1 **Reemployment of Kupffer's vesicle cells into axial and paraxial mesoderm**
2 **via transdifferentiation**

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4 Takafumi Ikeda¹, Kiichi Inamori¹, Toru Kawanishi¹, Hiroyuki Takeda^{1*}

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8 1 Laboratory of Embryology, Department of Biological Sciences, Graduate School of

9 Science, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan

10

11 *To whom correspondence should be addressed.

12 Fax: (+81) 3-5841-4434. e-mail: htakeda@bs.s.u-tokyo.ac.jp

13

14 **Abstract (150-250 words)**

15 Kupffer's vesicle (KV) in the teleost embryo is a fluid-filled vesicle surrounded by a
 16 layer of epithelial cells with rotating primary cilia. KV transiently acts as the left-right
 17 organizer but degenerates after the establishment of left-right asymmetric gene
 18 expression. Previous labelling experiments indicated that descendants of KV-epithelial
 19 cells are incorporated into mesodermal tissues after KV collapses (KV-collapse) in
 20 zebrafish embryos. However, the overall picture of their differentiation potency had
 21 been unclear due to the lack of suitable genetic tools and molecular analyses. In the
 22 present study, we established a novel zebrafish transgenic line with a promoter of
 23 *charon*, in which all KV-epithelial cells and their descendants are specifically labelled
 24 until the larval stage. We found that KV-epithelial cells underwent epithelial-
 25 mesenchymal transition upon KV-collapse and infiltrate into adjacent mesodermal
 26 progenitors, the presomitic mesoderm and chordoneural hinge. Once incorporated, the
 27 descendants of KV-epithelial cells expressed distinct mesodermal differentiation
 28 markers and contributed to the mature populations such as the axial muscles and
 29 notochordal sheath through normal developmental process. These results indicate that
 30 fully differentiated KV-epithelial cells possess unique plasticity in that they are
 31 reemployed into mesodermal lineages through transdifferentiation after they complete

32 their initial role in KV. (194 words)

33

34 **Keywords (4-6 words)**

35 charon, zebrafish, Kupffer's vesicle, transdifferentiation

36

37 Introduction

38 Kupffer's vesicle (KV) is a teleost-specific organ, which is transiently formed on the
39 ventral side of the embryonic tailbud (Brummett and Dumont, 1978; Kupffer, 1868).
40 KV consists of a fluid-filled lumen and its surrounding epithelial cells (hereafter
41 referred to as KV-epithelial cells), and functions as the left-right organizer (LRO),
42 playing a crucial role in left-right axis formation in the embryo (Essner et al., 2005). In
43 the zebrafish, KV-epithelial cells are derived from a special group of cells called dorsal
44 forerunner cells (DFCs; Melby et al., 1996; Oteíza et al., 2008). DFCs originally bear
45 endodermal character as they express endoderm-specific genes, *sox32* and *sox17*
46 (Alexander et al., 1999; Kikuchi et al., 2001; Warga and Kane, 2018). During epiboly
47 stages, DFCs are transformed into KV-epithelial cells through mesenchymal-epithelial
48 transition (Amack et al., 2007; Matsui et al., 2015; Oteíza et al., 2008; Zhang et al.,
49 2016). At early somite stages, each KV-epithelial cell protrudes a primary cilium on the
50 apical side, which rotates and generates the leftward fluid flow in the KV lumen (Essner
51 et al., 2005). Sensed by primary cilia themselves, the leftward flow induces left-right
52 asymmetric gene expression in and around KV, leading to left-sided expression of *nodal*
53 in the lateral plate mesoderm (LPM; Essner et al., 2005). Judged by their morphological
54 characteristics and specialized function, KV-epithelial cells are considered as fully

55 differentiated and functional cells.

56 In the light of the fate plasticity in matured cells, we were interested in the fate
57 of KV-epithelial cells after they complete their mission in left-right patterning. Indeed,
58 KV collapses and disappears soon after the establishment of the asymmetric gene
59 expression in the LPM (hereafter referred to as KV-collapse), but previous lineage-
60 tracing studies of DFCs demonstrated that upon KV-collapse, descendants of DFCs are
61 incorporated into mesodermal tissues such as notochord, somites and tail mesenchyme
62 (Cooper and D'Amico, 1996; Melby et al., 1996). However, detailed tracking and
63 molecular characterization of those incorporated cells have not been performed so far,
64 and thus we do not know yet whether ciliated KV-epithelial cells actually possess fate
65 plasticity to transdifferentiate into functional mesodermal cells.

66 Recently, several transgenic zebrafish lines that express fluorescent proteins in
67 DFCs and KV were generated for easier and more reliable tracing of DFC- and KV-
68 derived cells. Among them, *sox17* lines have been frequently used because *sox17*
69 promoter uniformly labels both DFCs and KV-epithelial cells from epiboly to
70 somitogenesis stages (Compagnon et al., 2014; Mizoguchi et al., 2008; Oteíza et al.,
71 2008; Sakaguchi et al., 2006). Furthermore, a recent work using *sox17:GFP-CAAX* line
72 reported the epithelial-to-mesenchymal transition (EMT) of KV-epithelial cells during

73 KV-collapse (Amack, 2021), but their fate after EMT has not been directly tracked yet.
 74 For specific fate tracking of KV-epithelial cells, the *sox17* lines are not necessarily
 75 suitable, because endodermal cells as well as DFCs are broadly labelled (Mizoguchi et
 76 al., 2008). Other transgenic lines marking KV-epithelial cells (summarized in Table 1)
 77 have a similar limitation (Caron et al., 2012; Chen et al., 2012; Du and Dienhart, 2001;
 78 Molina et al., 2007). Thus, for precise tracing, another transgenic line was required in
 79 which mature KV-epithelial cells are specifically and uniformly labelled.

80 Here, we established novel transgenic lines, in which entire KV-epithelial cells are
 81 specifically labelled, using the promoter of *charon*. *charon* encodes a secreted Nodal
 82 antagonist belonging to the DAN family (Hashimoto et al., 2004). Its expression starts
 83 shortly after the differentiation of KV-epithelial cells from DFCs, and is highly specific
 84 to KV-epithelial cells both in zebrafish and medaka (Hashimoto et al., 2004; Hojo et al.,
 85 2007). The expression of *charon* is initially symmetric, but gradually becomes right-
 86 sided under the control of the leftward flow in KV (Hojo et al., 2007; Lopes et al., 2010).
 87 The essential role of *charon* in the left-right patterning is conserved among vertebrates as
 88 *charon*-knockout mice and zebrafish both exhibit randomized left-right axis (Marques et
 89 al., 2004; Montague et al., 2018).

