1 Asymmetric cell volume changes regulate epithelial morphogenesis in zebrafish Kupffer's 2 vesicle

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12 ABSTRACT

13 How epithelial cell behaviors are coordinately regulated to sculpt tissue architecture is a
14 fundamental question in biology. Kupffer's vesicle (KV), a transient organ with a fluid-filled
15 lumen, provides a simple system to investigate the interplay between intrinsic cellular
16 mechanisms and external forces during epithelial morphogenesis. Using 3-dimensional (3D)
17 analyses of single cells we identify asymmetric cell volume changes along the anteroposterior
18 axis of KV that coincide with asymmetric cell shape changes. Blocking ion flux prevents these
19 cell volume changes and cell shape changes. Vertex simulations suggest cell shape changes
20 do not depend on lumen expansion. Consistent with this prediction, asymmetric changes in KV
21 cell volume and shape occur normally when KV lumen growth fails due to leaky cell adhesions.
22 These results indicate ion flux mediates asymmetric cell volume changes that contribute to
23 asymmetric cell shape changes in KV, and that these changes in epithelial morphology are
24 separable from lumen-generated forces.

25

26 INTRODUCTION

27 In an embryo, epithelial cells undergo tightly regulated shape changes to drive tissue
28 remodeling and organ development. Changes in epithelial cell morphology can be mediated by
29 intrinsic mechanisms, such as rearrangements of the actomyosin cytoskeleton that often occur
30 in response to biochemical signaling cascades (Fuss et al., 2004, Escudero et al., 2007).
31 Extrinsic biophysical forces can also influence epithelial morphogenesis (Navis and Nelson,
32 2016, Navis and Bagnat, 2015). For example, the mechanical properties of neighboring cells
33 can help shape how an epithelium develops (Luu et al., 2011, Sedzinski et al., 2016). Another
34 source of extrinsic force found in many organs is a fluid-filled lumen. Forces generated by
35 increased fluid pressure during lumen expansion can have an impact on individual cell shapes
36 and the overarching epithelial architecture (Bagnat et al., 2010). Conversely, movements of

37 fluids from epithelial cells to the lumen have been proposed to regulate both lumen growth and
38 thinning of the epithelium (Hoijsman et al., 2015). Thus, exactly how intrinsic molecular
39 mechanisms and extrinsic mechanical forces interact to regulate epithelial cell shape changes
40 during organogenesis remains an open and intriguing question.

41

42 In this study, we used the zebrafish Kupffer's vesicle (KV) as a model organ to
43 investigate mechanisms that regulate shape changes of single cells during epithelial
44 morphogenesis. KV, which functions as a 'left-right organizer' to determine left-right asymmetry
45 of the zebrafish embryo (Essner et al., 2005, Kramer-Zucker et al., 2005), is a transient organ
46 comprised of a single layer of ~50 epithelial cells that surround a fluid-filled lumen. Each KV cell
47 extends a motile monocilium into the lumen to generate asymmetric fluid flows that direct left-
48 right patterning signals. Fate mapping studies have identified precursor cells, called dorsal
49 forerunner cells (DFCs), that differentiate into epithelial KV cells at the end of gastrulation
50 stages of development (Melby et al., 1996, Cooper and D'Amico, 1996). These cells then form
51 the KV organ in the tailbud at the embryonic midline (marked by notochord) during early
52 somitogenesis stages (**Figure 1A**). Similar to other organs that develop a fluid-filled lumen—
53 such as the gut tube (Alvers et al., 2014) or pancreas (Villasenor et al., 2010)—KV cells form a
54 rosette-like structure to give rise to a nascent lumen that expands over time (Amack et al., 2007,
55 Oteiza et al., 2008). Previous 2-dimensional (2D) analyses of KV cells revealed that during
56 expansion of the KV lumen, KV cells at the middle focal plane undergo asymmetric cell shape
57 changes along the anteroposterior (AP) axis that sculpt the architecture of the mature KV organ
58 (Wang et al., 2012). A transgenic strain developed in this study, *Tg(sox17:GFP-CAAX)*,
59 provides bright labeling of KV cell membranes with green fluorescent protein (GFP) that shows
60 the AP asymmetric architecture of the whole organ (**Movie 1**) and the 2D shapes of epithelial
61 KV cells (**Figure 1A**). KV cells have similar shapes at early stages of development, whereas at
62 later stages the cells in the anterior half of KV (KV-ant cells) develop columnar morphologies
63 that allow tight packing of these cells and posterior KV cells (KV-post cells) become wide and
64 thin (Wang et al., 2012) (**Figure 1B**). This morphogenetic process, which we refer to as 'KV
65 remodeling', results in an AP asymmetric distribution of cilia that is necessary to drive fluid flows
66 for left-right patterning (Wang et al., 2012). Thus, KV is a simple and accessible organ that is
67 ideal for probing the relationship between intrinsic and extrinsic mechanisms that drive epithelial
68 morphogenesis.

69

70 Previous studies of KV have successfully contributed to our understanding of how
 71 epithelial cell shapes are regulated during embryogenesis, but the mechanisms that control KV
 72 cell shape changes are not fully understood. Experimental results and mathematical simulations
 73 from our group indicate that actomyosin contractility and differential interfacial tensions between
 74 KV cells mediate asymmetric cell shape changes (Wang et al., 2012). Additional studies
 75 identified an AP asymmetric deposition of extracellular matrix (ECM) implicated in restricting
 76 anterior KV cell shape during KV lumen expansion (Compagnon et al., 2014). We reasoned that
 77 these mechanisms likely work in concert with yet additional mechanisms to fully instruct
 78 epithelial morphogenesis during KV organ formation. Here, we developed methods to analyze
 79 single KV cells in 3-dimensions (3D) and created novel mathematical vertex models of KV
 80 development to identify mechanisms that contribute to asymmetric epithelial cell shape changes
 81 in KV. 3D analyses revealed that KV-ant cells increase their volume and KV-post cells decrease
 82 their volume during KV morphogenesis. These asymmetric cell volume changes occur at the
 83 same time as asymmetric cell shape changes. At the molecular level, KV cell volume and shape
 84 changes are mediated by ion channel activity that regulates ion flux and fluid transport. We next
 85 tested whether extrinsic biophysical forces had an impact on these cell morphology changes.
 86 Mathematical models indicate that mechanical properties of external cells surrounding the KV
 87 can impact cell shape changes in the KV. Models predicted that when external tissues are solid-
 88 like, asymmetric cell volume changes in KV cells contribute to cell shape changes even in the
 89 absence of lumen expansion. Consistent with mathematical model predictions, experimental
 90 perturbations of lumen expansion indicated that changes in KV cell volume and shape can occur
 91 independent of forces associated with lumen growth. Together, our results suggest ion channel
 92 mediated fluid flux serves as an intrinsic mechanism to regulate epithelial cell morphodynamics
 93 that create asymmetry in the KV organ. These findings shed new light on the interplay between
 94 lumenogenesis and epithelial morphogenesis and provide an example of cell morphology
 95 changes that can be uncoupled from mechanical forces exerted during lumen expansion.

96

97 **RESULTS**

98

99 **Mosaic labeling enables 3D analysis of single KV cells**

100 To investigate 3D behaviors of KV cells we first generated stable *Tg(sox17:GFP-CAAX)*
 101 transgenic zebrafish using a *sox17* promoter (Sakaguchi et al., 2006) to express membrane
 102 localized GFP (GFP-CAAX) in KV cells. This transgene marks all cells in the KV and is useful

103 for delineating 2D cell morphology (**Figure 1A**). However, due to difficulties in determining exact
 104 cell-cell boundaries in the KV (**Movie 1**), this strain is not ideal for visualizing individual KV cells
 105 in 3D. Therefore, we next developed a Cre-*loxP* based mosaic cell labeling method to visualize
 106 single KV cells. For this approach, we generated transgenic *Tg(sox17:Cre^{ERT2})* zebrafish that
 107 expresses Cre recombinase in the KV cell lineage that has inducible activity through the addition
 108 of 4-hydroxytamoxifen (4-OHT) (Feil et al., 1997). Transgenic *Tg(sox17:Cre^{ERT2})* fish were
 109 crossed with a previously described *Tg(ubi:Zebrafow)* ('zebrabow') strain (Pan et al., 2013) that
 110 can be used to generate differential fluorescent labeling of cells based on stochastic Cre-
 111 mediated recombination of the zebrabow transgene (**Figure 1C**). Double transgenic
 112 *Tg(sox17:Cre^{ERT2}); Tg(ubi:Zebrafow)* embryos were briefly treated with 4-OHT from the dome
 113 stage (4 hours post-fertilization or hpf) to the shield stage (6 hpf) (**Figure 1D**) to induce low
 114 levels of Cre activity that resulted in a switch from the default RFP (red fluorescent protein)
 115 expression to YFP (yellow fluorescent protein) expression in a subset of KV cells (**Figure 1E**).
 116 This strategy reliably created mosaic labeled KVs containing a few YFP⁺ KV cells with
 117 boundaries that are easily distinguished from surrounding RFP⁺ cells (**Movie 2**). Images of
 118 mosaic labeled KVs in living *Tg(sox17:Cre^{ERT2}); Tg(ubi:Zebrafow)* embryos were volume-
 119 rendered using Imaris (Bitplane) software to generate 3D reconstructions of the lumen and KV
 120 cells (**Figure 1F**). To assess the 3D morphology of individual KV cells residing in either the
 121 anterior or posterior region of the KV organ, we used the lumen surface as a reference point to
 122 ensure that all 3D datasets were analyzed from the same perspective. To make morphometric
 123 measurements, we defined three axes of KV cells: 1) cellular 'height (H)' is the length of the axis
 124 connecting dorsal and ventral surfaces of the cell, 2) cellular 'length (L)' is the length of the axis
 125 connecting apical (associated with the lumen) and basal surfaces of the cell, and 3) cellular
 126 'width (W)' is the length of the axis connecting two lateral sides of the cell (**Figure 1G,H**). The
 127 combination of mosaic labeling, live imaging and 3D cell morphometrics provides a new
 128 approach to investigate the cellular and mechanical mechanisms underlying KV epithelial
 129 morphogenesis at single cell resolution.

130

131 To assess the dynamics of KV cells in 3D, we first performed time-lapse imaging of
 132 mosaic labeled KVs in live *Tg(sox17:Cre^{ERT2}); Tg(ubi:Zebrafow)* embryos from the 2-somite
 133 stage (2 ss) when the lumen first forms to the 8 somite stage (8 ss) when the lumen is fully
 134 expanded (Amack et al., 2007, Wang et al., 2012, Gokey et al., 2016). These stages span the
 135 process of KV remodeling when KV cells change their shapes: 2 ss is pre-remodeling and 8 ss

136 is post-remodeling (Wang et al., 2012). Time-lapse images from these stages indicated KV cells
 137 are highly dynamic during KV morphogenesis (**Movie 3**). To quantify KV cell dynamics, we took
 138 several precautions in subsequent experiments to avoid artifacts. First, to avoid potential
 139 photobleaching from time-lapse imaging, live embryos were imaged only once at one stage of
 140 development—not continuously or at multiple stages. Second, to avoid differences in
 141 fluorescence signal due to differences in imaging depth, all KVs were visualized laterally (YZ
 142 orientation) and only mosaically labeled cells from the middle plane of the KV organ that is
 143 perpendicular to the dorso-ventral axis were selected for analysis (**Figure 2A**). We define the
 144 middle plane as the plane with the largest lumen diameter when viewed dorsally. Finally, we
 145 determined that the Cre activity was not spatially biased, but rather randomly labeled cells
 146 throughout the KV. By analyzing enough embryos, we sampled KV cells from all positions along
 147 the middle plane of KV at different stages of development (**Figure 2-figure supplement 1**).

148

149 To quantify the morphometric properties of KV-ant and KV-post cells during KV
 150 morphogenesis, live embryos with mosaic labeled KVs were imaged at a specific stage of
 151 development (2 ss, 4 ss, 6 ss and 8 ss) and then individual cells at the middle plane of the
 152 organ were volume-rendered (**Figure 2B**). We measured the height, length and width of KV
 153 cells from several wild-type embryos to determine the average parameters for KV-ant and KV-
 154 post cells. The results showed cell-to-cell variability, but pooling measurements from multiple
 155 cells at a specific developmental stage identified trends and statistically significant differences
 156 during KV morphogenesis. First, cell height of both KV-ant cells and KV-post cells increased
 157 from 2 ss to 8 ss (**Figure 2C**), reflecting the expansion of the apical surface of cells (see dotted
 158 line in **Figure 2B**) to accommodate lumen growth. There were no significant differences in cell
 159 height between KV-ant cells and KV-post cells during these stages (**Figure 2C**). Second, cell
 160 length decreased in both KV-ant cells and KV-post cells during KV morphogenesis, but a sharp
 161 reduction was observed from 4 ss to 6 ss in KV-post cells (**Figure 2D**). This resulted in the cell
 162 length of KV-post cells to become significantly different from KV-ant cells after 4 ss. Importantly,
 163 it is between 4 ss to 6 ss when cell shape changes associated with KV remodeling were
 164 previously observed (Compagnon et al., 2014, Wang et al., 2012). Third, analysis of cell width
 165 indicated KV-ant cells remained relatively constant during KV morphogenesis, whereas KV-post
 166 cells showed a distinctive increase in width between 4 ss and 6 ss that resulted in significant
 167 differences between KV-ant cells and KV-post cells after 4 ss (**Figure 2E**). In order to make
 168 comparisons with previous 2D studies, we calculated length-to-width ratios (LWR) to describe

169 cell morphology. We found that both KV-ant and KV-post cells had similar morphologies at 2 ss
170 and 4 ss and that these morphologies changed after 4 ss such that KV-ant cells had a
171 significantly larger LWR than KV-post cells (**Figure 2F**). This analysis of individual KV-ant and
172 KV-post cell shapes is consistent with asymmetric cell shape changes associated with KV
173 remodeling that were previously identified using 2D measurements of KV cell LWRs (Wang et
174 al., 2012) and provides for the first time a 3D quantification of epithelial cell morphodynamics
175 that occur during KV development.

