
155 hepatocytes. They constitute hemi-lumens that are structurally identical to true
156 canaliculi (**Figure 1a**). Although it was previously reported that cadherin and
157 integrins are necessary to elicit the development of apical poles (*2, 3*), the polarity in
158 our model developed without cell division (as in classical type of approaches (*20*)). It
159 resulted from single cell in contact with an inert substrate.

160 We then tested for bile secretion. Confocal imaging did not reveal any convincing
161 detachment of the membrane from the substrate suggesting a hemi-luminal cavity
162 below optical axial resolution (around 700nm). We thus used Reflection Interference
163 Contrast Microscopy (RICM) to measure the nanoscopic distance between the
164 substrate and the plasma membrane in living cells (*21*). It then revealed the average
165 concave shape of the membrane enclosed by the actin rings (**Figure 3 a**). The
166 membrane pulsated up and down at mean period of 6min/pulse. (N=9). The hemi-
167 lumens reached a maximal height of 171±5 nm (N=25). Upon partial inhibition of bile
168 salt synthesis by 10µm Ketoconazole, the lumen period increased by two fold to 12
169 min/pulse. It also enhanced lateral fluctuations of the intraluminal membrane
170 compared to the concentric pulsations observed in the control case (**Figure 3b** and
171 **Supplementary movies 1-2**). Additionally, actin filaments remained structured as a
172 patch in the center of the contact instead of organizing into a ring. We observed an
173 opposite effect when stimulating bile secretion using UDCA (Ursodeoxycholic acid,
174 40µM). The multiple circular interference fringes along the lumen indicated a three-
175 fold increase (633±54nm N=16) of the lumen maximal height. It strongly suggested
176 that the hemi-lumens formed between hepatocyte and cadherin-coated
177 micropatterns were functional and recapitulated the pulsatile behavior of canaliculi
178 observed *in vivo*. The reduced inflation of the lumens (around 200nm as compared
179 to 2 to 5 µm *in vivo*) likely results from (*22*) a large paracellular leak along lateral pole
180 lacking tight junctions and maintained solely by cadherins.
181 Our data demonstrated that the polarity program in mature hepatocytes does not
182 require the response of the neighboring cells or the remodeling of cadherin input
183 from substrate past the initial induction by cadherin-mediated contact. Interactions
184 with properly coated substrates suffice to induce the *bona-fide* polarization of single
185 hepatocytes (**Figure 1a**) in absence of tight junctions.
186 **Single hepatocytes develop secretory lumen with other cell types**
187 Drawing from the above conclusions, we reasoned that, provided initial contact
188 could be established, primary hepatocytes could polarized by mere adhesion to any
189 E or N cadherin presenting cells. To further test this hypothesis, we induced
190 heterotypic contacts between primary rat hepatocytes (*23, 24*) and epithelial cell
191 lines derived from various species and organs: EpH4, a murine breast cell line, Madin

Darby Canine Kidney cells (MDCK), a dog kidney cell line and Caco2, a human colorectal adenocarcinoma cell line. We tested that these simple epithelial cells did not self-polarize at the single cell level using the same microenvironment as for hepatocytes (**Figure 4a**). Monolayer co-cultures of these cell lines lead to spontaneous segregation of the population. However, constraining the cells in fibronectin coated microcavities (25x25x25µm) favored the establishment of stable heterotypic contacts (see **Material and Methods**). We previously established that such cavities forced cell-cell contacts to enable lumen formation (*9*). A large number of lumens formed along these heterotypic contacts. They presented the proper localization of the respective apical markers for each cell type (MRP2 for the hepatocytes, GBP35 for MDCK) and Golgi apparatus (**Figure 4b** and **Supplementary Figure 8**). All luminal membranes exhibited microvilli. ZO1 also localized at the lumen edge indicating a normal polarized state for both cells. The large inflation of the lumen revealed an efficient paracellular barrier and the development of transluminal osmotic gradients.

These results clearly demonstrate the ability of primary hepatocytes to self-polarize in contact with other epithelial cells. It suggests they could induce the polarization of their neighbours.

We then quantified the hetero-lumen formation efficiency with different epithelial cell types and compared it with the E-cad expression levels of those cell lines(MDCK < EPH4 < Caco2). The ratio of hetero-lumen formation inversely correlated with the cadherin expression levels in the cell line (**Figure 4c**). MDCK cells had the lowest expression levels of cadherin and proved the most efficient in creating hetero-lumens. We then overexpressed E-cadherin in EPH4 cells ($EPH4^{+}$) to reach the expression level found in Caco2 cells. It resulted in a lower occurrence of lumen formation matching that matching Caco2 hetero-doublets (**Figure 4c**). Similar overexpression in MDCK also led to the decrease of hetero-lumen formation. It strongly suggested a role of cadherin density in triggering apical lumen formation.