90 Using these *charon* lines, we first confirmed that after KV-collapse, KV-derived

91 cells (KVDCs) differentiate into mesodermal but not into ectodermal or endodermal
92 tissues. Detailed timelapse imaging and gene-expression analyses further revealed that
93 KVDCs undergo EMT and infiltrate into two types of undifferentiated mesoderm,
94 which are the presomitic mesoderm and the chordoneural hinge. Importantly, they
95 expressed differentiation markers similar to their surrounding mesodermal cells at later
96 stages. These findings demonstrate that KV-epithelial cells possess high plasticity after
97 completing their initial role in KV, and that they transdifferentiate into mesodermal
98 lineages during normal development.

99

100 Table 1: Transgenic lines in zebrafish for the visualization of DFCs and KV-epithelial
101 cells

Promoter	Expression area at epiboly and somite stage	Timing	Length	Literature
<i>crestin</i>	Dorsal enveloping layer and KV	From DFCs (~50% epiboly)	1 kb	Chen et al., 2012
<i>foxj1</i>	Pronephric duct, floor plate and KV	From DFCs (~95% epiboly)	0.6 kb	Caron et al., 2012
<i>dusp6</i>	Mid-hindbrain boundary, Rhombomere 4 and KV	From DFCs (bud stage)	10 kb	Molina et al., 2007
<i>sox17</i>	Endoderm and KV	From DFCs (~80% epiboly)	5.0 kb	Sakaguchi et al., 2006; Oteiza et al., 2008
<i>twhh</i>	Notochord and KV	From 12 hpf	5.2 kb	Du & Dienhart, 2001
<i>charon</i>	KV	From ~6 somite stage	5.0 kb	Gourronc et al., 2007; the present study

102

103

104 **Results**

105 Establishment of *charon:EGFP* to specifically label KV-epithelial cells

106 To establish transgenic lines in which KV-epithelial cells are specifically labelled, we
107 cloned the 5-kb upstream sequence of zebrafish *charon* (Fig.1a, Supplementary Fig.1).

108 A previous transient promoter assay demonstrated that the upstream sequence of
109 zebrafish *charon* drives the reporter expression around KV (Gourronc et al., 2007), but

110 any stable transgenic line using the *charon* promoter has yet to be established. After
111 confirming the previous report by the Tol2-mediated EGFP reporter assay

112 (Supplementary Fig.3a), we established three stable transgenic lines in which either
113 EGFP, Lck-mGreenLantern (mGL) or H2B-mEosEM (a nuclear-localizing form of a

114 photoconvertible fluorescent protein mEosEM; Fu et al., 2020) expression is driven by
115 the *charon* 5-kb promoter (Fig.1b, Supplementary Fig.4a). In all the lines established,

116 reporter gene expression was specifically detected in KV-epithelial cells (Fig.1c-e,
117 Supplementary Fig.4b, c). In *charon:EGFP* embryos, EGFP fluorescence started to be

118 detected from around the 6-somite stage (ss) just like endogenous *charon* (Hashimoto et
119 al., 2004), and became stronger as KV grows in size. DAB (3,3'-Diaminobenzidine,

120 tetrahydrochloride) immunohistochemistry against EGFP showed that at 6 ss and 12 ss,
121 EGFP expression is strictly limited to the entire KV-epithelial cells with no ectopic

122 expression in adjacent mesoderm (Fig.1d-f). Fluorescence *in situ* hybridization with
123 immunofluorescence (FISH-IF; He et al., 2020) of *charon* in *charon:EGFP* embryos
124 further showed that *charon* expression mostly overlaps with that of EGFP at 12 ss
125 (Fig.1g). However, unlike endogenous *charon*, EGFP expression did not exhibited left-
126 right bias (Fig.1d-f). This can be explained by the fact that 3' untranslated sequence of
127 *dand5* (a mouse ortholog of *charon*) is responsible for its right-sided expression
128 (Minegishi et al., 2021; Nakamura et al., 2012). Untranslated sequences of zebrafish
129 *charon*, which are not included in the plasmid construct of this study, could have a
130 similar function to that of mouse *dand5*.

131 Furthermore, we found that the activity of upstream sequences of *charon* in KV-
132 epithelial cells is conserved among teleosts. A transgenic medaka (*Olyzias latipes*),
133 which was established with the upstream sequence of medaka *charon* (Supplementary
134 Fig.2), exhibited EGFP-reporter expression in KV-epithelial cells from 4 ss with faint
135 ectopic expression in the notochord (Supplementary Fig.5c). Overall, these results show
136 that *charon* transgenic lines are best suitable for the study of development and function
137 of KV-epithelial cells.

138

139 KVDCs differentiate into mesodermal lineages

140 KV is a transient organ and starts to degenerate at mid-somite stages (14–16 ss) when
 141 asymmetric *nodal* expression is established in the LPM (Cooper and D’Amico, 1996;
 142 Long, 2003; Supplementary Fig.8). However, we observed that EGFP-expressing cells
 143 in *charon:EGFP* embryos survived after KV-collapse and even persisted in hatched
 144 larvae (Supplementary Fig.3). Hereafter we refer to the EGFP-positive cells in
 145 *charon:EGFP* after KV-collapse as “KV-epithelium-derived cells (KVDCs)” to
 146 distinguish them from KV-epithelial cells. To track KVDCs after KV-collapse, we
 147 histologically analyzed embryos at the mid-somite stage (22 ss) and larvae at 3 days
 148 post fertilization (dpf) using DAB immunohistochemistry and confocal microscopy. In
 149 22-ss embryos whose KVs have already diminished, most KVDCs were clustered
 150 around the collapsed lumen, but some of them were found in the adjacent notochord and
 151 presomitic mesoderm (PSM; Fig.2a, b). In 3-dpf hatched larvae, KVDCs were broadly
 152 distributed in the region posterior to the cloaca where no endodermal tissue exists
 153 (Fig.2c). KVDCs were detected in mesodermal tissues including both segmented and
 154 unsegmented posterior-most somites (Moriyama et al., 2012; Fig.2c, e1, e2), notochord
 155 cells near the posterior end and the notochordal sheath in the more anterior side (Fig.2d,
 156 e3), and fin mesenchymal cells with radial filopodia especially in the caudal fin fold

157 (Fig.2c, d and e4). We counted the number of KVDCs in each tissues using
158 *charon:H2B-mEosEM* 3-dpf larvae and found that they were most abundant in the
159 unsegmented posterior-most somites (56 ± 11 cells/embryo; Fig.2f). In contrast, no
160 KVDCs were observed in the ectoderm (Fig.2d, e).