176

177 **3D analysis of single cells reveals asymmetric cell volume changes during KV** 178 **morphogenesis**

179 We next used mosaic labeling to address whether the size of individual KV cells
180 changes during development. Measuring the volume of 3D reconstructed KV cells revealed
181 striking dynamics in cell size that mirrored changes in cell shape. At 2 ss and 4 ss, KV-ant and
182 KV-post cells showed similar cell volumes (**Figure 2G**). However, between 4 ss to 6 ss—when
183 KV cells change shape—the volume of KV-ant cells increased and the volume of KV-post cells
184 decreased (**Figure 2G**). Cell volumes were significantly different between KV-ant and KV-post
185 after 4 ss (**Figure 2G**). Thus, asymmetric cell volume changes along the AP axis occur during
186 the same stage as AP asymmetric cell shape changes. Overall, between 2 ss and 8 ss, KV-ant
187 cells increased volume from $2106 \pm 1014 \mu\text{m}^3$ to $2547 \pm 693 \mu\text{m}^3$ (shown are mean $\pm$ one
188 standard deviation) and KV-post cell decreased volume from $2180 \pm 1034 \mu\text{m}^3$ to 1564 ± 539
189 μm^3 (**Figure 2G**). The considerable standard deviations here likely reflect the high degree of
190 variability in KV size among wild-type embryos (Gokey et al., 2016). It is important to note that
191 while the cell sizes are variable, the direction of size changes during development is constant.
192 KV-ant cells always increase in volume and KV-post cells always decrease in volume. This
193 identification of asymmetric cell volume changes provides new insight into the mechanics of
194 epithelial morphogenesis in KV.

195

196 To better understand volume changes during KV development, we measured the volume
197 of the entire KV organ at different stages. To make these measurements in live embryos,
198 *Tg(sox17:GFP-CAAX)* transgenic fish were crossed with *Tg(actb2:myl12.1-MKATE2)* zebrafish
199 that have enriched apical membrane expression of the fluorescent mKate2 protein fused to
200 Myl12.1 (myosin light chain 12 genome duplicate 1) in KV cells. Double transgenic
201 *Tg(actb2:myl12.1mKATE2; Tg(sox17:GFP-CAAX)* embryos were used to visualize both KV

202 lumen and KV cells in living embryos (**Figure 2-figure supplement 2A**). The KV lumen was
 203 reconstructed in 3D using *Tg(actb2:myl12.1-MKATE2)* expression and then divided into two
 204 equal halves (hence equal volumes) along the AP axis (**Figure 2-figure supplement 2B,B'**).
 205 Next, the total KV cellular component of KV-ant and KV-post cells was reconstructed using
 206 *Tg(sox17:GFP-CAAX)* expression (**Figure 2-figure supplement 2B,B'**). Volume measurements
 207 indicated that both 'total KV-ant cellular volume' and 'total KV-post cellular volume' were similar
 208 at 2 ss, but then were significantly different at 8 ss (**Figure 2-figure supplement 2C**). Overall,
 209 'total KV-ant cellular volume' increased from $1.4 \times 10^5 \mu\text{m}^3$ to $1.7 \times 10^5 \mu\text{m}^3$ and 'total KV-post cell
 210 volume' decreased from $1.2 \times 10^5 \mu\text{m}^3$ to $0.98 \times 10^5 \mu\text{m}^3$ between 2 ss and 8 ss stages (**Figure 2-**
 211 **figure supplement 2C**). Thus, consistent with volume changes in single cells at the middle
 212 plane of KV, we observed asymmetric volume changes along the anterior and posterior axis of
 213 KV when the entire cellular component of the organ was analyzed. These results suggested that
 214 asymmetric cell volume changes might contribute to lumen growth and/or cell shape changes
 215 during the concurrent processes of lumen expansion and epithelial morphogenesis in KV.

216

217 Inhibiting ion flux disrupts asymmetric cell volume changes, lumen expansion and cell 218 shape changes in KV

219 We next sought to identify a mechanism that mediates KV cell volume changes between
 220 2 ss and 8 ss. At the molecular level, cellular volume can be controlled through coordinated flux
 221 of Na^+ , K^+ and Cl^- ions via specific ion channels and pumps that results in osmotically driven
 222 water transport (Wilson et al., 2007, Barrett and Keely, 2000, Frizzell and Hanrahan, 2012,
 223 Frizzell, 1995, Damkier et al., 2013, Spring and Siebens, 1988, Hoijman et al., 2015, Saias et
 224 al., 2015). To test whether cell volume changes in KV cells are mediated by ion flux, we
 225 inhibited either the sodium-potassium pump (Na^+/K^+ -ATPase) or the cystic fibrosis
 226 transmembrane conductance regulator (Cftr). Both have previously been shown to play a role in
 227 KV lumen expansion (Navis et al., 2013, Compagnon et al., 2014). We first treated mosaic-
 228 labeled (e.g. 4-OHT treated) *Tg(sox17:Cre^{ERT2}); Tg(ubi:Zebrafish)* embryos with the small
 229 molecule ouabain to inhibit the Na^+/K^+ -ATPase pump as previously described (Compagnon et
 230 al., 2014). The Na^+/K^+ -ATPase has a central role in generating an electrochemical gradient
 231 across the plasma membrane that drives transport of water and solutes (Wilson et al., 2007,
 232 Damkier et al., 2013). Ouabain treatments are known to disrupt ion flux, resulting in an increase
 233 in intracellular sodium and calcium concentrations. In these experiments, control embryos
 234 treated with vehicle (DMSO; dimethyl sulfoxide) had mosaic labeled KV-ant and KV-post cells

235 that had similar morphologies at 2 ss and quite different morphologies at 8 ss (**Figure 3A**).
 236 Similar to wild-type embryos, the KV lumen expanded in DMSO treated control embryos
 237 between 2 ss and 8 ss and mosaic labeled cells in these KVs underwent normal AP asymmetric
 238 changes in cell volume and shape (**Figure 3A,E**). Embryos treated with ouabain from the bud
 239 stage (10 hpf) to 8 ss (13 hpf) showed reduced KV lumen expansion (**Figure 3B,E**), which is
 240 consistent with previous results (Compagnon et al., 2014). We next tested whether inhibiting
 241 Na^+/K^+ -ATPase activity has an impact on KV cell volume dynamics. In control embryos, KV-ant
 242 cells increased volume from $2019 \pm 668 \mu\text{m}^3$ to $2304 \pm 618 \mu\text{m}^3$ and KV-post cells decreased
 243 volume from $1811 \pm 422 \mu\text{m}^3$ to $1479 \pm 323 \mu\text{m}^3$ between 2 ss and 8 ss (**Figure 3A**). In contrast,
 244 AP asymmetric cell volume changes were not observed in embryos treated with ouabain. In
 245 ouabain treated embryos both KV-ant and KV-post cells increased volume from $1582 \pm 818 \mu\text{m}^3$
 246 to $2328 \pm 1050 \mu\text{m}^3$ and from $1551 \pm 286 \mu\text{m}^3$ to $2407 \pm 493 \mu\text{m}^3$ respectively (**Figure 3B**).
 247 These results support a model in which ion flux mediates asymmetric cell volume changes
 248 during KV morphogenesis.

249

250 As a second approach to test the role of ion flux in regulating KV cell volumes, we
 251 interfered with Cftr activity. Cftr is an apically localized chloride channel that moves Cl^- ions out
 252 of the cell and establishes electrochemical gradients, which drive water into the lumen through
 253 osmosis (Navis and Bagnat, 2015). Cftr activity can also modulate several other ion-channels
 254 and transporters (Vennekens et al., 1999), making it a key driver of ion flux and fluid secretion
 255 (Braunstein et al., 2004, Valverde et al., 1995). Mosaic-labeled embryos were treated with the
 256 pharmacological compound CFTRinh-172 to inhibit Cftr activity (Roxo-Rosa et al., 2015) or a
 257 previously characterized antisense Cftr morpholino oligonucleotide (Cftr MO) (Gokey et al.,
 258 2016) to reduce Cftr protein expression. Treating embryos with $30 \mu\text{M}$ CFTRinh-172 from bud
 259 stage to 8 ss reduced KV lumen expansion (**Figure 3C,E**) as expected (Roxo-Rosa et al.,
 260 2015). To test whether Cftr has a role in modulating KV cell size, we performed a 3D analysis of
 261 mosaic labeled single cells. Similar to ouabain treatments, CFTRinh-172 treatments eliminated
 262 asymmetric volume changes in KV cells. In contrast to controls (**Figure 3A**), KV-post cells in
 263 CFTRinh-172 treated embryos did not lose volume, but rather increased in volume from $1878 \pm$
 264 $361 \mu\text{m}^3$ to $2079 \pm 857 \mu\text{m}^3$. KV-ant cells increased in volume from $1530 \pm 289 \mu\text{m}^3$ to $1917 \pm$
 265 $477 \mu\text{m}^3$ (**Figure 3C**). Reducing Cftr expression by injecting embryos with Cftr MO had effects
 266 that were similar to CFTRinh-172 treatments: KV lumen failed to expand and asymmetric cell
 267 volume changes were disrupted (**Figure 3D,E**). In Cftr MO treated embryos both KV-ant and

268 KV-post cells increased their volume from $2047 \pm 725 \mu\text{m}^3$ to $2252 \pm 842 \mu\text{m}^3$ and from $2039 \pm$
 269 $745 \mu\text{m}^3$ to $2367 \pm 770 \mu\text{m}^3$ respectively (**Figure 3D**). This suggests that in both CFTRinh-172
 270 and Cftr MO treated embryos KV-post cells fail to undergo volume loss due to inhibition of fluid
 271 efflux from these cells. Taken together, these results indicate ion flux regulated by Na^+/K^+ -
 272 ATPase and Cftr activity is a mechanism that drives asymmetric changes in KV cell volumes
 273 along the AP axis.

274 We next evaluated the impact of perturbing ion flux on cell shape changes during KV
 275 remodeling. The height (apical surfaces) of KV cells did not increase between 2 ss and 8 ss in
 276 embryos treated with inhibitors of Na^+/K^+ -ATPase or Cftr activity as they did in controls (**Figure**
 277 **3-figure supplement 1A**), which is consistent with a failure of lumen expansion. Analysis of
 278 length to width ratios (LWRs) of individual KV-ant and KV-post cells revealed that AP
 279 asymmetric cell shape changes observed in control embryos between 2 ss and 8 ss (**Figure**
 280 **3A**) failed to occur in embryos treated with ouabain or Cftr inhibitors (**Figure 3B-D**). These
 281 results indicated that ion flux is necessary for both cell volume changes and cell shape changes
 282 during KV remodeling.

283 Since our ion flux inhibitor treatments were global, we wanted to test whether blocking
 284 ion channels altered other tissues in the embryo, including cells surrounding KV that could have
 285 an impact on KV cell shapes. Since the effect of loss of Cftr function on KV has already been
 286 determined genetically (Navis et al., 2013), we focused on the effects of ouabain treatments on
 287 external cells. The overall morphology of ouabain treated embryos was similar to controls at 8
 288 ss, except the KV lumen was smaller (**Figure 3-figure supplement 2A**), indicating ouabain
 289 treatments did not cause severe developmental defects. To analyze cells surrounding KV, we
 290 ubiquitously expressed a membrane-localized mCherry (*mCherry-CAAX*) in *Tg(sox17:GFP-*
 291 *CAAX)* embryos that allowed us to simultaneously visualize both KV cells at the middle plane of
 292 KV and the surrounding external cells (**Figure 3-figure supplement 2B**). Due to lack of a
 293 reference frame (e.g. the lumen surface) to quantitate LWRs of surrounding cells, we used a
 294 different parameter called the 'cell shape index, q ' ($q = [(\text{cell cross-sectional perimeter}) / \sqrt{(\text{cell}$
 295 $\text{cross-sectional area})}]$) to define surrounding cell morphology (Bi et al., 2016). Analysis of
 296 external cells with clearly defined boundaries (*mCherry-CAAX* labeling) that were positioned
 297 either anterior or posterior of KV indicated that there was no significant difference in cell shapes
 298 at 2 ss or 8 ss stages between control or ouabain treated embryos (**Figure 3-figure**
 299 **supplement 2C**). These results indicate that ouabain does not alter the shapes of cells

300 surrounding KV and suggest that defects in KV cell shape changes result from altered ion flux in
 301 KV. This is consistent with a previous study (Compagnon et al., 2014), in which blocking ion flux
 302 suggested forces associated with lumen expansion drive KV remodeling. However, because our
 303 3D analyses showed that altering ion flux disrupts both lumen expansion and KV cell volume
 304 changes, it remained unclear whether failed cell shape changes were due to defects in lumen
 305 expansion or asymmetric cell volume dynamics or both.

306 **Mathematical simulations of KV cell shape changes**

307 To begin to tease apart how intrinsic cell size changes and extrinsic lumen expansion
 308 forces contribute to asymmetric KV cell shape changes, we developed and simulated a
 309 mathematical vertex model for cell shapes in KV. Vertex models, which have been used
 310 successfully by our group and others for predicting features of developing tissues (Fletcher et
 311 al., 2014, Bi et al., 2015, Farhadifar et al., 2007, Hufnagel et al., 2007, Wang et al., 2012),
 312 represent two-dimensional cross-sections of cells in a tissue as a network of edges and
 313 vertices, as shown in **Figure 4A-D**. Adhesion molecules and cytoskeletal machinery generate
 314 forces that affect cell shape in different ways. In the vertex model, the balance between these
 315 forces is represented by an effective interfacial tension parameter Λ . Positive interfacial
 316 tensions describe cytoskeletal forces that tend to decrease interface lengths, while negative
 317 interfacial tensions describe adhesion effects that tend to increase interface lengths.
 318 Additionally, cellular volume control is described in the 2D vertex model by a preferred cross
 319 sectional area A_0 that the cells strive to attain. Deviations of a cell's actual area from its
 320 preferred area correspond to cellular pressures. A so-called "conjugate gradient" computer
 321 algorithm was applied to alter the positions of the vertices based on the forces acting on them
 322 until a relaxed state is reached where all interfacial tensions are balanced by cellular pressures.
 323 Additional details about the model and the computer algorithm can be found in the supplemental
 324 materials.