Additionally, hetero-contacts between rat hepatocytes and rat embryo fibroblast REF52 did not result in any subsequent development of luminal cavities. We observed that despite the establishment of a cell-cell junction between those cell types (**Figure 4d**), the cadherin contacts and the underlying actin structures

224 remained punctate. It strongly contrasted with the homogeneous cadherin and actin
225 distribution along hepatocyte/epithelial cell junctions. Taken together, these
226 observations led us to hypothesize that the apical pole formation required fine-
227 tuned spatial distribution of cadherin adhesion along the initial contact to induce
228 polarity.

229 **Cadherin distribution and action organization trigger polarity development**

230 We further tested these hypotheses using our single cell approach.

231 We modulated the amount of cadherin adhesion by changing the shape and size of
232 the pattern while keeping cadherin density constant. Independent of the pattern's
233 shape and size (**Supplementary Figure 9**), hemi-lumens formed with identical rate of
234 occurrence, and remained circular with an area of 200 μm^2 ± 60. Unconstrained
235 hepatocytes left to spread on non-patterned homogeneously coated cadherin
236 substrates also polarized. The lumens were then more irregular, and could reach an
237 area of 600 μm^2 for very large contacts (2400 μm^2). We concluded that the cadherin
238 contact area did not influence the lumen size and shape.

239 Next, we varied cadherin density on homogeneously functionalized substrates
240 (**Material and methods**). **Figure 5a** shows that at low cadherin densities the
241 hepatocytes did not attach. The number of adhering hepatocytes increased and
242 plateaued as we varied the cadherin density on the substrate by reaching absorption
243 equilibrium with 1 µg/ml to 50 µg/ml soluble E-cadherin (**Material and Methods**)
244 However, the proportion of lumen formation peaked significantly for 10 µg/ml
245 cadherin. Lower and higher cadherin densities proved less efficient in promoting
246 lumen formation confirming an optimal density of cadherin to trigger apical surface
247 development.

248 We then tested if the spatial distribution of cadherin also affected lumen formation.
249 We seeded single hepatocytes on doughnut cadherin patterns (30 µm ⃠) with an
250 antifouling region in the center (15 µm ⃠) (**Figure 5b**). We matched the size of the
251 non-adhesive region to the average size of hemi-lumens. All hepatocytes failed to
252 polarize. The membranes remained suspended over the non-adhesive part of the
253 lumen as demonstrated by the stochastic fluctuations probed by RICM live imaging
254 (**Supplementary Video 4**). It resulted in a flat average profile of the membrane
255 height compared to the dome shape profile observed on circular patterns (**Figure**

256 **5b**). The actin retained a structure comparable to a free cortex (**Figure 5c**). It never
257 developed into a ring. ZO1 and Par-3, and MRP2 were diffusely present over the
258 central zone and did not organized into rings at the edges (**Figure 5c**). These data
259 suggested that the local adhesion/de-adhesion process of cadherins at the center of
260 the contact was essential to polarity establishment. Correlatively, signaling from
261 cadherins outside the future luminal area was not sufficient to trigger the formation
262 of apical poles. We concluded that the lumen development originated from the local
263 engagement/disengagement of cadherins rather than from an integrated signal over
264 the whole contact.
265 We then hypothesized that preventing cytoskeletal rearrangement by external
266 physical cues would inhibit the development of an apical pole, irrespective of the
267 polarity axis provided by ECM and E-cadherin signaling. The polarity development
268 was directly triggered by the reorganization of actin prompted by E-cadherin
269 engagement. We thus impaired the development of the P2 phase fibers by plating
270 the hepatocytes on homogeneously E-cadherin coated substrates studded with
271 topographical features (pillars or grids with dimensions ranging from 2um to 500 nm
272 in width, and 800nm in height, **Material and Methods**). The hepatocytes spread on
273 and in-between the topographical features. Despite the homogeneous E-cadherin
274 coating, all topographies resulted in an inhibited development of P2 like actin fibers
275 (**Figure 5d**). Instead, actin pined around the topographical features. Upon addition
276 of matrigel, most of the actin remained largely “clamped” by the topography. This
277 absence of actin reorganization resulted in a drastic reduction of hepatocytes with a
278 polarized phenotype (75% polarization in the absence of features, compared to 20%
279 polarization on micropillars, 25 % on nanopillars, and 15% on nanogrids, **Figure 5d**).
280 MRP2 was diffusively recruited at the contact with the substrate (**Figure 5d**). Even
281 the polarized hepatocytes (discriminated by an actin structure surrounded by ZO1
282 and Par3) developed much smaller apical area than control ones (**Figure 4e**). Our
283 data thus demonstrated that impinging actin restructuration at the lateral pole
284 (either by physical impairment or by local absence of cadherin adhesion) inhibits of
285 polarity development.
286 These conclusions are in agreement with our observations with the hetero-
287 doublets reported above. Although hetero-doublets may lack physiological