161 To further support the above findings, we directly examined the migration of
162 KVDCs into these mesodermal tissues by a photoconversion-based method (Shimada et
163 al., 2013). We photoconverted H2B-mEosEM transiently expressed in KV-epithelial
164 cells of *β actin:memCherry* embryos, whose cell membrane is labelled with mCherry
165 (Xiong et al., 2014). After photoconversion at around 13 ss, labelled KV-epithelial cells
166 were tracked at an interval of 1 hour during segmentation periods (n = 4 embryos,
167 Fig.2g). At 14 ss (0 hour after photobleaching), photoconverted H2B-mEosEM was
168 only detected in KV-epithelial cells, confirming the specificity of photoconverted
169 labelling (Fig.2h). The labelled KVDCs tended to stay together until 19 ss (4 hours after
170 photobleaching), and then gradually migrated from KV into the PSM and notochord
171 (Fig.2h, arrowheads in the 28-ss panel). When the same embryos were further observed
172 at 2 dpf, the labelled KVDCs were broadly distributed in segmented somites,
173 unsegmented posterior-most somites, and the notochord (Fig.2h, arrowheads in the 2-
174 dpf panel). We obtained a similar result using another transgenic line, *foxj1a:KikGR*, in

175 which photoconvertible protein KikGR (Tsutsui et al., 2005) is expressed in KV-
176 epithelial cells as well as in the floor plate (Caron et al., 2012; Supplementary Fig.7a).
177 Taken together, the results demonstrated that KVDCs migrate and contribute to the axial
178 and paraxial mesoderm.

179

180 Loss of the LRO and epithelial character in KVDCs

181 We next examined the molecular characteristics of KVDCs after KV-collapse. We first
182 examined whether any KVDCs are eliminated by apoptosis during and after KV-
183 collapse. Immunostaining of cleaved caspase-3, an executioner of apoptotic process
184 (Elmore, 2007), revealed that apoptosis was induced in KVDCs after KV-collapse, but
185 that the number of apoptotic KVDCs was limited (Fig.3a, b). We then asked how
186 survived KVDCs lost morphological and molecular characteristics of the LRO
187 components by examining *charon* (LRO marker) and *sox17* (DFCs marker) expression
188 and the protein distribution of ZO-1 (epithelial marker) and acetylated α -tubulin
189 (primary cilia marker) in KVDCs (Alexander and Stainier, 1999; Essner et al., 2005;
190 Hashimoto et al., 2004; Oteíza et al., 2008). FISH-IF showed that the expression of
191 *charon* was diminished at 20 ss and 26 ss compared to at 12 ss, and *charon*-negative
192 KVDCs were frequently detected (Fig.3c). Moreover, we did not detect *sox17*

193 expression in KV-epithelial cells or KVDCS at all stages examined (Supplementary
194 Fig.6), which is consistent with the previous report (Alexander and Stainier, 1999).

195 ZO-1 was accumulated on the surface of the KV lumen at 6 ss, confirming the
196 epithelial character of EGFP-positive cells in *charon:EGFP* (Fig.3d; Oteíza et al., 2008).

197 At 18 ss, shortly after KV-collapse, migrating KVDCs no longer expressed ZO-1,

198 although residual ZO-1 expression was detected in a cluster of KVDCs located on the

199 ventral side of the CNH. At 26 ss, a substantial number of KVDCs moved to the

200 posterior edge of the chordoneural hinge (CNH), a group of progenitors which are

201 located at the posterior end of the notochord and thought to give rise to the notochord,

202 floor plate and hypochord (Agathon et al., 2003; Row et al., 2015). Furthermore,

203 primary cilia of KVDCs were largely diminished after KV-collapse, particularly in

204 migrating ones (Fig.3e). Taken together, the results suggest that KV-epithelial cells

205 gradually lose their LRO and epithelial character, and acquire mesodermal fates through

206 mid-to late somite stages.

207

208 KVDCs undergo EMT at the onset of migration

209 We examined detailed cellular behavior of KVDCs at the onset of migration using a

210 double transgenic line, *charon:EGFP;βactin:memCherry*. KV initially protruded into

211 the yolk, then incorporated into the tailbud at around 10 ss, and finally collapsed
 212 (Supplementary Figure 8). At around 14 ss, KV-epithelial cells detached from the
 213 underlying yolk syncytial layer (YSL) (Fig.4, Supplementary Video 1). Interestingly, a
 214 ring-like structure labelled by strong mCherry signals appeared in the YSL just beneath
 215 KV (Fig. 4, magenta arrowheads). This YSL ring shrank when KV was detached from
 216 the YSL as if it pushes KV out into the tailbud and breaks the connection between KV
 217 and the YSL. Once KV was detached from the YSL, its lumen started to shrink (Fig. 4,
 218 the 01:20 panel). At the same time, KV-epithelial cells started to disperse and migrate,
 219 extending filopodia-like structures (Fig. 4, insets in the 02:40 panel, Supplementary
 220 Video 2). This observation, together with the loss of ZO-1 expression (Fig.3d), suggests
 221 that KV-epithelial cells undergo epithelial-mesenchymal transition during KV-collapse.

222

223 Differentiation of KVDCs into the PSM and somite derivatives

224 We next examined the developmental capacity of KVDCs which migrate into somites
 225 using lineage-specific differentiation markers. At 12 ss when KV is still present, KV
 226 epithelial cells were ventrally attached to the PSM as well as notochord (Fig.5a). A pan-
 227 PSM marker *msgn1* (Joseph and Cassetta, 1999; Yoo et al., 2003; Yoon et al., 2000) was
 228 strongly expressed in the PSM but not in KV-epithelial cells at 12 ss (Fig.5a),

229 confirming their distinct lineages. After KV-collapse, KVDCs were found in the PSM
230 (Fig.5b1) and later in the anterior segmented somites as well, where *msgn1* expression
231 is no longer detected (Fig.5b2). These results suggest that KVDCs are incorporated into
232 the PSM after KV-collapse and are intermingled with surrounding PSM cells.