325

326 Using the vertex model, we studied whether the observed AP asymmetry in KV cell
 327 volume changes could act upstream of the observed AP asymmetry in KV cell shapes as
 328 described by aspect ratios. To this end, we focused our modeling efforts on the middle plane of
 329 the KV (**Figure 4A-D**). We first measured the cross-sectional areas of KV cells within this plane
 330 and found that changes in cell cross-sectional areas correlated with the corresponding cell
 331 volume changes (**Figure 4 – supplement table 1**). Thus, to test whether KV cell volume

changes can be sufficient to induce changes in KV cell aspect ratios, we prescribed the measured cross-sectional areas for KV lumen and KV cells (**Figure 4 – supplement table 2**) as preferred areas A_0 in our model and then studied the induced AP asymmetry of KV cell aspect ratio.

336

To simulate KV cell shape changes, we initialized a vertex model where a lumen is surrounded by $2N$ adjacent KV cells (split into KV-ant and KV-post cells with N chosen between 8 and 12) and the KV organ is surrounded by 100 “external” cells. To understand how the volume changes between 2 ss and 8 ss affect cell shape, we do not take into account the full time-dependent evolution of the system. Rather, we performed quasi-static simulations consisting of two parts. Initially, preferred area values A_0 for lumen, KV-ant cells, and KV-post cells were set equal to the respective 2 ss values reported in the first column of **Figure 4-supplement table 2**, and the system was relaxed to a force-balanced state using our computer algorithm (upper panels in **Figure 4A-D**). Subsequently, the preferred area values A_0 were changed to their respective values at 8 ss chosen from the second column of **Figure 4-supplement table 2** and the system was relaxed again (lower panels in **Figure 4A-D**). Such a quasi-static approach is appropriate if in the zebrafish KV, relaxation to mechanical equilibrium is faster than the volume changes of lumen and KV cells. In other developing epithelia, this relaxation timescale has been measured using laser ablation and is on the order of seconds (Fernandez-Gonzalez et al., 2009), which is significantly faster than the rate of lumen volume expansion, which is on the order of hours. Note that the images in **Figure 4A-D** are cropped to focus on the KV rather than the surrounding cells (same for **Figure 4 – figure supplements 2,3A-D**). We show the full system for **Figure 4A** in **Figure 4 – figure supplement 1B**.

355

There are still additional free parameters in the model, corresponding to the interfacial tension values for each cell, but these are not constrained by experimental data. Therefore, we decided to perform a wide parameter sweep to determine how these parameters affect cell shapes. In previous work (Wang et al., 2012), we demonstrated that AP asymmetric interfacial tensions were sufficient to drive KV cell shape remodeling even in the absence of asymmetric volume changes. To analyze whether asymmetric area changes alone are sufficient to drive the asymmetric KV cell shape changes, we choose the interfacial tensions between KV cells, Λ^{KV-KV} , to be identical between KV-ant and KV-post cells. For simplicity, we assume that all

363

364 cells external to the KV all have the same interfacial tension $\Lambda^{\text{ext-ext}}$, which is allowed to differ
 365 from $\Lambda^{\text{KV-KV}}$. For the purpose of illustration, we show two example simulations for different te-
 366 nsion pairs $(\Lambda^{\text{KV-KV}}, \Lambda^{\text{ext-ext}})$ in **Figure 4A,B**. In **Figure 4A** with $\Lambda^{\text{KV-KV}} = -50$ and $\Lambda^{\text{ext-ext}} =$
 367 150, KV-ant cells at 8 ss appear narrow and elongated while KV-post cells appear wide and
 368 short, qualitatively reflecting the experimentally observed KV cell shape asymmetry at 8 ss.
 369 However, in **Figure 4B** with $\Lambda^{\text{KV-KV}} = 50$ and $\Lambda^{\text{ext-ext}} = -120$, KV-ant and KV-post cells at 8 ss
 370 have very similar morphology, suggesting no cell shape change. To quantitatively compare
 371 simulations with experimental data, we developed a metric that captures the anterior-posterior
 372 asymmetry (APA) of KV cell shapes that is characteristic of KV remodeling. APA is defined as
 373 the difference between the length-width ratios (LWRs) of KV-ant and KV-post cells: $\text{APA} =$
 374 $\text{LWR-ant} - \text{LWR-post}$ (for the definition of the LWR, see **Figure 4 – figure supplement 1A**).
 375 Note that because the definition of the APA is based on length-to-width ratios, it is size-
 376 independent. In our *in vivo* measurements, wild-type embryos at 8 ss correspond to an APA
 377 value of ~ 0.9 . For the simulation shown in **Figure 4B**, we found an APA value of 0, suggesting
 378 no asymmetry, while the simulation in **Figure 4A** has APA value of 0.29, which is clearly
 379 asymmetric but not as high as in wild-type experiments.

380

381 Because the interfacial tensions $\Lambda^{\text{KV-KV}}$ and $\Lambda^{\text{ext-ext}}$ cannot be determined from our
 382 experimental data, an obvious question is whether there is *any* choice of those tension values in
 383 our model that would allow area changes alone to drive the observed shape changes. **Figure**
 384 **4E** is a plot of APA as the interfacial tension in KV cells and external cells are varied. For each
 385 $(\Lambda^{\text{KV-KV}}, \Lambda^{\text{ext-ext}})$ parameter pair, the indicated APA value represents an average computed
 386 from 100 individual simulation runs. Blue areas indicate a positive APA corresponding to regions
 387 where KV-ant cells are more radially elongated than KV-post cells are, while red regions
 388 indicate negative APA corresponding to regions with more elongated KV-post cells. A first
 389 observation is that the APA is never above 0.31, which is much smaller than wild-type
 390 experimental observations. This suggests that changes to cross-sectional area may be an
 391 important contribution to shape remodeling, but alone they are not sufficient to generate the
 392 observed shape changes.

393

394 A second observation is that there are coherent regions in parameter space with similar
 395 values of APA, which suggests that our model may be able to identify a simple mechanism for

396 how changes to cross-sectional area drive shape change. It has recently been discovered that
 397 as cells increase their interfacial tension Λ or increase their preferred area A_0 , the tissue
 398 transitions from fluid-like to solid-like behavior, undergoing a so-called rigidity transition (Bi et al.,
 399 2014, Bi et al., 2015, Park et al., 2015). Moreover, some of us have recently reported that a
 400 similar fluid-solid transition also occurs in bulk three-dimensional tissues (Merkel and Manning,
 401 2017). Therefore, we expect that if we can identify a simple mechanism for 2D shape
 402 asymmetry that depends on the fluidity of the surrounding tissue, that same mechanism will also
 403 be present in 3D.

404

405 To do so, we map the results for 2D fluid-solid transitions onto our model, where black
 406 dashed lines in **Figure 4E** indicate phase boundaries between solid and fluid. In the upper right
 407 quadrant of **Figure 4E,F**, both the KV cells and external cells are solid-like (SS), in the lower
 408 right quadrant external cells are fluid-like while KV cells are solid-like (FS), in the upper left
 409 quadrant KV is fluid-like and external tissue is solid-like (SF), and in the lower left quadrant both
 410 tissues are fluid-like (FF). There is also a small, hatched region of parameter space where KV-
 411 ant cells are solid-like while KV-post cells are fluid-like. To test the significance of our APA
 412 values, we computed the standard error of the APA mean for each ($\Lambda^{KV-KV}, \Lambda^{ext-ext}$) parameter
 413 pair. We found that for solid-like KV cells, the error is typically on the order of 0.05, indicating
 414 that our results are robust in this regime. Conversely, for fluid-like KV cells, the standard error
 415 of the mean can become much larger, which is reflected in the higher APA fluctuations in this
 416 regime. They are a direct consequence of softer or floppier KV cells, leading to large
 417 fluctuations in their LWRs.

418

419 Interestingly, our model predicts that cross-sectional area changes drive the observed
 420 shape changes primarily in the solid-solid region, and our understanding of the fluid-solid
 421 transition helps us to understand this effect (**Figure 4G**). When the external cells are fluid-like,
 422 the KV cells are able to slide past the external cells, so that the interface between the KV-ant
 423 and KV-post cells (indicated by a thick black line in **Figure 4G**) moves towards the posterior as
 424 the area of the KV-post cells is reduced. In contrast, when the external cells are solid-like, the
 425 interface between the KV-ant and KV-post cells is pinned. In this second case, when the KV-
 426 post cells lose area they must maintain their lateral width and so the apico-basal extension must
 427 decrease. We note that this proposed mechanism works equally well in 3D as in 2D -- solid-like
 428 external tissue would pin the anterior-posterior interface so that cell volume changes would

429 affect the area of lateral interfaces between KV cells but not the apical area in contact with the
 430 lumen. Therefore, in 3D asymmetric cell volume changes would lead to asymmetric cell shape
 431 changes and similar APA values to the ones we identified in our 2D model. Although we cannot
 432 rule out a more complex model for KV cell shape changes (e.g. with additional parameters
 433 characterizing the mechanical heterogeneities in each cell), our simple model suggests that
 434 asymmetric cell volume changes contribute to cell shape changes, though additional
 435 mechanisms are necessary to explain the very high APA values that are observed in
 436 experiments.

437

438 The number of epithelial cells in KV can vary in a wild-type population (Gokey et al.,
 439 2016), therefore we checked the robustness of our result (APA values) with respect to small
 440 changes in N . In particular, while **Figure 4** shows the simulation results for $N = 10$ KV-ant and
 441 KV-post cells, **Figure 4-figure supplements 2-3** show the corresponding results for $N = 8$ and
 442 $N = 12$, respectively. In particular, independent of N , we observe positive APA only in the
 443 regime where both KV and external cells are solid-like. Moreover, there is a general trend of
 444 higher APA for a smaller KV cell number. Note that in addition to the mechanism creating the
 445 AP cell shape asymmetry illustrated in **Figure 4G**, which works largely independent of N , we
 446 have also discovered a quite different mechanism, which only works for small N if KV-ant cells
 447 are solid and KV-post cells are fluid (for details, see Supplemental Information). In this case,
 448 KV-post cells are more easily deformed and accommodate the lumen expansion by increasing
 449 their apical lumen interface, which leads to flatter KV-post cells and thus a high APA at 8 ss
 450 (see **Figure 4-figure supplement 2E**). Note however that this mechanism depends on lumen
 451 expansion (compare **Figure 4-figure supplement 2F**).

452

453 Another benefit of the model is that we can test specific hypotheses prior to exploring
 454 them experimentally. First, to investigate whether lumen expansion is necessary to create an
 455 asymmetry in KV cell elongation, we repeated the numerical simulations shown in **Figure**
 456 **4A,B,E** — which included both asymmetric cell cross-sectional area changes and increase in
 457 lumen cross-sectional area between 2 ss and 8 ss — except in this simulation we kept the
 458 lumen cross-sectional area fixed (**Figure 4C,D,F**). The APA values, shown in **Figure 4F** are
 459 generally smaller (max. APA value of 0.23) than those in **Figure 4E** (max APA value of 0.31).
 460 However, most of the regimes where both KV and external cells are solid-like still show positive
 461 APA values. These results suggest that in an environment in which cells have solid-like

462 mechanical properties, asymmetric volume changes in KV cells can partially drive asymmetric
463 KV cell shape changes even in the absence of lumen expansion (**Figure 4H**).

464

465 Second, using our model, we can explore whether heterogeneous mechanical properties
466 of the external cells can have a significant effect on KV cell shape changes. So far, we have in
467 our model described all external cells using the same parameters. However, given the presence
468 of morphogenetic gradients along the AP axis within the presomitic mesoderm that include FGF
469 and Wnt signals (Oates et al., 2012), it is plausible that the mechanical properties of the tailbud
470 cells surrounding the KV may also show an AP-oriented gradient. Moreover, the KV is
471 anteriorly abutting the notochord with likely different mechanical properties from the tailbud cells.
472 We thus wondered how our simulation results would depend on such heterogeneities of the
473 external cells. To study this question, we performed simulations similar to that shown in **Figure**
474 **4A** where we additionally allowed all external cells on either the posterior side or the anterior
475 side to be fluid (**Figure 4 – figure supplement 4A** and **B**, respectively). These fluid-like
476 subsets of the external cells have an interfacial tension of $\Lambda^{\text{ext-ext}} = -120$ (as in **Figure 4B**). All
477 other parameters were chosen as in **Figure 4A**, which included asymmetric cell cross-sectional
478 area changes and lumen expansion. In particular, the solid external cells had $\Lambda^{\text{ext-ext}} = 150$.
479 We found that AP cell asymmetry was much more pronounced if only the anterior external cells
480 were solid (**Figure 4 – figure supplement 4A**) than if only the posterior cells were solid (**Figure**
481 **4 – figure supplement 4B**). For the solid anterior external cells, the average APA computed
482 from 100 simulations was 0.39 with a standard error of the mean of 0.03. Thus, solidity of only
483 the anterior cells can be sufficient to create an asymmetry of KV cell shapes that can be slightly
484 stronger than if all external cells were solid. Conversely, the average APA with solid external
485 cells only posteriorly was only 0.07 with a standard error of the mean of 0.04. Thus, if there are
486 solid cells only posteriorly, the induced AP asymmetry in KV cell shape was much weaker.
487 Hence, asymmetry in the mechanical properties of the cells (and/or material) surrounding the
488 KV can support asymmetric cell shape changes in KV.