288 relevance, heterotypic junctions are very likely to form between cells with different
289 hepatic lineage at various maturation stages during the long *in vivo* maturations of
290 hepatocytes (over weeks). When and how canaliculi formation is triggered still
291 remains to be deciphered. We tested the possibility of establishing luminal
292 structures between hepatocytes at different maturation stages using hiPSCs (human
293 induced pluripotent stem cells) differentiated over 25 days (*25, 26*) into hepatocytes.
294 The hepatoblast-to-hepatocyte differentiation took 6 days in vitro (*27*). During this
295 hepatic lineage induction, we observed a heterogeneous population of cells
296 expressing different levels of apical polarity markers (*28*). We plated a mixed
297 population of MRP2 positive and MRP2 negative hepatocytes in the micro-fabricated
298 cavities bio-functionalized with fibronectin (see protocol). We found that homotypic
299 junction between two $MRP2^+$ hepatocytes expectedly developed a canaliculus
300 (**Supplementary Figure 10**a). The lumens displayed symmetric accumulation of
301 MRP2 exclusively at their apical membrane. The apical poles of both cells displayed
302 characteristic actin enrichment. The lumens were inflated due to the proper
303 localization of tight junctions at their edges. We also observed a sizeable fraction
304 (40%±5) of heterotypic junctions between mature and immature hepatocytes
305 ($MRP2^+$ / $MRP2^-$ cells). They displayed strikingly asymmetric apical lumens
306 (**Supplementary Figure 10a**). The luminal membrane of the mature hepatocyte
307 ($MRP2^+$) was identical to the canaliculus formed in homotypic junctions between
308 mature hepatocytes. By contrast, the membrane of the immature hepatocyte
309 ($MRP2^-$) lacked actin enrichment and was hardly curved. However, tight junctions, as
310 shown by the presence of ZO1, likely sealed the edges of the asymmetric lumens
311 (**Supplementary Figure 10b**). We propose that mature hepatocytes could self
312 polarize and favor the local maturation of their neighbors cell by initiating a
313 functional canaliculi. This hypothesis remains to be fully tested.
314
315 Our single cell model demonstrates that the development of apical basal polarity in
316 hepatocytes is independent of the nature of their epithelial neighbors. The minimal
317 cues affecting the development lumen formation are summarized in **Figure 6**. The
318 spatial segregation of ECM and cadherin adhesion suffices to define a polarizing
319 direction that triggers the emergent organization a fully polarized phenotype

320 irrespective of the response of the neighbor cells. The spatial distribution of
321 cadherins adhesion along the initial cell-cell junction largely regulates the
322 reorganization of the lateral actin cortex. In turns, the actin organization
323 orchestrates the spatial distribution of the apical markers and of the Golgi.
324 Our reductionist approach demonstrated that single hepatocytes could be fooled
325 into a polarized state by artificial microniches and thus constituted, as far as bile
326 secretion is concerned, the first realization of a single cell liver.
327

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342 **Author Contributions:**

343 Y.Z and V.A performed most of the single cell experiment and imaging. R.T and S.S
344 NG developed and provided the IPSC induced hepatocytes. H.Y and I.C.N isolated
345 and provided the primary rat hepatocytes. R.M and YZ performed the quantitative
346 analysis. R.M and P.T performed the E-cad titration experiment. C.M helped and
347 quantified the RICM experiments. F.L.C provided invaluable support for imaging.
348 N.V.H an Y.Z performed the experiments on mouse hepatocytes. Y.Z, R.M and V.V
349 conceived the experiments. V.V supervised the work. Y.Z, R.M, V.A and V.V wrote
350 the manuscript.
351

352 **Competing Financial Interest:**

353 The authors declare no competing financial interest.
354

355 **Data availability.**

356 The data supporting the findings of this study are available within this article and its
357 Supplementary Information, or from the corresponding author on reasonable
358 request.

359

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463
464
465
466 **Methods:**