233 The zebrafish somite consists of three populations with distinct fates, the
234 myotome, dermomyotome, and sclerotome (Stickney et al., 2000). The myotome is
235 further divided into the two subpopulations, slow muscle in the dorsal (outer), and fast
236 muscle in the ventral (deeper) regions. KVDCs were found to be distributed in all these
237 populations at later stages (Fig.5). At 3 dpf, KVDCs which are located near the surface
238 and in the deep layer of the myotome expressed slow and fast muscle markers,
239 respectively, as detected by F59 and F310 antibodies (Fig.5c, d). These muscle cells
240 persisted at least until 5 dpf (data not shown), suggesting that they differentiated into
241 functional muscles. Fast muscles are known to be derived from adaxial cells which are
242 large, cuboidal in shape, located adjacent to the notochord and express *myoD* (Devoto et
243 al., 1996). During slow muscle development, adaxial cells radially migrate from their
244 original position toward the superficial layer of the myotome and differentiate into slow
245 muscles there (Barresi et al., 2001; Cortés et al., 2003). At late somite stage (26 ss), we
246 identified adaxial cell-like KVDCs, judged by their shape and location, and found that

247 they indeed expressed *myoD* (Fig.5h). This observation suggests that KVDCs follow the
248 normal developmental process to differentiate into slow muscles.

249 KVDCs were also found in the dermomyotome layer (the outer-most surface of
250 the somite) and in the sclerotome region (the ventro-medial region near the notochord
251 and neural tube), where they expressed the dermomyotome marker, Pax3/7 (Hammond
252 et al., 2007) and sclerotome marker, *nkx3.1* (Ma et al., 2018), respectively (Fig.5e, i).

253 We also examined the positional relationship between ventral KVDCs and posterior
254 blood vessels, because the ventral sclerotome is known to differentiate into mural cells,
255 which surround blood vessels (Stratman et al., 2017). For this, we used double
256 transgenic fish of *charon:EGFP* and *drl:mCherry*, whose LPM derivatives are specifically
257 labelled with mCherry (Mosimann et al., 2015; Prummel et al., 2019). We found that
258 phalloidin-negative KVDCs were attached to the posterior-most part of the caudal vein
259 (Fig.5f, g), supporting the idea that KVDCs acquire the differentiation potency of the
260 sclerotome. Importantly, KVDCs did not overlap with mCherry-expressing LPM-
261 derivatives, indicating that they do not contribute to the LPM lineage (Fig.5f, g).

262

263 Differentiation of KVDCs into the notochord and tail mesenchyme

264 We further examined the differentiation process of KVDCs in the notochord and tail

mesenchyme. At 12 ss, KV-epithelial cells were ventrally attached to the *no tail* (*ntl*, the young notochord marker)-positive notochord (Schulte-Merker et al., 1994; Fig.6a). At 20 ss, some KVDCs were found in the posterior end of *ntl*-expressing area, i.e., the CNH (Fig.6b). These KVDCs were also positive for *ntl*, indicating that they had acquired the axial mesodermal fate. Some KVDCs further developed into *ntl*-negative mature notochord cells at 26 ss (Fig.6b), and later into the surrounding sheath cells (Fig.2d). These results show that KVDCs which infiltrate into the CNH possess the full capacity of notochord differentiation.

The migration of KVDCs into the axial mesoderm seemed to take place exclusively in the CNH. The developing and mature notochord is surrounded by the basement membrane formed by deposition of extracellular matrix (ECM) such as Laminin and Fibronectin (Scott and Stemple, 2004). However, accumulation of these two major ECM components was reduced around the CNH as indicated by immunostaining (Fig.6d, e), which may facilitate cell mingling and infiltration of KVDCs into the CNH.

Lastly, we tracked the fate of the posterior-most KVDCs cluster which stays around the CNH until the end of the segmentation period (Fig.3d). We found that these KVDCs are distinguished from CNH cells, because they did not express the CNH

283 markers, *noto* and (Dheen et al., 1999; Fig.6c). Using *charon:H2B-mEosEM*, we
 284 examined the fate of these KVDCs by photoconverting H2B-mEosEM-expressing cells
 285 located posterior to the CNH at around 28 ss and chased the labelled cells in 2 dpf
 286 larvae. The result demonstrated that they preferentially differentiated into fin
 287 mesenchymal cells in the caudal fin fold rather than into the CNH or notochord cells (n
 288 = 3 embryos, Fig.6f). These results imply that these KVDCs occupy a posterior-most
 289 part of the paraxial (but not the axial) mesoderm and directly differentiate into caudal
 290 fin mesenchyme.

291

292 Discussion

293 During differentiation, the potency of cells becomes restricted as they are specified into
 294 certain lineages. In some contexts, however, differentiated cells can be converted to
 295 other cell types; a phenomenon called adult cell plasticity (Merrell and Stanger, 2016).
 296 Such plasticity is often observed in the regeneration process, but it is still a matter of
 297 debate whether it takes place commonly during normal development of animals
 298 (Merrell and Stanger, 2016). The present study demonstrated that derivatives of KV-
 299 epithelial cells (KVDCs) join mesodermal progenitors after KV-collapse and
 300 differentiate into mature cell types according to their location. Such “reemployment” of
 301 KV-epithelial cells is a rare example of cell plasticity of fully differentiated cells during
 302 normal development in vertebrates, which is achieved through transdifferentiation
 303 (Fig.7).

304
 305 KVDCs are a multipotent mesodermal cell population in the tailbud
 306 Our results revealed that KVDCs in the zebrafish tailbud possess unique differentiation
 307 potency. The zebrafish tailbud contains three undifferentiated cell populations, the PSM,
 308 CNH, and neuromesodermal progenitors (NMPs; Beck, 2015; Sambasivan and
 309 Steventon, 2021). KVDCs occupy a part of the PSM and CNH, and differentiate into the
 310 paraxial and axial mesoderm. In contrast, KVDCs never contribute to NMPs as they do

311 not give rise to the neural tube (Henrique et al., 2015). Importantly, KVDCs are unique
312 among these progenitors in the tailbud in that they are the descendants of functional
313 epithelial cells with primary cilia, which exerted a crucial role in left-right patterning.