489

490 **Interfering with Junction plakoglobin function inhibits KV lumen expansion**

491 We were intrigued by our modeling results that predicted that given the right environment
492 of surrounding cells, changes in KV cell volumes contribute to changes in KV cell shapes even
493 when the lumen fails to expand. To test this prediction experimentally, we wanted to take an
494 approach that would allow us to monitor cell shape changes in KVs in which ion flux and cell

495 volume changes occur normally but lumen expansion is inhibited. Since coordinated remodeling
 496 of adherens junctions between epithelial cells plays important roles during lumen formation
 497 (Alvers et al., 2014), we chose to interfere with junctions between adjacent KV cells to disrupt
 498 lumen growth. The adherens junction component E-cadherin has been linked to cell junction
 499 stability and barrier function that maintains luminal and tubular structures (Tay et al., 2013,
 500 Tunggal et al., 2005). E-cadherin is expressed in KV cells (Matsui et al., 2011, Tay et al., 2013),
 501 but loss of E-cadherin function in mutant embryos leads to early developmental defects during
 502 epiboly that preclude analysis of KV formation (Kane et al., 2005). We therefore needed tools
 503 that allow junctions to form, but with weakened integrity that allows fluid to leak out of the lumen.
 504 Transcriptome analysis of zebrafish KV cells (unpublished data) indicated that Junction
 505 plakoglobin (Jup; also called γ -catenin) is expressed in KV cells. Jup interacts in complexes at
 506 cell-cell adhesions (Fukunaga et al., 2005, Lewis et al., 1997) and is thought to link cadherins to
 507 the cytoskeleton (Kowalczyk et al., 1998, Leonard et al., 2008, Holen et al., 2012). Previous
 508 studies in cell cultures indicated Jup plays an essential role in maintaining cell-cell adhesions
 509 (Fang et al., 2014) and that perturbing Jup function results in increased epithelial permeability
 510 (Nottebaum et al., 2008). The zebrafish genome contains two *jup* genes, *jupa* and *jupb*. RNA *in*
 511 *situ* hybridizations confirmed *jupa* expression in KV cells and the population of precursor cells
 512 that give rise to KV called dorsal forerunner cells (DFCs) (**Figure 5-figure supplement 1**).
 513 Immunofluorescence experiments using Jup antibody (Martin et al., 2009) indicated Jupa
 514 protein is localized to lateral membranes of KV cells that are marked by GFP expression in
 515 *Tg(sox17:GFP-CAAX)* embryos (**Figure 5A**). Thus, we predicted that interfering with Jupa
 516 function would perturb KV cell-cell junction integrity such that the KV lumen would fail to expand
 517 properly.

518

519 To test the function of Jupa in KV morphogenesis we used an antisense morpholino
 520 (MO) to block *jupa* pre-mRNA splicing (*jupa* MO-1) or a previously reported MO that blocks
 521 translation (*jupa* MO-2) of *jupa* mRNA (Martin et al., 2009). Injection of either *jupa* MO
 522 efficiently reduced Jupa protein levels at KV cell junctions, as compared to embryos injected
 523 with a negative control MO (**Figure 5A; Figure 5-figure supplement 2A**). Reduction of Jupa
 524 expression was also confirmed using immunoblotting. Jupa antibody detected a prominent band
 525 (arrowhead) around 75 kDa—consistent with Jupa proteins (~75-80 kDa) in other vertebrates
 526 (McKoy et al., 2000)—that was significantly reduced in *jupa* MO injected embryos (**Figure 5B**).
 527 Interfering with Jupa expression with MOs did not alter the gross morphology of embryos at 8

528 ss, but did disrupt KV lumen expansion relative to controls (**Figure 5C,D**) as predicted. ZO-1
 529 immunostaining of apical tight junctions was used to assess the severity of lumen expansion
 530 defects *jupa* MO treated embryos (**Figure 5C**). Delivering *jupa* MO specifically to the DFCs
 531 (Amack and Yost, 2004) that give rise to KV also disrupted lumen expansion (**Figure 5C,D**),
 532 indicating Jup functions cell-autonomously during KV morphogenesis. Importantly, the effect on
 533 KV lumen expansion caused by MO injection was significantly rescued by co-injection of full-
 534 length *jupa* mRNA (**Figure 5C,D; Figure 5-figure supplement 2B**), indicating specificity for this
 535 phenotype. As a second approach to compromise Jupa function, we injected a human *JUP*
 536 mRNA with a mutation that causes naxos disease (McKoy et al., 2000). This mutant '*JUP*-
 537 naxos' mRNA has previously been shown to encode a dominant-negative protein that interferes
 538 with Jup function in zebrafish (Asimaki et al., 2014). Similar to *jupa* MO treatments, expression
 539 of the *JUP*-naxos mRNA reduced KV lumen expansion without inducing other overt defects
 540 (**Figure 5C,D**). Importantly, the number of ciliated cells in KV in *jupa* MO and *JUP*-naxos mRNA
 541 treated embryos was similar to controls (**Figure 5E; Figure 5-figure supplement 2C**), which
 542 demonstrates that small KV lumen area was due to reduced lumen expansion rather than a
 543 reduced number of KV cells.

544

545 We next tested our prediction that loss of Jupa weakens cell-cell adhesions that are
 546 necessary for KV lumen expansion. First, we found that reducing Jupa expression moderately
 547 reduced E-cadherin enrichment (~22% decrease) along KV cell lateral domains relative to
 548 controls (**Figure 5-figure supplement 3**), which is consistent with previous results in cell culture
 549 studies (Fang et al., 2014). This finding suggested that although E-cadherin is maintained at
 550 levels sufficient for epiboly movements and KV formation, the cell-cell adhesions in KV might be
 551 weaker in Jupa depleted embryos than in wild-type. To test this functionally, we treated embryos
 552 with a small molecule activator of the Cftr channel (CFTRact-09) that increases Cl⁻ ion flux and
 553 can over-inflate the KV lumen (Gokey et al., 2016). Treatment of control MO embryos with
 554 CFTRact-09 significantly increased KV lumen area (~50%) as compared to DMSO, but a similar
 555 increase was not observed in Jupa depleted embryos (**Figure 5F**). This suggested that fluids
 556 entering the lumen were leaking out through compromised cell-cell junctions. To test this
 557 directly, we injected a solution containing fluorescent dextran into the KV lumen. In 4 out of 5
 558 control MO embryos the dextran remained in the lumen over a 1-hour time period. Conversely,
 559 in most Jupa depleted embryos the dextran gradually leaked out and only 1 out of 5 embryo
 560 retained significant amounts of dye (**Figure 5G**). Together, these results indicate Jupa functions

561 to maintain KV cell-cell adhesion integrity that is critical for KV lumen expansion, and suggest
562 Jupa depletion could provide a useful approach to block lumen expansion without affecting ion
563 flux mediated cell volume changes in KV.

564

565 **Asymmetric KV cell shape changes occur independent of lumen expansion**

566 We next used Jupa depleted embryos as a tool to test our hypothesis that KV cell
567 volume changes impact KV cell shape changes in the absence of forces exerted by the process
568 of KV lumen expansion. Morphometric analyses of individual KV cells in mosaic-labeled
569 *Tg(sox17:Cre^{ERT2}); Tg(ubi:Zebrawow)* embryos treated with *jupa* MO-1 revealed that
570 asymmetric KV cell volume changes occurred between 2 ss and 8 ss in Jupa depleted embryos
571 (KV-ant cells increased volume from $2015 \pm 534 \mu\text{m}^3$ to $2283 \pm 414 \mu\text{m}^3$ and KV-post cells
572 decreased volume from $1800 \pm 595 \mu\text{m}^3$ to $1491 \pm 310 \mu\text{m}^3$ that were similar to controls (KV-ant
573 cells increased volume from $2108 \pm 719 \mu\text{m}^3$ to $2709 \pm 774 \mu\text{m}^3$ and KV-post cells decreased
574 volume from $2275 \pm 883 \mu\text{m}^3$ to $1422 \pm 703 \mu\text{m}^3$)(**Figure 6A,B**). 3D analysis of cell shapes—
575 assessed using LWRs—indicated that even though the lumen failed to expand (**Figure 6D**;
576 **Figure 6-figure supplement 1A**), KV-ant and KV-post cells underwent normal asymmetric cell
577 shape changes in Jupa depleted embryos just as observed in control embryos between 2 ss to
578 8 ss (**Figure 6A,B**). These results, which are consistent with predictions of the vertex models,
579 provide *in vivo* evidence that cell shape changes can occur normally during KV remodeling in
580 the absence of KV lumen expansion.

581

582 To corroborate results obtained using Jupa depleted embryos, we took a second
583 approach to inhibit lumen expansion by interfering with cell-cell adhesion. We chose to use a
584 previously characterized MO that inhibits expression of the zebrafish Lgl2 (Lethal giant larvae 2)
585 protein (Tay et al., 2013). Similar to Jupa depletion, loss of Lgl2 moderately reduces the
586 accumulation of E-cadherin at lateral KV membranes and blocks KV lumen expansion (Tay et
587 al., 2013). Analyses of mosaic-labeled KV cells in Lgl2 depleted embryos yielded results that
588 were very similar to Jupa depleted embryos. Lgl2 depletion inhibited KV lumen expansion in
589 mosaic labeled embryos (**Figure 6D, Figure 6-figure supplement 1A**), but KV cells completed
590 normal asymmetric volume changes between 2 ss to 8 ss (KV-ant cells increased volume from
591 $2057 \pm 303 \mu\text{m}^3$ to $2329 \pm 847 \mu\text{m}^3$ and KV-post cells decreased volume from $2187 \pm 457 \mu\text{m}^3$
592 to $1617 \pm 336 \mu\text{m}^3$) and normal asymmetric cell shape changes during KV remodeling (**Figure**
593 **6C**). These results are consistent with Jupa knockdown results and indicate that asymmetric

epithelial cell shape changes that sculpt the KV organ are separable from the process of lumen expansion.

596

597 **DISCUSSION**

598 The collective behavior of epithelial cells plays a key role in determining the architecture
599 of tissues and organs. Studies of developmental processes in animal models have provided
600 important insights into the biochemical signals and mechanical forces that regulate epithelial
601 morphogenesis (Quintin et al., 2008, Schock and Perrimon, 2002). The zebrafish Kupffer's
602 vesicle (KV) is a simple organ that provides a useful model system to investigate mechanisms
603 that regulate epithelial cell shape changes in vivo. Using a mosaic labeling approach and 3D
604 morphometric analyses of single KV cells, we identified dynamic epithelial cell volume changes
605 during morphogenesis that are asymmetric along the anteroposterior body axis: KV-ant cells
606 become larger during development, whereas KV-post cells become smaller. Results from
607 mathematical simulations (summarized in **Figure 4G-H**) indicate that mechanical properties of
608 external cells surrounding the KV can impact cell shape changes in the KV, and that when
609 external cells are solid-like, asymmetric cell volume changes in KV cells contribute to cell shape
610 changes even in the absence of lumen expansion. Results from experimental perturbations
611 (summarized in **Figure 7**) indicated that interfering with ion flux prevents AP asymmetric KV cell
612 volume changes and shape changes during KV remodeling, and that these asymmetric
613 morphology changes can occur even when expansion of the fluid-filled KV lumen fails due to
614 weakened KV cell-cell junction integrity. Together, these studies identify asymmetric cell volume
615 regulation as an intrinsic mechanism that guides cell shape changes during epithelial
616 morphogenesis in KV. We propose this is a genetically programmed process that depends on
617 the properties of surrounding cells, but can be separated from the biophysical forces of
618 lumenogenesis.

619

620 **Asymmetric changes in cell size during KV epithelial morphogenesis**

621 The finding that KV cells change volume during development is insightful for thinking
622 about mechanisms of epithelial morphogenesis in KV since previous analyses (Compagnon et
623 al., 2014, Wang et al., 2012) that were limited to 2D did not predict differences in KV cell size.
624 Our previous analysis of 2D cell cross-sectional area (Wang et al., 2012) suggested cells
625 slightly reduce their size during morphogenesis, but did not detect differences between KV-ant
626 and KV-post cells. It is therefore striking that 3D analysis shows that KV cells do indeed change

627 volume, and do so asymmetrically along the AP axis. We recently reported that the size of the
 628 KV organ is not under tight control during development, but rather must only exceed a size
 629 threshold to function normally during left-right patterning (Gokey et al., 2016). Thus, KV size can
 630 vary among wild-type embryos. Consistent with these findings, we observed variable KV cell
 631 sizes. However, it is clear that wild-type KV cells always change size in an asymmetric way
 632 along the AP axis. Anterior KV cells always increase their size, whereas posterior cells always
 633 decrease their size. Interfering with the asymmetry of these size changes by blocking ion flux
 634 prevents asymmetric cell shape changes that we know from previous studies (Compagnon et
 635 al., 2014, Wang et al., 2012) are critical for KV function. These results indicate the AP
 636 asymmetry of volume changes is important for KV morphogenesis and function.

637

638 The decrease in KV-post size is mediated by ion channel activity that regulates fluid
 639 movement. Decrease in cell volume has also been observed during morphogenesis of zebrafish
 640 otic vesicle (Hojman et al., 2015), where it was suggested that movement of fluids from
 641 epithelial cells into the lumen contributes to lumen expansion. It is generally thought that ion flux
 642 in epithelial cells sets up a transepithelial flow of fluids from outside the tissue into the lumen
 643 (Gin et al., 2007, Frizzell and Hanrahan, 2012). When ion flux was blocked in KV via Na^+/K^+ -
 644 ATPase or Cfr inhibitors, KV-post cells did not shrink (but swelled) and the lumen failed to
 645 expand. This finding is consistent with a model in which intraepithelial fluid movement directly
 646 from KV-post cells into the lumen promotes lumen expansion. Since the amount of volume lost
 647 by KV-post cells does not fully account for the increase in lumen size, we propose ion flux in KV
 648 establishes both transepithelial flows and intraepithelial flows from KV-post cells to fill the
 649 lumen. The AP asymmetry of cell volume changes during KV morphogenesis suggests different
 650 mechanisms regulate the size of KV-ant vs. KV-post cells. The increase in KV-ant cell size could
 651 involve cell growth. Previous studies have uncovered a role for TOR signaling in cell growth
 652 (hypertrophy) in non-dividing cells (Guertin and Sabatini, 2006). Interestingly, TOR signaling has
 653 been implicated in the morphogenesis of KV (Casar Tena et al., 2015, DiBella et al., 2009, Yuan
 654 et al., 2012), but localized TOR activity has not been investigated. It will be interesting in future
 655 work to test for asymmetric expression/function of TOR pathway components in KV cells.