467 **Generation of Micropatterned substrates**

468 The 2D patterns were generated by microserigraphy method (*30*). 100µl of 10 µg/ml
469 fibronectin (Sigma, P1141) or E-cadherin (R&D System, 8875-EC) or N-cadherin (R&D
470 System, 1388-NC) was applied to a 2X2 cm NoA 74 membrane on a polymer bottom
471 dish (ibidi, 81156), and incubated overnight at 4°C. The membrane was peeled off
472 right before usage and the dish was treated with 0.2% pluronic acid for 30min at RT.
473 Employing the Alveole PRIMO system, E-cadherin coated doughnut patterns were
474 produced as recommended by the vendor. 10µl of 100µg/ml E-cadherin solution was
475 applied to each PDMS stencil, incubated for 2 hours at room temperature before
476 rinsing with PBS 3 times. The dish was then treated with 0.2% pluronic acid for
477 30min (Sigma, P2443-250G).
478
479 Generation of substrates of different rigidity
480
481 To fabricate soft (~10 kPa) substrates, we mixed polydimethylsiloxane (Dow Corning,
482 lot no. 0008602722) CY 52-276 component A and B at 1:1 ratio. To generate stiff
483 (~90 kPa) substrate, we made a mixture of CY52-276 components A and B (ratio =
484 1:1, total weight is x g), in which x µl (x is the net weight of A and B mixture) of
485 Sylgard 184 cross-linker was added. The above mixtures were degassed in a vacuum
486 chamber and spin-coated on clean coverslip. Subsequently, the coated coverslip was
487 placed in 80°C to let the PDMS cure for 1 hour. The surfaces of the cured substrates

488 were then silanized with (3-aminopropyl) triethoxysilane for 2 hours. Finally, the
489 coverslips were incubated with 10 µg ml^{-1} of E-cadherin solution (dissolved in
490 bicarbonate buffer) overnight at 4°.
491
492 **Microwell fabrication**
493 Microwells with dimensions of 25µm in diameter and 25µm in height were
494 fabricated using an established method (*29*). The functionalization of the microwell
495 top, side, and bottom surfaces was achieved by coating with 10µg/ml fibronectin for
496 1hour, followed by flipping into a fibronectin coated coverslip to passivate the new-
497 top surface with a solution of 0.2% pluronic acid.
498
499 **Topographical obstacles fabrication:**
500 Replicas of silicon molds containing the different features (750nm in height) was
501 made by double-casting PDMS (mixed at 10:1 base and curing agent, Sylgard184,
502 Dow Corning) cured at 80°C for 3 hours, passivated overnight at low pressure with a
503 solution of Trichloro(1H,1H,2H,2H-perfluorooctyl)-silane (Sigma, 448931).
504 The textured substrates were generated by UV curing (6 min, 185 and 253nm,
505 30mW/cm2, UVO Cleaner 342-220, Jelight) a drop of low refractive index polymer
506 premix (MY134, MyPolymers), sandwiched between a glass coverslip and the PDMS
507 mold, and immersed in water. After peeling off the mold, the features were coated
508 overnight with 10µg/ml E-cadherin solution (RnD, 8875-EC-50) in PBS at 4°C and
509 washed twice with PBS before cell seeding.
510
511 **hiPSC differentiation, seeding and culturing**
512 hiPSC-derived hepatic progenitor or hepatocyte-like cells were generated using an
513 established protocol (*25, 26, 31*). To generate hetero-doublets of iPSC-derived
514 hepatocytes at mature and immature stages, hepatocyte-like cells after 25 days of
515 differentiation were detached and suspended in Hepatozyme medium (Thermo,
516 17705021), supplemented with Oncostatin M 0.01 mg/ml (Bio-Techne, 295-OM-050)
517 and Hepatocyte Growth Factor 0.05 mg/ml (Peprotech, 100-39-100) to reach a final
518 cell density of $0.5 * 10^6$ cells/ml. Approximately 1 ml cell suspension was then
519 pipetted onto microwells in a 35mm glass bottom dish and placed in an incubator for

520 at least 2 hours to allow cell attachment. Extra cells that were not trapped in the
521 wells were removed by rinsing the dish with PBS buffer. The system was then
522 replenished with fresh culture medium. Cells were left in 5% CO_2 at 37°C and 95%
523 humidity for 1 day to develop polarity.
524
525 **Hepatocyte isolation, seeding and culturing**
526 Hepatocytes were isolated from male Wistar rats by a two-step in situ collagenase
527 perfusion method, as previously published (*32*). Animals were handled according to
528 the IACUC protocol approved by the IACUC committee of the National University of
529 Singapore. With a yield of >10^8 cells/rat, hepatocyte viability was tested to be >90%
530 by Trypan Blue exclusion assay.
531 In order to co-culture primary rat hepatocytes with another cell lines, e.g. MDCK,
532 EpH4, Caco2 and REF52 in a microwell array, freshly isolated rat hepatocytes (0.5
533 million) were seeded onto the microwell in the glass bottom dish and cultured in 2
534 ml of William's E culture medium supplemented with 2 mM L-Glutamine, 1 mg/ml
535 BSA, 0.3 µg/ml of insulin, 100 nM dexamethasone, 50 µg/ml linoleic acid, 100
536 units/ml penicillin, and 100 mg/ml streptomycin (Sigma-Aldrich). After 1 hour
537 incubation, the floating hepatocytes were removed by washing with PBS buffer and
538 culture medium were replenished. 0.5 million MDCK cells expressing histone-GFP
539 (generous gift from Dr Benoit Ladoux, Institut Jacques Monod, Paris), EpH4 or Caco2
540 or REF52 cells stained with CellTracker™ Green CMFDA Dye (ThermoFisher, C2925)
541 following manufacturer's instruction were subsequently detached and seeded into
542 the microniches. After 1 hour incubation, excess cells were removed and culture
543 medium were replenished. The system was left in incubator for 24 hours to develop
544 polarity.
545 For micropatterning experiments, 0.5 million rat hepatocytes were added onto E-
546 cadherin coated micropatterns in a 35mm glass bottom dish and cultured in 2ml of
547 William's E medium with all seven supplements as described before. Cells were
548 incubated with 5% CO_2 at 37°C and 95% humidity. After a 3-hour incubation, the
549 system was rinsed with PBS medium to remove hepatocytes that did not attach to
550 the micropatterns. The petri dish was subsequently replenished with fresh culture
551 medium. 3-hours later, the culture medium was replaced by medium supplemented