314 The contribution of the LRO cells to the mesodermal tissues could be a common
315 feature among vertebrates, although the origin and structures of the LRO are diverse
316 (Blum et al., 2014). The gastrocoel roof plate in amphibians and node in mammals,
317 which function as the LRO in these species (Blum et al., 2014), are suggested to
318 differentiate into the notochord and somites, and into the posterior notochord,
319 respectively (Brennan et al., 2002; Shook et al., 2004). Thus, it is likely that the
320 vertebrate LRO cells generally behave as mesodermal cells at later stages, although
321 DFCs in the zebrafish are transiently endodermal at the beginning of their emergence
322 (Alexander and Stainier, 1999).

323

324 Transdifferentiation of KVDCs

325 Transdifferentiation of mature cells is generally achieved through dedifferentiation into
326 a potent state followed by redifferentiation into a new lineage (Jopling et al., 2011). The
327 development of KVDCs appears to go through this process: KVDCs redifferentiate into
328 somite-derivatives and notochord after being integrated into their progenitors. We
329 observed that KV-epithelial cells undergo EMT during KV-collapse. EMT frequently

occurs in association with dedifferentiation as seen in cancer metastasis and neural crest delamination (Yang and Weinberg, 2008). Indeed, EMT in KV is accompanied by the loss of differentiated epithelial characters such as primary cilia and tight junctions, which lead to KV-collapse and cell migration. Factors triggering this EMT are yet to be determined, although potent signalling ligands, Wnt, BMP and FGF are produced in the nearby PSM (Hubaud and Pourquié, 2014; Row and Kimelman, 2009). Furthermore, we described the shrinkage of the YSL underlying KV in the present study (Fig.4c). This shrinkage might generate mechanical force which triggers EMT in cooperation with those signalling ligands (Gjorevski et al., 2012).

In general, mature epithelial cells maintain their fate and integrity by cell-cell junctions and attachment to the basement membrane (Yang et al., 2020). Then, why can KV-epithelial cells undergo transdifferentiation and what makes them so unique? Despite the presence of evident apico-basal polarity and cell-cell junctions, KV epithelium seems to lack the basement membrane, as revealed by our immunostaining and a previous report on the atypical localization of Laminin β 1a to their apical side (Hochgreb-Hägele et al., 2013). Thus, KV-epithelial cells may not be of typical epithelium in character, which could reflect their transient function. Lack of the basement membrane would allow KV-epithelial cells to dedifferentiate and migrate

348 without a need of ECM remodelling.

349 Some vertebrate tissues consist of cells derived from different lineages. The
350 prominent examples are the zebrafish pituitary and mammalian vascular endothelium,
351 where undifferentiated cells from different origins converge at the transcription level
352 once they are intermingled (Fabian et al., 2020; Plein et al., 2018). We confirmed here
353 that KVDCs, whose origin is distinct from the authentic mesoderm, differentiate into the
354 notochord and somite-derivatives in the caudal region. KVDCs are thus a good model
355 for the study of such lineage convergence during normal development. Single-cell
356 multiomics using *charon* transgenic lines will provide insights into the mechanism of
357 how and to what extent cells from different lineages converge together to form a
358 functionally integrated tissue.

359

360 **Figure legends**

361 **Figure 1.** Establishment of *charon:EGFP* for the specific labeling of KV-epithelial
362 cells

363 **a**, Location of the 5-kb *charon* promoter in the zebrafish genome. *charon* resides on
364 chromosome 1, and the 5-kb *charon* promoter spans from the direct upstream position
365 of its start codon.

366 **b**, Construction of *charon:EGFP* transgene flanked by Tol2 excision sites.

367 **c**, Lateral view of the EGFP fluorescence in a *charon:EGFP* embryo at 9 somite stage
368 (ss). Fluorescence image was merged with the DIC (differential interference contrast)
369 image. KV, Kupffer's vesicle.

370 **d**, Dorsal view of a *charon:Lck-mGL* embryo at 9 ss injected with H2B-mCherry
371 mRNA. Black arrowhead indicates the level of the sagittal optical section on the right
372 side.

373 **e**, Dorsal view of a DAB-stained *charon:EGFP* embryo at 12 ss.

374 **f**, Cross section of the tailbud of a DAB-stained *charon:EGFP* embryo at 12 ss. Nuclei
375 were counterstained with hematoxylin. Dorsal side to the top.

376 **g**, FISH-IF of *charon* in a *charon:EGFP* embryo at 12 ss. Magenta signal is for FISH of
377 *charon* mRNA whereas green is for immunostaining of EGFP proteins. Lateral view of

the tailbud region is shown. White box indicates the enlarged area. Dorsal to the bottom,
posterior to the left. Scale bars, 200 μ m (c, e), 100 μ m (f) and 50 μ m (d, h).

Figure 2. Histology of KVDCs

a, Lateral view of a DAB-stained *charon:EGFP* embryo at 22 ss. White arrowheads
indicate KVDCs which have started to migrate.

b, Cross section of the tailbud of a DAB-stained *charon:EGFP* embryo at 22 ss. Nuclei
were counterstained with hematoxylin. Dorsal side to the top. PSM, presomitic
mesoderm. nc, notochord.

c, Lateral view of a *charon:EGFP* embryo at 22 ss counterstained with phalloidin and
DAPI. Dorsal to the top. Green arrowheads indicate EGFP-positive cells in the CNH
(chordoneural hinge).

d, Cross sections of a DAB-stained *charon:EGFP* larva at 3 dpf. Nuclei were
counterstained with hematoxylin. Dorsal side to the top. Asterisk indicates a melanocyte.
dmu, dorsal muscle. fm, fin mesenchyme. nc, notochord. ncs, notochordal sheath. nt,
neural tube. vmc, ventral muscle.

e, Lateral view of the caudal region of a *charon:EGFP* embryo at 3 dpf counterstained
with phalloidin. White boxes indicate areas magnified in the panels 1-4. White dashed

396 line indicates the edge of the fin fold. Panel 1, EGFP-positive muscles in segmented
397 somites. Panel 2, EGFP-positive muscles in non-segmented somites. Panel 3, EGFP-
398 positive notochord cells. Panel 4, EGFP-positive fin mesenchymal cells in the caudal fin
399 fold. Scale bars, 200 μm (a, e), 100 μm (b, d), 50 μm (c).

400 **f**, Number of H2B-mEosEM-positive KVDCs in *charon:H2B-mEosEM* in four
401 populations shown in (e). Horizontal bars, mean. Vertical bars, standard deviation. N =
402 20 larvae.