656

657 What makes KV-ant cells behave different from KV-post cells? This asymmetry likely
 658 results from a combination of intrinsic and extrinsic factors that differentially regulate KV-ant and
 659 KV-post cells. One possible intrinsic mechanism is that different KV cells develop different

660 mechanical properties. Our previous mathematical models suggest that differential cell-cell
 661 interfacial tensions along the AP axis can generate AP asymmetric cell shape changes in KV
 662 (Wang et al., 2012). Interestingly, a recent study in *Drosophila* showed that contractile force
 663 induced cell shape changes are instituted via cell volume reduction (Saia et al., 2015), which
 664 indicates a link between cell volume regulation and mechanical force generation. In the KV
 665 system, it will be interesting to test in future work whether AP asymmetric volume changes result
 666 in differential cytoskeletal contractility between KV-ant and KV-post cells. Another possible
 667 contributing factor to asymmetric KV cell size is differential activation of ion channels in KV-ant
 668 and KV-post cells. For example, it is known that the Cfr localizes to the apical surface of all KV
 669 cells at all stages of KV development (Navis and Bagnat, 2015). A recent study has uncovered
 670 mechanosensitive activation of Cfr in response to membrane stretch (Zhang et al., 2010).
 671 Stretching the plasma membrane increased ion conductance and also the probability of open
 672 Cfr channels at cell membranes. During KV remodeling, apical membrane stretch in KV-post
 673 cells may lead to increased Cfr activity and higher ion-efflux with a loss of volume in these cells
 674 (**Figure 7**). An alternative possibility is that different KV cell fates (e.g. KV-ant and KV-post
 675 cells) may be determined early in development. By tracking the DFCs that give rise to KV, we
 676 have found that these cells maintain their relative spatial positions throughout KV development
 677 (Dasgupta and Amack, 2016). This suggests subpopulations of KV cells may differentiate early
 678 in development and become biochemically distinct during KV morphogenesis. Additional studies
 679 are warranted to test the hypothesis of distinct KV-ant and KV-post subpopulations of cells that
 680 have differential gene expression and/or ion channel activity.

681

682 **The impact of mechanical forces on KV epithelial morphogenesis**

683 Our vertex model simulations suggest that asymmetric volume changes are not alone
 684 sufficient to fully induce KV cell shape changes. In addition to cell-intrinsic mechanisms,
 685 biophysical forces likely guide the formation of the KV epithelium. These mechanical forces can
 686 also arise from extrinsic sources that stem from the mechanical properties of surrounding
 687 tissues or extracellular matrix (ECM) (Campas et al., 2014, Chanet and Martin, 2014, Serwane
 688 et al., 2016, Etournay et al., 2015). Localized ECM deposition around anterior region of KV has
 689 been found to be important for asymmetric cell shape changes during KV morphogenesis
 690 (Compagnon et al., 2014). Interestingly, our simulations indicated that asymmetric cell shape
 691 changes are more pronounced when anterior external cells had solid-like properties and
 692 posterior external cells were fluid-like. An AP gradient of ECM may contribute to differential

693 mechanical properties surrounding KV. Other mechanisms, which remain unexplored, are
 694 mechanical forces generated by cells surrounding the KV as it advances towards the tailbud via
 695 convergent extension movements. Tissue fluidity in the tailbud plays an important role in
 696 controlling body elongation in zebrafish (Lawton et al., 2013) and may have an impact on KV.
 697 Our mathematical modeling suggests that solid-like behavior of surrounding tissue may play an
 698 important role in KV remodeling. In a previous study, we used DFC/KV specific knockdown of
 699 the Rho kinase Rock2b to test whether actomyosin contractility in KV cells vs. surrounding cells
 700 is involved in KV cell shape changes (Wang et al., 2012). KV cell shape changes failed to occur
 701 in embryos with Rock2b knocked down in KV cells, even though surrounding cells were normal,
 702 indicating that cell-autonomous actomyosin activity is important for KV cell shape changes.
 703 However, future studies are needed to explore how the mechanical properties of neighboring
 704 cells impact the establishment of asymmetric KV-ant and KV-post cell behaviors.

705

706 Lumen expansion occurs synchronously with changes in epithelial cell shapes during KV
 707 morphogenesis (Compagnon et al., 2014, Wang et al., 2012), which raises the possibility that
 708 contractile forces and/or intraluminal pressure contributes to KV cell shape changes. This idea
 709 is supported by the observation that blocking lumen expansion with ion channel inhibitors
 710 prevents KV shape changes (Compagnon et al., 2014). However, blocking ion channel activity
 711 also disrupts the previously unrecognized KV cell volume changes, making it unclear whether
 712 the lack of cell shape changes are due to reduced luminal forces or absence of cell volume
 713 changes. Our mathematical models suggest that KV cells can undergo asymmetric cell shape
 714 changes even in the absence of forces associated with lumenogenesis. This prediction was
 715 experimentally tested by perturbing KV cell-cell adhesions, which allowed us to block lumen
 716 expansion without altering ion channel activity or cell volume changes. 3D morphometric
 717 analyses of cell behaviors revealed that KV cells undergo normal cell shape changes in the
 718 absence of lumen expansion. These results provide new mechanistic insight into KV epithelial
 719 morphogenesis and suggest a working model in which asymmetric KV cell shape changes
 720 depend on intrinsic ion flux-mediated fluid movements and do not depend on extrinsic forces
 721 generated by lumen expansion.

722

723 We propose that luminal forces have a nominal impact on KV cell shape changes.
 724 Previous experimental results support this idea. First, KV cells can fail to change shape even
 725 when lumen expansion is normal. Inhibiting Rock2b function or non-muscle myosin II activity

726 had no effect on KV lumen expansion, but prevented cell shape changes during KV remodeling
 727 (Wang et al., 2011, Wang et al., 2012). This indicates that the mechanical forces generated
 728 during lumenogenesis are not sufficient to drive KV cell shape changes without active
 729 cytoskeletal contractility. Second, the degree of KV lumen expansion is highly variable in a
 730 population of wild-type embryos. Correlations between KV lumen size and KV function show
 731 that the lumen only needs to exceed a relatively low size threshold for the KV to be functional
 732 (Gokey et al., 2016). Thus, full expansion of the lumen is not required for effective KV activity.
 733 Together, these findings indicate that forces exerted by expansion of the lumen play a minor
 734 role in cell shape changes during KV epithelial morphogenesis.

735

736 **Programmed cell volume changes in epithelial morphogenesis**

737 Cell size is regulated by ion flux, but can also depend on progression through the cell
 738 cycle. Thus it is important to note that KV cells are post-mitotic epithelial cells that assemble a
 739 cilium (Amack et al., 2007). As discussed above, we consider the robust AP asymmetric
 740 changes in KV cell size that occurs with precise developmental timing (between 4 ss and 6 ss)
 741 as genetically ‘programmed cell volume changes’ that regulate KV organ architecture. In the
 742 zebrafish KV epithelium, we propose that programmed cell volume changes work in concert with
 743 other mechanisms to drive KV remodeling. Other recent studies have also identified links
 744 between cell volume changes and epithelial morphogenesis (Kolahi et al., 2009, Saias et al.,
 745 2015, Hoijman et al., 2015). However, little is known about the influence of cell volume changes
 746 on cell shape regulation. As mentioned previously, during zebrafish otic vesicle development
 747 epithelial cells become thinner, suggesting intraepithelial fluid movement contributes to both
 748 lumen growth and cell/tissue shape change (Hoijman et al., 2015). This finding is consistent with
 749 studies in cell culture systems (Braunstein et al., 2004, Vazquez et al., 2001) that have shown
 750 that epithelial cells indeed undergo cellular fluid loss to regulate cell volume and cell shape. In
 751 the mouse embryo, a group of nonproliferative epithelial cells in the tooth primordium also
 752 decrease their volume and become thinner during tooth budding morphogenesis (Ahtiainen et
 753 al., 2016). Another recent study in *Drosophila* uncovered that during dorsal closure cells within
 754 the amnioserosa lose their volume by ~30% and change their shape (Saias et al., 2015).
 755 Additionally, in the egg chamber of *Drosophila* the follicle cell epithelium undergoes volume
 756 changes during oocyte development to attain distinct cell shapes (Kolahi et al., 2009). Taken
 757 together with our experimental results and mathematical models in KV, these examples suggest

758 that programmed cell volume change might be a common mechanism that regulates cell shape
759 during epithelial morphogenesis in several tissues and organs.

760

761 MATERIALS AND METHODS

762

763 Zebrafish husbandry and strains

764 Zebrafish strains were maintained using standard procedures. Wild-type TAB zebrafish were
765 obtained from the Zebrafish International Resource Center. In addition, the following transgenic
766 zebrafish lines were used: *Tg(ubi:Zebrawow)* (Pan et al., 2013), *Tg(sox17:GFP-CAAX)^{sny101}* (this
767 study), *Tg(actb2:myl12.1-MKATE2)^{sny102}* (this study), *Tg(sox17:Cre^{ERT2})^{sny120}* (this study).
768 Embryos were staged as described (Kimmel et al., 1995).

769

770 Generation of transgenic lines

771 Transgene constructs were generated using the Gateway-based Tol2 kit (Kwan et al., 2007). To
772 generate *Tg(sox17:Cre^{ERT2})* and *Tg(sox17:GFP-CAAX)* transgenics, gateway cloning was
773 performed by combining p5E-sox17 (a generous gift from Stephanie Woo) (Woo et al., 2012),
774 pME-Cre-ERT2 or pME-GFP-CAAX (Tol2 Kit v2), p3E-SV40-polyA (Tol2 Kit v2), and pDest-
775 Tol2CG4 (Tol2 Kit v2) plasmids and LR Clonase II Plus (Invitrogen). Verified constructs (25
776 ng/μl plasmid DNA) were injected separately with Tol2 Transposase mRNA (~25 ng/μl) into one
777 cell stage TAB zebrafish embryos to generate *Tg(sox17:Cre^{ERT2})^{sny120}* or *Tg(sox17:GFP-
778 CAAX)^{sny101}* F₀ founders. Adult F₀ animals were then crossed with wild-type fish to generate F₁
779 heterozygotes. *Tg(sox17:Cre^{ERT2})^{sny120}* fish were then crossed with homozygous
780 *Tg(ubi:Zebrawow)* (Pan et al., 2013) animals to generate a double *Tg(sox17:Cre^{ERT2});
781 Tg(ubi:Zebrawow)* transgenic strain. To generate a *Tg(actb2:myl12.1-MKATE2)* transgenic fish,
782 p5E-actb2 (Tol2 Kit v2), pME-myl12.1-MKATE2 (see supplementary materials and methods),
783 and p3E-SV40-polyA (Tol2 Kit v2) plasmids were recombined into pDestTol2CG4 destination
784 vector as described above. Wild-type TAB embryos were injected with verified constructs and
785 Tol2 Transposase mRNA to generate *Tg(actb2:myl12.1-MKATE2)^{sny102}* F₀ fish. Adult
786 *Tg(actb2:myl12.1-MKATE2)^{sny102}* F₀ fish were crossed with wild-type TAB to generate F₁
787 heterozygotes. *Tg(actb2:myl12.1-MKATE2)* fish were crossed with homozygous *Tg(sox17:GFP-
788 CAAX)* animals to generate a double *Tg(actb2:myl12.1-MKATE2); Tg(sox17:GFP-CAAX)*
789 transgenic strain.

790

791 Whole-mount in situ RNA hybridization

792 A plasmid encoding full-length *jupa* was kindly provided by Maura Grealy's lab (NUI, Galway)
 793 (Martin et al., 2009). It was subcloned into a pCS2⁺ vector and PCR amplified using following
 794 primers: *jupa*L- 5'-GGCTGGCCCTGTGTCCAGCC-3' and *jupa*R- 5'-
 795 GTAGCCATCAAGCTCTTCAT-3'. The amplicon was TA cloned into pCRII TOPO vector and
 796 used to generate sense and antisense mRNA probes (DIG RNA labeling kit, Sigma) to detect
 797 *jupa* expression by in situ hybridization. RNA in situ hybridizations were performed as described
 798 (Wang et al., 2011).

799

800 Embryo injections

801 Morpholino oligonucleotides (MOs) were obtained from Gene Tools, LLC. We designed *jupa*
 802 MO-1 (5'-TTATGATTGTGTCTTCTCACCTGCA-3') to interfere with *jupa* pre-mRNA splicing of
 803 exons 2 and 3. *jupa* MO-2 (5'-GAGCCTCTCCCATGTGCATTTCCAT-3') designed to block *jupa*
 804 mRNA translation was previously described (Martin et al., 2009). Other previously characterized
 805 MOs used in this study were *cfr* MO (5'-CACAGGTGATCTCTGCATCCTAAA-3') (Gokey et al.,
 806 2016), *lgl2* MO-1 (5'-GCCCATGACGCCTGAACCTCTTCAT-3') (Tay et al., 2013) and a
 807 standard negative control MO (5'-CCTCTTACCTCAGTTACAATTTATA-3') (Gene Tools). MOs
 808 were injected into wild-type TAB embryos between the 1- and 2-cell stages. Dose curves were
 809 performed to determine optimal MO treatments: 2.5 ng of *jupa* MO-1, 2.5 ng of *jupa* MO-2, 1 ng
 810 of *cfr* MO (Gokey et al., 2016), 4.4 ng of *lgl2* MO-1 (Tay et al., 2013) and 2.5 ng of control MO.
 811 All MOs were co-injected with 4 ng p53 MO (5'-GCGCCATTGCTTTGCAAGAATTG-3') to
 812 diminish off-target effects (Tay et al., 2013). To conduct rescue experiments, pCS2⁺ vector
 813 containing full-length *jupa* was digested with NotI restriction enzyme and the linearized plasmid
 814 was used as a template to synthesize capped *jupa* mRNA using SP6 mMessage mMachine kit
 815 (Ambion). For *Jup* rescue experiments, *jupa* MO-1 was co-injected with 75 pg *jupa* mRNA. A
 816 construct that encodes a dominant negative JUP-naxos protein (Asimaki et al., 2014) was a kind
 817 gift from the Saffitz Lab. To over-express the JUP-naxos protein, 120 pg of *JUP-naxos* mRNA
 818 was injected into 1-cell stage TAB embryos.