552 with 6% Matrigel (BD Bioscience, 356230) or 100 µg ml^{-1} soluble laminin (Invitrogen,
553 23017-015) . The matrigel was handled according to the protocol as described
554 previously[Martın-Belmonte, 2013]. Collagen I gel (0.75 mg ml^{-1}; Advanced
555 BioMatrix, 5005-100ML) was prepared following the manufacturer's instruction, and
556 1 ml of mixture was added to a 35-mm dish, and 1 ml medium without collagen was
557 replenished after gel formation. The system was then left in incubator for 24 hours.
558
559
560
561 **Pharmacological treatment**
562 To inhibit actomyosin contractility or block bile acid synthesis, culture medium
563 supplemented with blebbistatin (50µM in DMSO; Merck, 203390) or ketoconazole
564 (10µM in DMSO; Sigma, K1003) was administered 7 hours after matrigel overlay until
565 cell fixation. To stimulate bile acid secretion, Ursodeoxycholic acid (UDCA, 50µM in
566 DMSO; Sigma, U5127) was added at the same time as the matrigel overlay.
567
568 **Immunostaining and image acquisition**
569 Cells were fixed with 4% paraformaldehyde (PFA) for 30 minutes at 37°C. After
570 fixation, the cells were rinsed with PBS and permeabilized for 30 min in PBST (0.1%
571 Triton-X diluted in TBS). Permeabilized cells were blocked with 5% BSA diluted in PBS
572 for 4 h at 4^oC and incubated overnight with pan-Cadherin antibody (Sigma, C1821,
573 1:500), MRP2 antibody (Sigma, M8316, 1:200), BSEP antibody (Sigma, HPA19035,
574 1:250), DPP-IV antibody (SantaCruz, SC52642, 1:200), NTCP antibody (BosterBio,
575 PB9745, 1:500), Na^+/K^+ ATPase (Invitrogen, MA5-32184, 1:200), Par-3 antibody
576 (Millipore, 07-330, 1:200), ZO1 antibody (Life Technology, 61-7300,1:100), ZO-2
577 antibody (ThermoFisher, 38-9100, 1:100), Claudin-1 antibody (Invitro, 717800,
578 1:100), Claudin-3 antibody (Abcam, ab15102, 1:40), Occludin antibody (Invitrogen,
579 711500, 1:200), MyosinIIA antibody (Sigma, M8064, 1:200), Grasp65
580 antibody(Abcam, ab102645, 1:200), α-tubulin antibody(Abcam, ab15246, 1:200),
581 paxillin antibody (Abcam, ab32084, 1:200), 6x-His-tag antibody (ThermoFisher, MA1-
582 21315, 1:500) at 4^oC as instructed in the manufacturer's protocol. After rinsing with
583 PBS, cells were incubated with secondary antibodies (Alexa Fluor 546 Donkey Anti-

584 Rabbit IgG, A10040 and Alexa Fluor 647 Donkey Anti-Mouse IgG, A-31571, Life
585 Technologies, 1:200) and Alexa Fluor 488 Phalloidin (Life Technologies, A12379,
586 1:200) or ATTO-565 Phalloidin (Sigma 94072, 1:500) for 1 h in dark at room
587 temperature. After rinsing with PBS again and incubation with DAPI (Sigma, D9564),
588 cells were mounted in mounting medium (DAKO, S3023). 3D stacks of confocal
589 images were acquired with 60X NA1.3 water lens on a Nikon Eclipse Ti Microscope
590 equipped with Yokogawa CSU-X1 spinning disc unit. Structured Illumination
591 Microscopy images was acquired on the same microscope equipped with Live-SR
592 module (https://www.cairn-research.co.uk/product/live-sr/). The cells were chosen
593 purely based on criteria of cell adhesion. Typically, more than 70% of patterns
594 contained single cells that occupied the entire pattern, and these were selected for
595 imaging.
596