403 **g**, Scheme for the labelling of KV-epithelial cells by photoconversion. H2B-mEosEM is
404 expressed in KV-epithelial cells in *β actin:memCherry* embryos by injection of
405 *charon:H2B-mEosEM* plasmid. H2B-mEosEM was photoconverted at around 13 ss
406 using a confocal microscopy and time-lapse images were taken every 1 hour.

407 **h**, Time-lapse images of a *β actin:memCherry* embryo injected with *charon:H2B-*
408 *mEosEM*. Images taken every 2 hours after photoconversion and from 2 dpf treated
409 larvae are shown. Arrowheads indicate the migrated KVDCs labelled with
410 photoconverted H2B-mEosEM.

411

412 **Figure 3. Loss of the LRO character in KVDCs**

413 **a**, Immunostaining of cleaved caspase-3 in *charon:EGFP* embryos at 12, 20 and 26 ss.

414 Top, the signal of cleaved caspase-3. Bottom, merged view with EGFP and DAPI signal.

415 Magenta arrowheads indicate KVDCs which is positive for cleaved caspase-3.

416 **b**, Quantification of cleaved caspase-3-positive KVDCs. Horizontal bars, mean. Vertical

417 bars, standard deviation. *p*-values from two-tailed Welch's t-test are shown. N = 9 (12

418 ss), 11 (20 ss), and 10 (26 ss) embryos.

419 **c**, FISH-IF of *charon* in *charon:EGFP* at 20 and 26 ss. Magenta, signal of FISH for

420 *charon* mRNA. Green, signal of immunostaining for EGFP. Lateral view of the tailbud

421 region is shown. Dorsal to the top.

422 **d**, Immunostaining of ZO-1 in *charon:EGFP*. KV is shown for 6 ss whereas the tail

423 region is shown for 18 and 26 ss. Magenta and white arrowheads indicate KVDCs with

424 and without ZO-1 expression, respectively. nc, notochord. CNH, chordoneural hinge.

425 **e**, Immunostaining of acetylated α -tubulin in *charon:EGFP*. Magenta and white

426 arrowheads indicate KVDCs with and without acetylated α -tubulin signal, respectively.

427 Scale bars, 50 μ m (a, c, d, e).

428

429 **Figure 4. KVDCs migrate to mesodermal tissues through EMT**

430 Time-lapse images of a *charon:EGFP; β actin:memCherry* embryo taken every 20

431 minutes for 3 hours from 13 ss. Magenta arrowheads indicate the shrinkage of the YSL

underlying KV. White boxes in the panel 02:40 indicate inset areas. Green arrowhead in the inset indicates a filopodium-like structure formed in KVDCs. Scale bars, 50 μ m.

Figure 5. Differentiation of KVDCs into somite derivatives

a, FISH-IF of *msgn1* in a *charon:EGFP* embryo at 12 ss. Left, dorsal view. Top, an optical section at the level of the dashed line in the panel below. Right, a more dorsal stack than the left one. PSM, presomitic mesoderm.

b, FISH-IF of *msgn1* in *charon:EGFP* embryos at 20 ss and 26 ss. Left, dorsal view. Anterior to the top. Right, optical sections at the level of the dashed lines in the left panels. White arrowheads indicate KVDCs in the PSM (20 ss and 26 ss) and in the segmented somites (26 ss).

c, d, Vibratome cross sections of *charon:EGFP* 3 dpf larvae immunostained with F59 (c) and F310 (d) antibodies to show the distribution of slow and fast muscles, respectively. Counterstaining was performed with DAPI. White arrowheads indicate EGFP-positive slow and fast muscle cells. Dorsal to the top. nt, neural tube. nc, notochord.

e, Immunostaining of dermomyotomes by anti-Pax3/7 antibody in a *charon:EGFP* 2 dpf larva. Lateral view and optical sections at the level of dashed lines are shown. White

450 arrowheads indicate EGFP-positive dermomyotomes.

451 **f**, Lateral view of a *charon:EGFP;drl:mCherry* 3 dpf larva. White arrowheads indicate

452 EGFP-positive sclerotomal cells surrounding mCherry-positive cells derived from the

453 LPM. nc, notochord. LPM, lateral plate mesoderm.

454 **g**, Left, cross section of a *charon:EGFP;drl:mCherry* 3 dpf larva immunostained with

455 anti-EGFP and anti-mCherry antibodies. Counterstaining was performed with phalloidin

456 and DAPI. Right, a magnified view of the white rectangular area in the left panel. White

457 arrowheads indicate KVDCs surrounding mCherry-positive LPM derivatives.

458 **h, i**, FISH-IF of *myod1* in a *charon:EGFP* embryo at 26 ss (h) and *nkx3.1* in a 24 hpf

459 (hours post fertilization) embryo (i). Left panel, lateral view. Anterior to the left, dorsal

460 to the top. Right panel, optical sections at the level of the dashed lines in the left panels.

461 White arrowheads indicate KVDCs which differentiated in *myod1*-positive adaxial cells

462 (h) and *nkx3.1*-positive sclerotomes (i). Scale bars, 50 μ m (a-i).

463

464 **Figure 6. Differentiation of KVDCs into the notochord**

465 **a**, FISH-IF of *ntl* in a *charon:EGFP* embryo at 12 ss. Top, dorsal view. Anterior to the

466 top. Bottom, an optical section at the level of the dashed line in the upper panel. nc,

467 notochord.

468 **b**, FISH-IF of *ntl* in *charon:EGFP* embryos at 20 ss and 26 ss. Dorsal views. Anterior to
469 the left. White arrowheads, KVDCs in the CNH (20 ss) and in the notochord (26 ss).
470 CNH, chordoneural hinge.

471 **c, d**, Immunostaining of Laminin (c) and Fibronectin (d) in *charon:EGFP* embryos at
472 12 ss and 20 ss. For 12 ss, dorsal views of KV are shown. Anterior to the top. For 20ss,
473 lateral views of the tailbud are shown. Dorsal to the top. Anterior to the right.

474 **e**, FISH-IF of *noto (flh)* in a *charon:EGFP* embryo at 26 ss. Lateral view. Dorsal to the
475 top. Anterior to the left.