819

820

821 Fluorescent dextran injections into KV

822 Control MO or *jupa* MO-1 treated *Tg(sox17:GFP-CAAX)* embryos were dechorionated and
 823 mounted in 1% low melting agarose at 6 ss. KV lumens were microinjected with ~1nL of 10 kDa

824 dextran, Alexa Fluor-568 (Molecular Probes; Lot: 1120095) at 6 ss and imaged using a Zeiss
825 Imager M1 microscope immediately (at 6 ss). Successfully injected embryos were then
826 incubated at 28.5 degrees for one hour and then imaged again at 8 ss.

827

828 **Immunofluorescence and microscopy**

829 For whole mount immunofluorescent staining experiments, embryos were fixed in 4%
830 paraformaldehyde in 1X PBS with 0.5% Triton X-100 at 4°C overnight and then dechorionated in
831 1X PBS. Embryos were permeabilized in blocking solution containing 1X PBS, 0.1% Triton X-
832 100, 0.1%DMSO, and 5% goat serum for 4 hours. Primary antibodies were diluted in fresh
833 blocking solution and incubated with embryos at 4°C overnight. Primary antibodies used: mouse
834 anti-junction plakoglobin (1:200, BD Transduction Laboratories), mouse anti-ZO-1 (1:200,
835 Invitrogen), mouse anti-E-cadherin (1:200, BD Transduction Laboratories), mouse anti-
836 acetylated tubulin (1:200, Sigma) and rabbit anti-GFP (1:200, Molecular Probes). Embryos
837 were then washed in 1X PBS with 0.1% Triton X-100, 0.1% DMSO, and 1% BSA at room
838 temperature. AlexaFluor 488- and 568- conjugated anti-rabbit and anti-mouse secondary
839 antibodies (Invitrogen, Molecular Probes) were used at 1:200 dilutions in blocking solution
840 overnight. Stained embryos were then washed in 1X PBS with 0.1% Triton X-100, 0.1% DMSO,
841 1% BSA at room temperature. Embryos were imaged using either Zeiss Imager M1 microscope
842 or a Perkin-Elmer UltraVIEW Vox spinning disk confocal microscope. Quantification based on
843 fluorescent immunostaining was performed using ImageJ software. KV lumen areas were
844 measured using maximum projections of ZO-1 staining. E-cadherin levels at KV cell junctions
845 were measured by determining the mean gray level (per pixel) along KV cell lateral membranes
846 as described (Tay et al., 2013). This mean gray level (fluorescence intensity) was normalized to
847 GFP intensity along lateral membranes of KV cells.

848

849 **Immunoblotting**

850 Protein extracts from approximately 30 zebrafish embryos at 8 ss were prepared as described
851 (Martin et al., 2009). 30 µL of 2X SDS sample buffer was added and samples were boiled for 5
852 min. Extract from approximately 10 embryos was loaded into each lane of commercially
853 prepared 12% gels (Bio-Rad laboratories 456-1044) and ran at 100 V for 2 hrs. Semi-dry
854 transfers were performed at 15 V for 45 min. onto a nitrocellulose membrane (Millipore
855 HATF00010). Membranes were blocked in blocking solution (3% BSA, 100mM NaCl, 20mM Tris
856 with pH 7.6, 0.2% Tween-20 in distilled water) over night at 4°C. Membranes were cut and anti-

857 Jup (BD Transduction Laboratories) and anti-alpha tubulin antibodies (Sigma T-6199) were
 858 used at 1:1000 dilutions in primary antibody block (0.3% BSA and tris-buffer saline with Tween-
 859 20 or TBST) and incubated at 4°C over night. Membranes were washed 4×15 min in TBST.
 860 Anti-mouse (Bio-Rad laboratories 166-2408) secondary antibodies were used at a 1:10,000
 861 dilution in TBST for 2 hrs at room temperature. After 4 washes for 15 min in TBST (10mM Tris
 862 with pH 8, 150mM NaCl, 0.05% Tween-20 in distilled water) membranes were incubated in ECL
 863 (Bio-Rad laboratories 170-5060) for 1 min and imaged on a ChemiDoc MP (Bio-Rad
 864 laboratories) imager. Band intensities were quantified using ImageJ software.

865

866 **Pharmacological treatments**

867 To induce low levels of Cre recombinase activity in *Tg(sox17:Cre^{ERT2}; Tg(ubi:Zebrawow)* double
 868 transgenic embryos, these embryos were treated with a working concentration of 5μM 4-
 869 hydroxy tamoxifen (Sigma) in 0.1% DMSO from the dome stage to the shield stage. To inhibit
 870 ion channels, embryos were either treated with a working concentration of 1mM ouabain
 871 (Sigma) dissolved in water or 30μM CFTRinh-172 (Tocris, Catalog No. 3430) in 0.1% DMSO
 872 from the bud stage to 2 ss or 8 ss. To activate Cftr channels, control MO and *jupa* MO injected
 873 embryos were treated with a working concentration of 10 μM CFTRact-09 (Chem Bridge) from
 874 the bud stage to 8 ss. 0.1% DMSO was used as a vehicle control for all experiments. After
 875 pharmacological treatments, embryos were thoroughly washed with embryo medium, mounted
 876 in 1% low melting agarose and imaged using either a Perkin-Elmer UltraVIEW Vox spinning disk
 877 confocal microscope or a Zeiss Imager M1 microscope.

878

879 **Live imaging and morphometric analysis of KV cells**

880 To image live KV cells, embryos were dechorionated and mounted in 1% low-melting point
 881 agarose on a glass-bottom MetTek dish at specific stages. Time-lapse imaging of KV was
 882 performed using 2 μm step-scan captured at 5 min. intervals for 105 min. using a Perkin-Elmer
 883 UltraVIEW Vox spinning disk confocal microscope. The acquired 3D datasets were processed
 884 and volume rendered using surface evolver tool in Imaris (Bitplane). Imaris was used to
 885 measure the length, width and height of reconstructed KV cells. The surface of the lumen was
 886 used to establish the axes of KV cells such that lateral axis (cell width) is parallel to the tangent
 887 of the curved lumen surface. To measure KV-lumen, KV-ant and KV-post cell cross-sectional
 888 areas, captured 3D images were oriented and maximum cross-sectional area from the middle

889 plane perpendicular to the DV axis of individual cells were measured using clipping plane
890 function in Imaris (Bitplane).

891

892 **KV cell volume measurements**

893 Single mosaic labeled cells (YFP⁺) in the KV were 3D reconstructed using ‘Create Surface’ tool
894 in Imaris (BITPlane) software. From 3D reconstructed cells the ‘cell volume’ was measured. To
895 measure total KV cellular volume, double transgenic *Tg(actb2:myl12.1mKATE2);*
896 *Tg(sox17:GFP-CAAX)* embryos were used to 3D reconstruct the KV lumen and the total KV
897 cellular component. The 3D lumen was split into equal anterior and posterior halves and the
898 cellular component associated with the two halves of the lumen were defined as the ‘total KV-
899 ant cellular volume’ and ‘total KV-post cellular volume.’

900

901 **Analysis of cells external to the KV**

902 55 pg of mRNA encoding a membrane-targeted mCherry (*mCherry-CAAX*) was injected into
903 *Tg(sox17:GFP-CAAX)* embryos at the 1-cell stage. Confocal images were captured from live
904 embryos at 2 ss and 8 ss. The cell shape index, $q = [(cell\ cross\ sectional\ perimeter)/\sqrt{(cell\ cross\ sectional\ area)}]$ (Bi et al., 2016) was used to define morphology of cells surrounding KV at
905 the middle plane of the KV organ in control and ouabain treated embryos at 2 ss and 8 ss. On
906 average, 5 cells were measured from the anterior and posterior regions per embryo.

908

909 **Statistics**

910 The significance of pairwise differences between groups of biological data was computed by
911 Student’s two-tailed t-tests with two-tailed distribution and two-sample unequal variance.

912

913 **SUPPLEMENTARY MATERIALS AND METHODS**

914

915 **Vertex Model simulation of KV**

916 We simulate KV morphogenesis using the Vertex Model with periodic boundary conditions (Bi et
917 al., 2015, Farhadifar et al., 2007, Fletcher et al., 2014, Hufnagel et al., 2007). Because fully 3D
918 models introduce a larger number of variables and parameters, we choose a two-dimensional
919 description where each cell i is represented as a polygon with area A_i and perimeter P_i . We
920 focus our description on the plane through the center of the KV perpendicular to the dorso-

921 ventral axis, and represent lumen, KV-ant cells, KV-post cells, and cells external to the KV as
 922 different cell types, which may differ in their mechanical properties. We choose to have an
 923 equal number N of KV-ant and KV-post cells, respectively, and 100 external cells. Force-
 924 balanced states are defined by minima of the following effective energy functional

$$925 \quad E = \frac{1}{2} \sum_i [K_A (A_i - A_0)^2 + K_P P_i^2] + \sum_{\langle ij \rangle, i < j} \Lambda_{ij} l_{ij}. \quad (1)$$

926 Here, the first sum is over all cells. The first term in it describes a cell area elasticity, where K_A
 927 is the associated spring constant and A_0 is the preferred area. The second term in the first sum
 928 describes cell perimeter elasticity, where K_P is the associated spring constant. The second sum
 929 in Eq. (1) is over all interfaces $\langle ij \rangle$ between adjacent cells i and j . It accounts for the interfacial
 930 tensions between cells, where Λ_{ij} denotes the interfacial tension between cells i and j and l_{ij}
 931 denotes the interface length. Note that in order to facilitate comparison with experimental data,
 932 we choose micrometers as length units for our vertex model simulations.

933 For our simulations, we choose the values of A_0 displayed in **Figure 4 – supplement**
 934 **table 2**. The listed values for lumen and KV cells are experimentally measured average cross-
 935 sectional areas (see separate supplement section). We assumed the external cells to be about
 936 as big as the KV cells, so we set the value of the external cells at 2 ss to the average of KV-ant
 937 and KV-post cells. The total preferred area is computed as the total sum of the preferred areas
 938 A_0 of all cells at 2 ss. The preferred area of the external cells at 8 ss is chosen such that the
 939 total preferred area stays constant between 2 ss and 8 ss, which corresponds to only a small
 940 preferred area change of these cells (**Figure 4 – supplement table 2**). We set $K_A = 1000$ and
 941 $K_P = 1$ for all cell types. We have chosen a very high ratio $(K_A L^2)/K_P$ with L being a typical cell
 942 diameter in order to ensure that the measured cross-sectional area values in **Figure 4 –**
 943 **supplement table 2** are largely fulfilled by the cells.

944 The values for the line tensions depend on both involved cell types. We have set the
 945 line tension between any two KV cells i and j to the same value $\Lambda_{ij} = \Lambda^{\text{KV-KV}}$, independent of
 946 whether the KV cells are anterior or posterior cells. Similarly, the line tension between two
 947 external cells i and j is set to $\Lambda_{ij} = \Lambda^{\text{ext-ext}}$. Since we have no measured values for these
 948 interfacial tensions, we vary both interfacial tension parameters, $\Lambda^{\text{KV-KV}}$ and $\Lambda^{\text{ext-ext}}$, in **Figure**
 949 **4, Figure 4-figure supplement 2,3**. The interfacial tension between a KV cell i and an external
 950 cell j is set to the average of both homotypic interfacial tensions with an additional offset:

951 $\Lambda_{ij} = \Lambda^{\text{KV-ext}} = (\Lambda^{\text{KV-KV}} + \Lambda^{\text{ext-ext}})/2 + 200$. The tension offset serves to prevent KV cells from
 952 being extruded from the KV epithelium and to allow for a smoother basal interface between KV
 953 and external cells. Between KV cells and lumen, the interfacial tension is set to a positive value
 954 of $\Lambda_{ij}^{\text{lumen-KV}} = 100$ to ensure that the lumen surface is roughly spherical.

955 The system is initialized using the Voronoi tessellation of a pattern of cell positions. The
 956 $2N$ KV cell positions are arranged equidistantly on a circle around the central lumen “cell”
 957 position. The radius of this circle is computed as the estimated lumen radius plus half of the
 958 estimated KV cell height. The positions for the 100 external cells are drawn randomly from a
 959 uniform distribution with the condition of having at least a distance of lumen radius plus
 960 estimated KV cell height from the lumen cell position. Then, preferred cell areas are set to their
 961 2 ss values and the system is relaxed by minimizing the energy functional. Afterwards, the
 962 preferred areas are set to their respective 8 ss values and the system is relaxed again. Note
 963 that the Voronoi tessellation is only used to facilitate the initialization. The subsequent energy
 964 minimizations are carried out using varying vertex positions. Also note that the dimensions of
 965 the periodic box were also allowed to vary during the minimizations. We use the conjugated
 966 gradient algorithm from the GSL library (<https://www.gnu.org/software/gsl/>) for the energy
 967 minimization (Press, 2007).

968

969 Computation of the KV cell length-width ratio (LWR) in the simulations

970 We compute the LWR of a given KV cell as the quotient of its length L divided by its width W
 971 (**Figure 4-figure supplement 1**). We define the width W as the distance between the midpoints
 972 of the respective interfaces with the two adjacent KV cells. The length L is defined as the
 973 distance from the midpoint of the interface with the lumen to the midpoint between points P and
 974 Q , which are the respective endpoints of the interfaces with the adjacent KV cells. In **Figure 4E-**
 975 **F** we plot the respective average LWR. During the energy minimizations, KV cells occasionally
 976 lose contact with the lumen. For the averaging, we thus only take into account the KV cells that
 977 are in still contact with the lumen.