597 **RICM analysis:**
598

599 RICM analysis was performed considering the theory of partial coherent light,
600 following the description of cell adhesion analyses reported in Limozin and Sengupta
601 (*21*). Relative heights were reconstructed using the intensity-height relation

602 $$I(h) = \frac{S}{2} - D\frac{\sin(y)}{2y}\cos\left(2kn_i[hcos^2(\frac{\alpha}{2} - h_{off}]\right) \quad (1)$$

603 where

604 $$y = 2khsin^2\left(\frac{\alpha}{2}\right) \quad (2)$$

605 k = $2\pi/\lambda$ is the wave vector for the illumination light for a wavelength $\lambda = 546 \pm$
606 $10\ nm$, n1 = 1.335 is the refractive index of the outer buffer, S and D are the sum
607 and difference of the maximal and minimal intensity in the experimental fringe
608 pattern, respectively, and h_{off} is a phase shift arising from the reflection at different
609 interfaces.
610 The illumination numerical aperture (INA), which is given by the half-angle of the
611 cone of illumination, α, was set to a maximum value to minimize the depth of focus
612 and thereby to avoid reflections from organelles or other intracellular structures.
613 The measured INA amounted to $INA = n_1\, sin\, sin\,(\alpha) = 0.73$. Cell contact areas
614 of constant dark intensity were considered 'adhered' and of clostest proximity to the

615 substrate. These areas served as starting point for the reconstruction of relative
616 membrane heights.
617 Data were analyzed using self-written routines in Matlab (version 9.3 (R2017b), The
618 MathWorks, Inc. MA, USA) and FIJI (version 1.52s, Rasband, W.S., NIH, Bethesda,MD,
619 USA).
620
621
622 **Image analysis**
623 To analyze the relative position of each protein, a homemade program was written
624 with Matlab (MathWorks, Natick, Mass). The position of the lumen center was
625 determined by using fit-circle function implemented in Matlab with a radius range
626 lower than the cell size on the thresholded actin image by Otsu's method acquired at
627 the membrane/substrate interface. The radial profile was then performed on every
628 channel. The intensity profile for each cell was then aligned by normalizing the
629 distance between the centre of the lumen and the outer point of the actin ring. This
630 outer point was determined by finding the maximum of the second derivative of the
631 actin intensity profile. As there was no ring formed on E-cadherin doughnut pattern,
632 the distance was normalized between the centre of the pattern and the inner border
633 of the adhesive area. The relative distance of the different ring was then calculated
634 by measuring the distance between the peaks of the average curve of each staining.
635
636 To assess Golgi localization in relation to lumen position, the degree of overlay of
637 these two structures was measured. Z direction maximum intensity projection was
638 applied to all the stacks containing Grasp65 signal to extract Golgi structure, while
639 projection of selected frames of Phalloidin staining at the cell/substrate interface
640 was used to extract lumen localization. ROIs of Golgi and lumen structures were
641 created by thresholding the corresponding Z-projection images. The ratio of the
642 number of pixels of the intersection over that of actin mask was finally used to
643 describe the degree of overlaying.
644
$$Overlay(Actin, Golgi) = \frac{Mask_{Actin} \cap Mask_{Golgi}}{Mask_{Actin}} \quad (3)$$
645

646 To measure the size and circularity of hemi-lumens and cells, 3 frames of phalloidin
647 staining images at the hepatocyte/substrate interface were selected and
648 reconstructed using maximum intensity projection. The contour of the hemi-lumen
649 and cells was drawn manually based on F-actin signal using ImageJ. The Area and
650 Circularity were measured with the ImageJ measurement plugin. A circularity of 1.0
651 indicates a perfect circle. As the value approaches 0, it indicates an elongated
652 polygon.
653
654 To evaluate the MRP2 distribution and actin structure size for hepatocytes cultured
655 on textured substrate, selected frames imaged at the hepatocyte/substrate interface
656 were reconstructed using maximum intensity projection. The areas of MRP2, actin
657 structure and cells were then manually measured using ImageJ.
658
659 Statistical analysis was performed using GraphPad Prism 6
660 (https://www.graphpad.com/scientific-software/prism/). The statistical significance
661 between two groups was analyzed by Unpaired Student's t-test unless otherwise
662 stated. In all cases, a P value of less than 0.05 was considered statistically significant
663 and P value is specified in each captions.
664
665
666

Figure captions:

Figure 1: Actin and hepatic polarity related proteins undergo extensive reorganization when single hepatocytes are cultured in defined microenvironment. **a,** Left, a schematic showing the microenvironment in which single primary hepatocytes autonomously acquires apical basal polarity. Right, side view illustration summarizing the segregation of 3 distinctive domains and the position of the different proteins at the mature stage of hemi-lumen development. The apical pole is delimited by the actin ring whose inner part is rich in myosin IIA. Moving outwards, rings of ZO1/ZO2 and Par3 are found, followed by cell-cell contacts labelled by E-cadherin. E-cadherin is not observed from the centre of the lumen out to the ZO1/ZO2 ring. Mrp2, BSEP is mostly located above the apical pole. In this system, claudins and occludins did not exhibit any specific localisation. **b,** Representative Structured Illumination Microscopy (SIM) images showing the reorganization of cortical actin around the apical pole of single hepatocytes situated on E-cadherin circular patterns fixed at 3 hours before, and then 0, 4, 7, 10 and 14 hours after matrigel addition. Scale bar, 5µm. **c,** Representative SIM images of adherens junction associated proteins (Cadherins and Myosin II), cytosolic and transmembrane components of tight junction (ZO-1, claudin-3 and, occludin), apical markers (Par3 and MRP2) at five stages of polarity development. **d,** Fraction of cells displaying typical phenotypes of each development phase fixed at different time points. Based on the actin organization, five phases are defined as indicated. The number of cells analyzed was pooled from n = 3 independent experiments (n= 42 for P1, n= 65 for P2, n= 56 for P3, n= 66 for P4, n= 72 for P5).

Figure 2: Characterisation of protein organization of single-cell hemi-lumen. **a** and **b**, Montage and quantification of confocal images of the hemi-lumen stained for structural and polarity markers shows the spatial localisation of the different rings of proteins around the lumen. For quantification, the centre of the lumen is considered as 0 while the edge of the actin ring is considered as 1. The red, dark green, light green and blue background correspond to the region described in **e**. $N_{ActZO1Cad}$=9, $N_{ActMyosin}$=14, $N_{ActZO1Par3}$=17, $N_{ActCldn3}$=10, $N_{ActZO1ZO2}$=5, $N_{ActMrp2}$=11. **c.** Representative images showing relative location of actin structure (green) and maximum intensity projection of Golgi staining (by Grasp65 in red) in the cases when the hemi-lumens at the centre of cell/substrate interface. 3D reconstruction of image stack from left panel showing the Golgi(red) is situated right above lumen area (Cyan). Plots showing the projection of Golgi structure highly overlapped with lumen area. We do not observe any significant difference between both cases of lumen location. n=21 for centred lumen at centre, n=19 for off-centred lumen. Scale bar = 5µm. **d, left:** Confocal images of immunostained α-tubulin (red), actin (green) and nucleus (blue) in the hepatocyte with hemi-lumen showing the enrichment of microtubule arrays above central actin ring. **Right:** 3D reconstruction of image stack from left panel with orthogonal views. Scale bar = 5µm. **e,** Montage of typical immunofluorescent images of apical and basolateral markers shows that apical proteins, Mrp2, BSEP and DDP-IV, are predominantly found at the central actin region whereas NTCP and Na^+/K^+ ATPase are absent from apical domain. **Right:** en

713 face view illustrates the segregation of three zones at the cell-substrate interface
714 based on the distribution of cortical actin and polarity markers. Scale bar = 5μm.
715
716 **Figure 3: Functional characterisation of bile secretion of single-cell hemi-lumen.**
717 **a, Left:** Typical immunostaining and RICM images of single-cell liver. **Right**:
718 quantification of the lumen shape, maximum height and pulsation behaviour in
719 control condition. **b,** characterization of the actin structure, lumen shape, height and
720 pulsation behaviour after ketoconazole and UDCA treatment. Reduction of bile
721 secretion by ketoconazole leads to a homogeneous bright region that is shallower,
722 and with a slow pulsation compared to control. Boosting secretion by UDCA induces
723 an inflation of the lumen resulting in multiple interference rings on the RICM images.
724 Scale bar = 5μm.
725
726 **Figure 4: Hepatocytes form lumens at different rates with other epithelial cell lines**
727 **but not with fibroblast.**
728 **a,** Typical images of single MDCK (**left**) or EpH4 (**right**) plated on E-cadherin island
729 and embedded with 6% matrigel. Immunostaining of actin (green) and respective
730 apical marker (red), GP135 and moesin, suggests single MDCK and EpH4 cells don't
731 develop central apical pole. Scale bar = 5μm. **b**, Representative confocal images of
732 the lumen (L) created inside a microwell between primary hepatocyte (PH) and
733 different cell lines such as kidney cells (MDCK, KC) or mammary epithelial cells (Eph4,
734 ME) stained with Mrp2 (red), actin (green) and cell tracker/histones (magenta). The
735 well wall and cell junction has been delimited by dashed lines. Scale bar = 5μm. **c,**
736 **Left:** Quantification of the ratio of hetero-lumen formation to the hetero-doublets
737 between primary hepatocyte and another epithelial cell lines including Caco-2, EpH4
738 overexpressing E-cadherin (EpH4+), EpH4 wildtype, MDCK overexpressing E-cadherin
739 (MDCK+) and MDCK wildtype. **Right:** assessment of E-cadherin expression level in
740 epithelial cell lines monolayer via three independent Western blot. The hemi-lumen
741 occurrence is inversely correlated to expression levels of E-cadherin. Scale bar, 5μm.
742 N_{Caco2} = 29 (38%), N_{Eph4+} = 20 (35%), N_{Eph4} = 56 (59%), N_{MDCK+} = 84 (42%), N_{MDCK} = 53
743 (87%) **d, top:** representative side view images of hetero-doublets between primary
744 hepatocyte (PH) and rat embryo fibroblast cell line REF 52 (REF52) confined in micro-
745 cavities stained with actin (green), pan-cadherin (red) and cell tracker (magenta).
746 **Bottom:** the max intensity projection along the junctional volume indicated by dash
747 lines in the top panel. No lumen structures are observed and junctional cadherin and
748 actin remains punctate. Scale bar = 5μm. To distinguish cell types in heterodoublets,
749 Eph4 and REF52 cell lines were pre-stained with cell tracker and MDCK expressed
750 H2B-GFP.
751
752 **Figure 5: Cadherin-distribution-dependent actin organization is critical for apico-**
753 **basal polarity establishment.**
754 **a,** Low magnification images of primary hepatocytes stained for actin, ZO-1 and Pan-
755 cadherins after seeding on different concentrations of E-cadherin. A minimum
756 threshold of E-cadherin concentration is required for the cells to attach, illustrated
757 by the shaded region on the right graph. The number of cells attaching increases
758 with the concentration of E-cadherin above this threshold value. The ratio of
759 hepatocytes forming hemi-lumens to the total cells reaches an maximum at 10μg/ml