476 **f**, Lineage tracing of the KVDCs cluster at the posterior tip of the CNH. H2B-mEosEM
477 was expressed in KVDCs by *charon:mEosEM* plasmid injection into *βactin:memCherry*
478 embryos. Photoconversion was performed in 28 ss and the labelled KVDCs were chased
479 at 2 dpf. White arrowheads indicate the labelled KVDCs which differentiated into fin
480 mesenchymal cells in the caudal fin fold. Scale bars, 50 μm (a-f).

481

482 **Figure 7. An overview of KVDCs differentiation**

483 Schematic diagram depicting the process of the transdifferentiation-mediated
484 reemployment of KV-epithelial cells. After KV-collapse, KV-epithelial cells
485 dedifferentiate and undergo EMT. Mesenchymalized KVDCs infiltrate into mesodermal

486 progenitors, the PSM and CNH, and acquire new fates according to their new locations.

487 Note that they do not directly migrate to the segmented somites or the mature notochord.

488

489 Supplementary Figure 1. Sequence of the zebrafish 5-kb *charon* promoter

490 Nucleotide sequence of the 5-kb *charon* promoter in the zebrafish.

491

492 Supplementary Figure 2. Sequence of the medaka 5-kb *charon* promoter

493 Nucleotide sequence of the 5-kb *charon* promoter in medaka. Red, partial coding

494 sequences of *charon* gene.

495

496 Supplementary Figure 3. Fluorescence stereomicroscope images of

497 *charon:EGFP*

498 **a**, Lateral view of a *charon:EGFP* embryo at 6 ss.

499 **b**, Ventral view of a *charon:EGFP* embryo at 20 ss .

500 **c**, Lateral view of a *charon:EGFP* larva at 2 dpf. Scale bars, 200 μ m (a-c).

501

502 Supplementary Figure 4. Fluorescence of *charon:Lck-mGreenLantern*

503 **a**, The construction of *charon:Lck-mGreenLantern* transgene.

504 **b, c**, Lateral view of a *charon:Lck-mGreenLantern* embryo at 8 ss (b) and 22 ss (c). KV,
505 Kupffer's vesicle. Scale bars, 200 μ m.

506

507 Supplementary Figure 5. Medaka *charon* promoter is active in medaka KV-
508 epithelial cells

509 **a**, Location of the *charon* 5-kb promoter in the medaka genome. *charon* resides on
510 chromosome 1, and the *charon* 5-kb promoter spans from the direct upstream position
511 of its start codon.

512 **b**, Construction of *Ol_charon:EGFP* transgene flanked by I-SceI recognition sites.

513 **c**, Expression of EGFP in *Ol_charon:EGFP* embryos. Lateral views on the top row,
514 dorsal views on the bottom row. White dot lines indicate embryonic bodies. Red circle
515 indicates the position of KV. Scale bars, 500 μ m.

516

517 Supplementary Figure 6. FISH-IF of *sox17* in *charon:EGFP* embryos

518 FISH-IF of *sox17* in *charon:EGFP* embryos at 6 ss, 12 ss, 20 ss and 26 ss. Lateral views
519 of tailbud regions are shown. Scale bars, 50 μ m.

520

521 Supplementary Figure 7. *foxj1a:KikGR* supports KV-epithelial cell migration

522 **a**, Scheme for the photoconversion labelling of KV-epithelial cells in *foxj1a:KikGR*.

523 KikGR in KV-epithelial cells was selectively photoconverted at around 13 ss and time-

524 lapse images were taken every 1 hour.

525 **b**, Time-lapse images of a photoconverted *foxj1a:KikGR* embryo. Single stacks from

526 images taken every 2 hours after photoconversion are shown. When photoconverted at

527 around 13 ss, labelled KVDCs migrated into the PSM and the notochord by 28 ss (6

528 hours after photoconversion). Scale bars, 50 μ m.

529

530 Supplementary Figure 8. Time-lapse imaging of the incorporation of KV into the

531 tailbud

532 Time-lapse imaging of the KV development in a TL2E embryo. Arrowheads indicate

533 the position of KV. Staring from 4 ss, images are taken every 15 minutes for 10 hours.

534 Scale bars, 50 μ m.

535

536 Supplementary Video 1. Time-lapse imaging of KV-collapse

537 A video showing the process of KV-collapse in a *charon:EGFP; β actin:memCherry*

538 embryo. Time-lapse images were taken every 5 minutes for 3 hours from 13 ss. Note

539 that a ring-like structure is formed beneath KV during KV-collapse. Scale bar, 50 μm .

540

541 Supplementary Video 2. Formation of filopodia-like structures in KVDCs

542 Green channel is extracted from Supplementary Video 1 to show the formation of

543 filopodia-like structure in KVDCs. Scale bar, 50 μm .

544

545 **Author Contributions**

546 Conceptualization, T.I., K.I., and H.T.; Methodology, T.I., K.I., and T.K.; Investigation,
547 T.I., K.I., and T.K.; Resources, T.I., K.I., and T.K.; Writing - Original Draft, T.I.; Writing
548 - Review & Editing, T.I., T.K., and H.T.; Supervision, H.T.; Funding Acquisition, T.I.,
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550

551

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561

562

563 **Competing interests**

564

565 **Materials & Correspondence**

566

567

568 **Material and Methods**

569 Zebrafish strain and manipulation of embryos

570 The RW (RIKEN WT) and TL2E (Tüpfel long fin 2E) strains were used as the wild-
571 type zebrafish strains. Adult fish and embryos were maintained under standard
572 conditions. Fertilized embryos were incubated at 23-28°C in 1/3×Ringer's solution
573 (38.7 mM NaCl, 0.97 mM KCl, 1.67 mM HEPES, 1.80 mM CaCl₂) to obtain the stage
574 of interest. For imaging hatched larvae, 0.003% N-phenylthiourea (PTU) was added to
575 1/3×Ringer's solution during somitogenesis to prevent pigmentation. Staging of
576 embryos is based on Kimmel et al. (1995). All experimental procedures and animal care
577 were carried out according to the animal ethics committee of the University of Tokyo
578 (Approval No. 20-2).