978

979

980

981 **Definition of separation of solid from fluid regimes**

982 Earlier work on the vertex model suggested that a shape index computed from cell perimeter
 983 and area can be used to differentiate between solid and fluid regime (Bi et al., 2015). However,
 984 these simulations only studied vertex model tissues with a single cell type randomly arranged,
 985 while in our simulations, there are several cell types and a very distinct geometrical
 986 arrangement. Thus, to differentiate solid cell from fluid ones, we choose a different
 987 measurement, which is based on the actual cell perimeter P_i and the interfacial tensions Λ .
 988 Based on the interfacial tensions, one can define another parameter, which characterizes a
 989 preferred perimeter $P_0 = -\Lambda/(2K_P)$. It has been observed that fluidity also correlates with the
 990 difference between actual and preferred perimeter $P_i - P_0$ (Bi et al., 2015). Solid vertex model
 991 tissues have $P_i - P_0 > 0$ while fluid vertex model tissue has $P_i - P_0 = 0$. Correspondingly, we
 992 use this criterion to differentiate between solid and fluid cells to define the positions of the
 993 dashed black lines in **Figure 4E,F** and in **Figure 4 – figure supplements 2E,F and , Figure 4 –**
 994 **figure supplements 3E,F**. Note that as a consequence, the positions of these lines slightly
 995 vary for different conditions.

996

997 **Case of solid anterior and fluid posterior KV cells**

998 In our vertex model we have discovered a second mechanism that can lead to a positive APA
 999 for small N , which is different from the mechanism illustrated in **Figure 4G**. This mechanism is
 1000 at work for instance in the hatched region in **Figure 4-figure supplement 2E**, where anterior
 1001 KV cells are solid-like and posterior KV cells are fluid-like. This difference arises even though
 1002 both anterior and posterior KV cells have the same interfacial tension Λ^{KV-KV} , because anterior
 1003 KV cells have a much higher preferred area at 8ss than posterior KV cells (**Figure 4-**
 1004 **supplement table 2**). Because the condition of fluidity in the vertex model depends on both
 1005 preferred area and interfacial tension ((Bi et al., 2015), there is an intermediate regime when
 1006 increasing Λ^{KV-KV} where anterior KV cells are already solid, but posterior KV cells are still fluid.
 1007 When the lumen area increases in this regime, both anterior and posterior cells together have to
 1008 accommodate a larger total apical interface with the lumen. However, because the anterior cells
 1009 are solid while the posterior cells are fluid, the latter are more easily stretched laterally. This
 1010 induces an asymmetry in cell shape that corresponds to a positive APA. Note that the effect of
 1011 this mechanism appears to extend further into the region where also the posterior KV cells are

solid, likely because close to the hatched region they are still more easily deformable than the anterior KV cells.

Simulations with asymmetric properties of the external cells

To simulate asymmetric properties of external cells (see **Figure 4 – figure supplement 4**), we proceeded as before with the following changes. We divide all external cells into an anterior and a posterior subset based on the randomly drawn initial Voronoi cell positions. If the initial Voronoi position of a cell is anterior (posterior) of the initial lumen Voronoi position, we regard it as one of the anterior (posterior) external cells. The interfacial tensions between two anterior (posterior) external cells are defined by the parameter $\Lambda^{\text{ext,A-ext,A}}$ ($\Lambda^{\text{ext,P-ext,P}}$). To set the anterior cells solid (fluid) and the posterior cells fluid (solid), we choose the parameter values $\Lambda^{\text{ext,A-ext,A}} = 150$ and $\Lambda^{\text{ext,P-ext,P}} = -120$ ($\Lambda^{\text{ext,A-ext,A}} = -120$ and $\Lambda^{\text{ext,P-ext,P}} = 150$). The interfacial tension between an anterior and a posterior external cell was set to the average: $\Lambda^{\text{ext,A-ext,P}} = (\Lambda^{\text{ext,A-ext,A}} + \Lambda^{\text{ext,P-ext,P}})/2$.

Percentage cell volume, cell cross-sectional area and cell height change quantifications

Percentage changes were measured using the following method: If we consider, cellular properties at 8 ss = y with standard deviation δy and cellular properties at 2 ss = x with standard deviation δx , then ‘% change (z)’ = $\{(y - x)/x\} \cdot 100$. The standard deviations can be used as the uncertainty in the measured values. Thus, the uncertainty δz in z can be represented as:

$$\delta z = \frac{100}{x} \left[\delta y^2 + \delta x^2 \left(\frac{y}{x} \right)^2 \right]^{1/2}$$

Generation of pME-myl12.1-MKATE2 construct

The myl12.1 ORF was PCR amplified from cDNA pool generated from 8 ss zebrafish embryos using following primers:

myl12.1F: 5'-ATTAATGGATCCATGTCGA GCAAACGCGC CAA-3'

myl12.1R: 5'-ATTAATGAATTCTGCATCGTCTTTGTCTTTGGCTC-3'

1039 The PCR amplified myl12.1 ORF was sub-cloned into pCS2⁺MKATE2 vector using BamH1 and
1040 EcoR1 restriction enzymes to construct pCS2⁺ myl12.1-MKATE2 plasmid.

1041 The myl12.1-MKATE2 construct was PCR amplified from pCS2⁺ myl12.1-MKATE2 plasmid
1042 using following primers:

1043 attB1: 5'-GGGGACAAGTTTGTACAAAAAGCAGGCTATGTCGAGCAAACGCGCCAA-3'

1044 attB2: 5'-GGGGACCACTTTGTACAAGAAAGCTGGGTTCATCTGTGCCCCAGTTT-3'
1045

1046 The PCR amplified myl12.1-MKATE2 construct was then cloned into pDONR221 vector using
1047 BP recombination to generate the middle entry pME-myl12.1-MKATE2 vector.
1048

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1059

1060 **COMPETING INTERESTS**

1061 The authors declare no competing financial and non-financial interests.

1062

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FIGURES AND FIGURE LEGENDS

Figure 1

Figure 1. Mosaic labeling of KV cells. (A) A dorsal view of the tailbud in a live zebrafish embryo at the 8-somite stage (8 ss) of development. Kupffer's vesicle (KV) is positioned at the end of the notochord. The inset shows GFP-labeled KV cells surrounding the fluid-filled KV lumen in a *Tg(sox17:GFP-CAAX)* transgenic embryo at 8 ss. This is the middle plane of the KV. Scale = 10μm. (B) Schematic of cell shape changes during KV remodeling. KV-ant cells (blue) and KV-post cells (red) have similar shapes at 2 ss, but then undergo regional cell shape changes such that KV-ant cells are elongated and KV-post cells are wide and thin by 8 ss.

1342 These cell shape changes result in asymmetric positioning of motile cilia that generate fluid
1343 flows for left-right patterning. **(C)** Structure of the *ubi:zebrabow* and *sox17:Cre^{ERT2}* transgenes
1344 and the possible recombination outcomes of the ‘zebrabow’ transgene by Cre recombinase
1345 activity in KV cell lineages. **(D)** Time course of mosaic labeling of KV cells. Brief treatment of
1346 double transgenic *Tg(sox17:Cre^{ERT2}); Tg(ubi:Zebrabow)* embryos with 4-OHT from the dome
1347 stage to the shield stage generates low levels of Cre activity that changes expression of default
1348 RFP to expression of YFP in a subset of KV cells. **(E)** Mosaic labeled YFP⁺ KV cells at the
1349 middle plane of KV at 8 ss. **(F)** 3D reconstructed KV cells (green) and KV lumen (pink) at 8 ss.
1350 Scale = 10µm. **(G-H)** Morphometric parameters of 3D rendered KV-ant **(G)** and KV-post **(H)**
1351 cells: length = axis spanning from apical to basal side of the cell, height = axis spanning from
1352 dorsal to ventral side of the cell, and width = axis connecting lateral sides of the cell. Scale =
1353 5µm.

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Figure 2. 3D morphometric analysis of single cells reveals asymmetric cell volume changes during asymmetric KV cell shape changes. (A) A lateral view of a mosaic labeled KV in a *Tg(sox17:Cre^{ERT2}); Tg(ubi:Zebrawow)* at 2 ss. The embryo diagram represents the orientation of the image. The notochord and KV are outlined in blue. Yellow lines mark the KV lumen. Examples of 3D reconstructed KV-ant and KV-post cells along the middle plane of KV are shown. Scale = 10µm. (B) Representative snapshots of 3D rendered KV-ant and KV-post cells at different stages of KV development between 2 ss and 8 ss. The parameters including height (H), length (L) and width (W) were used to quantify cell morphology. Yellow lines indicate the KV luminal surface. Scale = 10µm. (C-E) Quantification of height (C), length (D) and width (E) of individual KV-ant and KV-post cells during development. (F) A length-to-width ratio (LWR)

1408 was used to describe KV cell shapes. KV-ant and KV-post cells change shape between 4 ss
 1409 and 6 ss. **(G)** Volume measurements of individual KV cells at different stages of development.
 1410 Similar to cell shapes, KV-ant and KV-post cells change volume between 4 ss and 6 ss. All
 1411 measurements presented in C-G were made on the same group of reconstructed cells. The
 1412 number of KV-ant and KV-post cells analyzed is indicated in the graph in C. N = number of
 1413 embryos analyzed at each stage. Graphs show the mean + SD. Results were pooled from three
 1414 independent experiments. *p < 0.01 and ns = not significant (Student's T-Test).

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Figure 2-figure supplement 1. Single KV cells were sampled from all positions along the middle plane of KV for morphometric analysis. (A,B) Representative mosaic labeled KVs at pre (2 ss) and post-remodeling (8 ss) stages. YFP⁺ cells along the middle plane of the organ are numbered. KV lumen and cell boundaries are outlined. Yellow lines divide the KV lumen into anterior and posterior halves. **(A',B')** Pooled images of KV lumen boundaries and cell numbers show no bias in the distribution of mosaic labeled cells. Scale = 20μm.

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Figure 2-figure supplement 2

Figure 2-figure supplement 2. Total changes in KV volume during KV morphogenesis. (A)

A double transgenic *Tg(sox17:GFP-CAAX); Tg(actb2:myl12.1-MKATE2)* embryo, in which GFP expression marks KV cells and MKATE2 expression marks the KV lumen. (B) 3D reconstruction of the entire KV organ at 2 ss (B) and 8 ss (B'). The KV lumen was split into two equal halves (purple and orange) along anteroposterior axis. This line bisecting the KV was then used to define the anterior (blue) and posterior (red) regions of the cellular component of KV. (C) The graph represents total volumes of anterior and posterior KV cellular components at 2 ss and 8 ss. Shown is the mean + SD. Results are pooled from two independent experiments. N = number of embryos analyzed at each stage. *p < 0.01 and ns = not significant (Student's T-Test).

1571 function using the small molecule inhibitor CFTRinh-172 (C) or *cfr* MO (D) also blocked KV
 1572 lumenogenesis and disrupted asymmetric cell volume changes and shape changes of KV cells.
 1573 (E) Quantification of 3D reconstructed KV lumen volumes (insets depict lumen in YZ axis) in
 1574 control and treated live embryos at 8 ss. For all quantitative analyses, the mean + SD is shown.
 1575 The number of KV-ant and KV-post cells analyzed is indicated in the graphs in A-D. N = number
 1576 of embryos analyzed. Results were pooled from two independent trials. Scale = 20μm, *p < 0.01
 1577 and ns = not significant (Student's T-Test).

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Figure 3-figure supplement 1

Figure 3-figure supplement 1. KV cell heights in embryos treated with ion channel inhibitors. (A-B) Quantification of individual KV-ant and KV-post cell heights from different treatments at 2 ss (A) and 8 ss (B). Results were pooled from two independent experiments at each stage. The number of KV-ant and KV-post cells analyzed is indicated within the graph. N = number of embryos analyzed at each stage. Shown is the mean + SD, *p < 0.01 and ns = not significant (Student's T-Test).

Figure 3-figure supplement 2

Figure 3-figure supplement 2. Morphology of external cells surrounding KV in ouabain treated embryos. (A) Ouabain treatment did not cause gross developmental defects as compared to control embryos, except the KV lumen (arrow) was smaller at 8 ss. (B) Ubiquitous

1670 expression of membrane-targeted mCherry (*mCherry-CAAX*) in *Tg(sox17:GFP-CAAX)* embryo
 1671 allowed visualization both KV cells at the middle plane of KV and the surrounding external cells
 1672 in control and ouabain treated embryos. Surrounding cells with clear boundaries were selected
 1673 for cell shape analysis. Representative surrounding cells at 2 ss in the anterior (blue) or
 1674 posterior (red) region of KV are shown. Yellow line divides KV lumen into anterior and posterior
 1675 halves. Scale = 20 μ m. (C) Quantitative analysis of cell shape index (*q*) shows no significant
 1676 difference in surrounding cells between control and ouabain treated embryos at 2 ss and 8 ss.
 1677 Results were pooled from two independent experiments at each stage. The number of
 1678 surrounding anterior cells (blue) and surrounding posterior cells (red) analyzed is indicated in
 1679 the graph. N= number of embryos analyzed. Shown is the mean + SD. ns = not significant
 1680 (Student's T-Test).

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Figure 4

Figure 4. Vertex model simulations for cell shapes during KV remodeling.