760 E-cadherin, above which the cells preferentially form lumens between each other.
761 White arrows point to representative hemi-lumens. Experiments have been
762 performed on 12 fields of views in 4 independent experiments for each
763 concentration. Scale bar = 10µm. **b, Left panel**: Schematic of the geometry and
764 dimensions of the E-cadherin coated doughnut pattern. **Middle panel**:
765 Representative heat map showing the height of plasma membrane to the coverslip
766 measured from reflective interference contrast microscopy (RICM) image on
767 doughnut pattern of e-cadherin. **Right panel**: Maximum height of plasma membrane
768 quantified from RICM images of primary hepatocytes on E-cadherin circular (n=28)
769 and doughnut (n=14) patterns. ****, p<0.0001. **c,** Representative fluorescence
770 images showing the actin, ZO-1, Par3 (left) and actin, MRP2 (middle) localized on the
771 plasma membrane/E-cadherin interface. Scale bar: 5µm. Quantification of actin, ZO-
772 1, Par3, MRP2 distribution in relation to the position of the non-adhesive region
773 (white) and E-cadherin coated region (Purple), n= 16 for cells stained with actin, ZO-1
774 and Par3, n=11 for cells stained with actin and MRP2. Despite the creation of a
775 lumen-like structure on doughnut pattern, no specific localization of apical markers
776 has been identified.
777 **d,** Representative immunofluorescence images showing perturbation of Actin, MRP2
778 and ZO-1 distribution in hepatocytes with either a disorganized central actin
779 phenotype (top, non-polarized NP) or with central actin ring phenotype (below,
780 polarized P) when seeded on micropillar, nanopillar, and nanogrid substrates coated
781 with E-cadherin. Scale bar, 5µm. Quantification of hemi-lumen occurrence in cells on
782 flat surface and on textured substrates as indicated (N= 53, 82, 81 and 100 for flat,
783 micropillars, nanopillars, and nanogrids, respectively). Schematics show the
784 dimension of each texture. The number of cells analysed was pooled from 4
785 independent experiments.
786 **e,** Quantification of the ratio of the area of the central actin ring and MRP2 ($A_{structure}$)
787 to the cell area (A_{cell}) for polarized primary hepatocytes seeded on flat surface
788 (n=22), micropillars (n=25), nanopillars (n=20) and nanogrids (n=24). Black lines pair
789 the ratio measured for actin and MRP2 in the same hepatocyte. Quantification of the
790 ratio of the area of MRP2 to the cell area of polarized (P) and non-polarized cells
791 (NP) seeding on micropillars, nanopillars and nanogrids (n=24-35), ****, p<0.0001.
792
793 **Figure 6: Graphical summary of the minimal external cues required to trigger the**
794 **development of the polarity of hepatocytes at different stages of hemi-lumen**
795 **formation.**
796 **a,** Important cues to trigger polarity as a whole. Details of the cues that we tested
797 and that do not appear to influence polarity development. Details of the biophysical
798 cues that inhibit the establishment of polarity. **b**, Promoting and inhibitory cues to
799 transition between the 5 steps of polarity development depicted in Figure 1. Actin
800 fibres are in green, Myosin II microfilaments are in red.
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