(A-D) Vertex model simulations with N=10 KV-ant and KV-post cells. Upper and lower panels respectively show force-balanced states at 2 ss and 8 ss. All shown simulations start from the

1736 same initial cell positions, but the mechanical parameters differ. The full simulation box is
1737 cropped in order to focus on the KV. For the example of panel A, **Figure 4 – figure supplement**
1738 **1B** shows the respective full state. **(A)** Both KV and external cells are solid-like (interfacial
1739 tensions $\Lambda^{KV-KV} = -50$ and $\Lambda^{ext-ext} = 150$), and the lumen cross-sectional area expands
1740 according to experimental measurements between 2 and 8 ss. **(B)** KV cells are solid-like
1741 ($\Lambda^{KV-KV} = 50$), external cells are fluid-like ($\Lambda^{ext-ext} = -120$), and the lumen cross-sectional area
1742 expands. **(C)** Both KV and external cells are solid-like ($\Lambda^{KV-KV} = -50$ and $\Lambda^{ext-ext} = 150$) and
1743 the lumen cross-sectional area stays constant between 2 and 8 ss. **(D)** KV cells are solid-like
1744 ($\Lambda^{KV-KV} = 50$), external cells are fluid-like ($\Lambda^{ext-ext} = -120$), and the lumen cross-sectional area
1745 is constant. **(E,F)** Parameter scan for the anterior-posterior asymmetry, APA (LWR-ant – LWR-
1746 post), depending on the respective interfacial tensions of KV cells and external cells, which
1747 defines whether these cells are solid-like or fluid-like (FS = external cells fluid-like, KV cells
1748 solid-like; SS = both external and KV cells are solid-like; FF = both external and KV cells fluid-
1749 like; SF = external cells solid-like, KV cells fluid-like; hatched region = KV-ant cells solid-like and
1750 KV-post cells fluid-like). For each pair of interfacial tensions, the APA was computed from the
1751 average of 100 separate simulation runs. When KV cells are solid-like, the standard error of the
1752 mean APA is typically on the order of 0.05. However, for fluid-like KV cells standard error of the
1753 mean APA can become much larger, which is reflected by the large mean APA fluctuations in
1754 this regime. **(E)** The lumen cross-sectional area changes normally between 2 and 8 ss. **(F)** The
1755 lumen cross-sectional area is fixed at a constant value between 2 and 8 ss. The parameter
1756 values corresponding to panels A-D are marked in E,F. For both E and F, a positive APA is
1757 robustly obtained only when KV and external cells are both solid-like. **(G,H)** Illustrations of how
1758 mechanical properties of external cells affect APA values in our simulations. For solid external
1759 cells, the interface between KV-ant and KV-post cells is prevented from moving posteriorly upon
1760 decreasing KV-post cell cross-sectional areas between 2ss and 8ss. As a consequence, the
1761 posterior KV cells flatten and obtain a smaller LWR-post value, which results in a positive APA.
1762 Conversely for fluid external cells, a decrease in KV-post cell cross-sectional area is
1763 accommodated by a posterior sliding of the interface between KV-ant and KV-post cells.
1764 Consequently, the APA does not increase and may even decrease. These mechanisms work
1765 both for increasing lumen cross-sectional area (G) and for constant lumen cross-sectional area
1766 (H).

Figure 4-figure supplement 1

Figure 4-figure supplement 1. Definition of the length-width ratio (LWR) in the simulations, and example for full force-balanced state. (A) The width W is the distance between the midpoints of the respective interfaces with the two adjacent KV cells. The length L is the distance from the midpoint of the interface with the lumen to the midpoint between points P and Q . The LWR is defined by the quotient L/W . **(B)** Non-cropped force-balanced state for the example case shown in **Figure 4A**. We use periodic boundary conditions for all our simulations (for details see Supplemental Information).

Figure 4-figure supplement 2

Figure 4-figure supplement 2. Vertex model simulations for cell shapes during KV remodeling (N=8). Results as in Figure 4 for N=8 KV-ant and KV-post cells.

Figure 4-figure supplement 3

Figure 4-figure supplement 3. Vertex model simulations for cell shapes during KV remodeling (N=12). Results as in Figure 3 for 12 KV-ant and KV-post cells.

Figure 4-supplement table 1

Relative anteroposterior (AP) differences of:	at 2 ss	at 8 ss
Cell volume	$(-7 \pm 22)\%$	$(90 \pm 40)\%$
Cell cross-sectional area	$(11 \pm 17)\%$	$(80 \pm 30)\%$
Cell height	$(-1 \pm 20)\%$	$(18 \pm 14)\%$

Figure 4-supplement table 1. Percentage differences of cell volume, cell cross sectional area, and cell height between anterior and posterior cells [(Post – Ant)/Post] at 2 ss and 8 ss for DMSO control embryos. The cell volume is proportional to the product of cross-sectional area and height. As a consequence, if the AP difference in cell volume was fully due to the difference in the cross-sectional areas, then the percentage differences of both volumes and cross-sectional areas should be the same. Similarly, if the cell volume difference was fully due to the height difference, then the percentage differences of volumes and height should be the same. We find that at 2ss, the AP differences are not high in all three quantities. However, at 8ss the AP volume difference is very large and is mostly accounted for by the AP difference in cross-sectional areas, while the height difference stays comparably small.

Figure 4-supplemental table 2

Cell type/lumen	Preferred area A_0 at 2 ss (μm^2)	Preferred area A_0 at 8 ss (μm^2)
KV-ant cells	199	238
KV-post cells	179	134
External cells	189	170 (lumen area changes) 190 (no lumen area change)
Lumen	1657	3602 (lumen area changes) 1657 (no lumen area change)

Figure 4-supplement table 2. Preferred areas A_0 prescribed in our vertex model simulations for the different cell types at 2 ss and 8 ss. The listed values for lumen and KV cells are experimentally measured average cross-sectional areas from DMSO-treated control embryos. The 2 ss values for the external cells were set to the average of KV-ant and KV-post cells. The preferred area of the external cells at 8 ss is chosen such that the total preferred area stays constant between 2 ss and 8 ss. Note that in **Figure 4A,B,E**, the lumen area changes between 2 ss and 8 ss (same in **Figure 4 – figure supplements 2,3A,B,E**), while in **Figure 4C,D,F**, the lumen area at 8ss is set to the value measured at 2 ss (same in **Figure 4 – figure supplements 2,3C,D,F**). In order to facilitate comparison with experimental data, we choose micro-meters (μm) as length units for our vertex model simulations.

Figure 5. Interfering with Junction plakoglobin inhibits KV lumen expansion. (A) Immunostaining with Jup antibodies shows Jup enrichment at lateral membranes of KV cells marked by membrane-targeted GFP expression in *Tg(sox17:GFP-CAAX)* embryos. Embryos

1991 injected with *jupa* MO-1 showed reduced Jup protein levels. Boxes indicate enlarged regions
1992 shown as individual channels. Arrows point out representative lateral membranes. Scale =
1993 20µm. **(B)** Immunoblotting confirmed reduction in Jup protein level (arrowhead) in *jupa* MO-1
1994 injected embryos relative to wild-type (WT) and control MO injected embryos. The graph shows
1995 normalized Jupa band intensities. Shown is the mean + SD for three independent experiments.
1996 **(C)** At 8 ss, control embryos showed an inflated KV lumen (red arrow) that was labeled using
1997 ZO-1 antibody staining. Embryos injected with *jupa* MO-1 to knockdown Jup expression in all
1998 cells (global knockdown) or specifically in DFC/KV cells (DFC^{*jupa* MO-1}) appeared normal at 8 ss
1999 except that the KV lumen failed to expand. Interfering with Jup by injecting *JUP-naxos* mRNA
2000 also inhibited KV lumen expansion. Scale = 10µm. **(D)** Quantification of KV lumen area in
2001 control and treated embryos at 8 ss. Co-injecting *jupa* MO-1 with *jupa* mRNA significantly
2002 rescued lumenogenesis defects. Shown are mean + SD for three independent experiments. **(E)**
2003 The number of ciliated KV cells was not different among the treatment groups. Shown is the
2004 mean + SD for results pooled from three independent experiments. **(F)** Representative images
2005 of at 8 ss in control and *jupa* MO injected embryos treated with vehicle (DMSO) or CFTRact-09.
2006 The graph shows KV lumen area (outlined by yellow line) in control and treated embryos. Scale
2007 = 10µm. Shown is the mean + SD for two independent experiments. **(G)** Representative images
2008 of KV lumens of contro MO and *jupa* MO embryos injected with rhodamine-dextran. Scale =
2009 20µm. The graph shows percentage of embryos retaining and losing the fluorescent dye
2010 between 6 ss and 8 ss from two independent trials. N = number of embryos analyzed. *p < 0.01
2011 and ns = not significant (Student's T-Test).

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Figure 5-figure supplement 1. *jupa* is maternally supplied and enriched in precursor dorsal forerunner cells (DFCs) and KV. (A-E) RNA *in situ* hybridizations of *jupa* during early zebrafish development. (A) Antisense *jupa* probe revealed maternal *jupa* mRNA in 2-cell stage embryo. (B) A control *jupa* sense probe showed no staining. (C-D) *jupa* mRNA was detected in DFCs (black arrow) and KV cells (red square) during epiboly and early somite stages. (E) Enlarged region depicting *jupa* staining in KV (black arrow).

Figure 5-figure supplement 2

Figure 5-figure supplement 2. Interfering with Junction plakoglobin inhibits KV lumenogenesis but not ciliated cell number. (A) Immunostaining with Jup antibodies showed that embryos injected with *jupa* MO-2 had reduced Jup protein levels in KV relative to control embryos (image of control MO embryo reproduced from **Figure 5A** for comparison). Boxes indicate enlarged regions shown as individual channels. Lateral membranes of KV cells are marked by membrane-targeted GFP expression in *Tg(sox17:GFP-CAAX)* embryos. Arrows

2086 point out representative lateral membranes. Scale = 20μm. **(B)** Representative KV lumens
 2087 labeled using ZO-1 antibody staining at 8 ss from different treatments. Scale = 10μm. **(C)** KV
 2088 cilia labeled using acetylated-tubulin antibody staining at 8 ss from different treatments. Scale =
 2089 10μm.

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Figure 5-figure supplement 3

Figure 5-figure supplement 3. Jupa knockdown results in reduced E-cadherin levels at lateral membranes of KV cells. Immunostaining with E-cadherin (E-cad) antibodies showed E-cad enrichment at lateral membranes of KV cells marked by membrane-targeted GFP expression in *Tg(sox17:GFP-CAAX)* embryos. Embryos injected with *jupa* MO-1 showed a moderate reduction of E-cad protein enrichment at KV membranes. Arrows point to

2152 representative lateral membranes in individual channels. The graph represents normalized E-
2153 cad intensities from two experiments. N= number of embryos analyzed. Scale = 5 μ m. *p < 0.05
2154 (Student's T-Test).

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Figure 6

Figure 6. Asymmetric cell shape changes in KV are separable from lumen expansion. (A)

Mosaic labeled KV cells in control MO injected embryos showed asymmetric changes in cell volumes and cell shapes between at 2 ss and 8 ss. (B-C) Perturbing cell-cell junction integrity in KV by interfering with *jupa* (B) or *Igf2* (C) expression inhibited KV lumen expansion, but asymmetric cell volume changes occurred that were similar to controls. In addition, asymmetric KV cell shape changes occurred normally in *jupa* and *Igf2* MO embryos. (D) Quantification of 3D reconstructed KV lumen volumes (insets depict KV lumen in YZ axis) in control and treated live embryos at 8 ss. For quantitative analyses, the mean + SD is shown. The number of KV-ant and KV-post cells analyzed is indicated in the graphs in A-C. N = number of embryos analyzed. Data for control MO and *jupa* MO experiments are pooled from three independent experiments and *Igf2* MO data are pooled from two experiments. Scale = 20µm. *p < 0.01 and ns = not significant (Student's T-Test).

Figure 6-figure supplement 1

Figure 6-figure supplement 1. KV cell heights in embryos with KV cell-cell adhesion perturbations. (A-B) Quantification of individual KV-ant and KV-post cell heights from different treatments at 2 ss (A) and 8 ss (B). Data for control MO and *jupa* MO experiments are pooled from three independent experiments and *lgl2* MO data are pooled from two experiments. The number of KV-ant and KV-post cells analyzed is indicated within the graph. N = number of embryos analyzed at each stage. Shown is the mean + SD, *p < 0.01 and ns = not significant (Student's T-Test).

Figure 7

Figure 7. Summary and working model for epithelial cell shape changes during KV morphogenesis.

Results from experiments and modeling suggest AP asymmetric cell volume changes contribute to asymmetric cell shape changes in the KV epithelium. Inhibiting ion flux blocks asymmetric cell volume changes, lumen expansion and shape changes in KV-ant (blue) and KV-post (red) cells. Vertex simulations predict that asymmetric volume (cross-sectional area) changes in KV cells can introduce AP asymmetry in KV cell shapes without lumen expansion. Consistent with this

2283 prediction, asymmetric changes in KV volume and shape occurred in the absence of lumen
2284 expansion in embryos with weakened KV cell junction integrity. These results suggest a model
2285 in which asymmetric cell volume changes contribute to cell shape changes in KV and that this
2286 process is separable from lumen growth.

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2316 **Movie 1.** KV organ architecture in 3D in a live *Tg(sox17:GFP-CAAX)* embryo at 8 ss. The
 2317 membrane-localized GFP marks all cells in KV. KV is rotating along its anteroposterior (AP)
 2318 axis. Scale = 20µm.

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2320 **Movie 2.** 3D projection of KV in a live mosaic labeled *Tg(sox17:Cre^{ERT2});*
 2321 *Tg(ubi:Zebrafish)* embryo at 8 ss. Stochastic Cre-mediated recombination labels only few cells
 2322 with YFP expression with clear boundaries that are easily distinguishable from non-recombined
 2323 RFP⁺ cells. The KV organ is rotating along its anteroposterior (AP) axis. Scale = 20µm.

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2325 **Movie 3.** Time-lapse imaging of KV cells in a live mosaic labeled *Tg(sox17:Cre^{ERT2});*
 2326 *Tg(ubi:Zebrafish)* embryo treated with 4-OHT from dome stage to shield stage. Images were
 2327 collected every 5 min from 2 ss to 8 ss. The movie spans 105 min of development. Single 3D
 2328 rendered KV-ant (blue) and KV-post (red) cells are followed during KV morphogenesis. Scale =
 2329 20µm.

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