579

580 Plasmid construction

581 The sequences of *charon* and *foxj1a* promoters (5 kb and 5.2 kb, respectively) were
582 amplified from the genomic DNA of the RW strain and were cloned into pCR Blunt II-
583 TOPO vector (Invitrogen). pDestTol2pA2-*charon:EGFP* (*charon:EGFP* for short) was
584 constructed by replacing the *drl* promoter in pDestTol2pA2-*drl:EGFP* (Mosimann et al.,
585 2015) with the cloned *charon* promoter. Medaka *charon* promoter was cloned from

586 genomic DNA of medaka d-rR strain and was inserted into pBlueScript II SK(+)-EGFP
587 carrying an I-SceI cassette to make pBSSK-I-SceI-*Ol_charon:EGFP* (*Ol_charon:EGFP*
588 for short). pDestTol2pA2-*charon:Lck-mGreenLantern* was constructed as follows: the
589 CDS of mGreenLantern (Campbell et al., 2020) whose codon usage is optimized for
590 zebrafish was purchased from Integrated DNA Technologies (IDT), and was inserted
591 into pCSf107mT vector (Mii et al., 2009) with an N-terminal membrane localization
592 signal of mouse Lck (Chertkova et al., 2017) to make pCSf107-Lck-mGreenLantern.
593 Then, the CDS of Lck-mGreenLantern was subcloned into pDestTol2pA2 vector
594 carrying the *charon* promoter. pDestTol2pA2-*charon:H2B-mEosEM* was constructed as
595 follows: the CDS of mEosEM was synthesized through mutagenesis of mEos4b (Paez-
596 Segala et al., 2015) as described (Fu et al., 2020), and was assembled together with the
597 CDS of human Histone H2B type 1-J (H2BC11) into pCSf107mT to make pCSf107-
598 H2B-mEosEM. Then, the CDS of H2B-mEosEM was subcloned into pDestTol2pA2
599 vector carrying the *charon* promoter. To construct template plasmids for the antisense
600 probe synthesis, full-length coding sequences (CDSs) of zebrafish *charon*, *sox17*, *msgn1*,
601 *myod1*, *nkx3.1*, *ntl* and *noto* cloned from 16 ss cDNA were inserted into pCSf107mT.
602 These cloning and subcloning were performed using PrimeSTAR GXL polymerase,
603 PrimeSTAR MAX polymerase, In-fusion HD kit and In-Fusion Snap Assembly kit

604 (TaKaRa). pRSETa_mEos4b was a gift from Loren Looger (Addgene plasmid #51073;
605 <http://n2t.net/addgene:51073>; RRID:Addgene_51073). Lck-mScarlet-I was a gift from
606 Dorus Gadella (Addgene plasmid #98821; <http://n2t.net/addgene:98821>;
607 RRID:Addgene_98821). Digital plasmid maps are available upon request.

608

609 Transgenesis

610 Zebrafish transgenesis was based on the Tol2 transposase method (Kikuta and
611 Kawakami, 2009). To obtain founders, Tol2 mRNA (25 ng/μL) and plasmids (12.5
612 ng/μL) were injected into one-cell stage embryos of the RW strain. Founders carrying
613 transgenes were screened by crossing with the TL2E strain. For medaka transgenesis,
614 the I-SceI method (Rembold et al., 2006) was used. Following digestion with I-SceI,
615 *Ol_charon:EGFP* plasmid was injected into one-cell stage embryos of the d-rR
616 wildtype strain. Founders were screened by crossing with the d-rR strain. Fluorescence
617 image of transgenic embryos was captured by M165 FC fluorescence stereomicroscope
618 and DFC7000T digital camera (Leica).

619

620 Time-lapse imaging and photoconversion

621 To perform time-lapse imaging, embryos were mounted laterally in the chamber of 1%

622 agarose in 1/3×Ringer's solution with their left side down. Embryos were imaged by
623 LSM710 confocal microscopy (Carl Zeiss) from 13 ss for 3 hours or longer at an
624 interval of 5 min. In cell-tracking experiments, the photoconversion of H2B-mEosEM
625 was performed with a 405 nm diode laser at the power of 10%. After photoconversion,
626 embryos were released in 1/3x Ringer's solution with 0.003% PTU and were grown
627 until they reach the stages of interest in a shaded chamber. The 3D rendering of time-
628 lapse images was performed with Imaris (version 8.1.2, Bitplane).

629

630 Histology and immunostaining

631 Embryos were fixed with 4% paraformaldehyde (PFA) in PBST at 4°C overnight or at
632 room temperature for 2 hours, washed with PBST for three times and were stored at 4°C
633 until use (except for Laminin and Fibronectin, see below). Fixed embryos were
634 permeabilized with 1% Triton X-100 in PBS, blocked with 2% BSA in PBSDT
635 (PBS/1% DMSO/0.1% Triton X-100) and were immunostained with following
636 antibodies: mouse monoclonal anti-GFP (1/100, A-11120, Invitrogen), rabbit poly-
637 clonal anti-GFP (1/800, 632592, Clontech), anti-Myosin heavy chain (1/10, F59,
638 deposited to the DSHB by Stockdale, F.E.), anti-Myosin light chain 1 slow and 3 fast
639 (1/10, F310, deposited to the DSHB by Stockdale, F.E.), anti-Pax3/7 (1/500, DP312, by

640 courtesy of N. H. Patel), anti-DsRed (1/500, 632496, Clontech), anti-cleaved caspase-3
641 (1/500, 9661, Cell Signaling Technology), anti-phospho Histone H3 (1/500, 06-570,
642 Merck), and anti-ZO-1 (1/50, 33-9100, Invitrogen). For immunostaining of Laminin and
643 Fibronectin, embryos were fixed by 2% PFA for 2 hours at 25°C, then for overnight at
644 4°C. After quenching PFA with 50 mM glycine in PBSTT (PBS/0.5% Triton X-
645 100/0.5% Tween 20) and subsequent washing, embryos were blocked with 5% BSA in
646 PBSTT, and were stained with anti-Laminin (1/100, L9393, Sigma-Aldrich) or anti-
647 Fibronectin (1/100, F3648, Sigma-Aldrich). Secondary antibodies for
648 immunofluorescence were anti-rabbit IgG labelled with Alexa Fluor 488, 555, or 568
649 (A11008, A21429 or A10042, Invitrogen) and anti-mouse IgG labelled with Alexa Fluor
650 488 or 568 (A11001 or A10037, Invitrogen). For counterstaining, Phalloidin (labelled
651 with Rhodamine or Alexa Fluor 647) (R415 or A22287, Invitrogen) and DAPI (4',6-
652 diamidino-2-phenylindole) were added to the secondary antibody solution. Stained
653 embryos were mounted with 1% LMP agarose/PBS (16520-050, Invitrogen) in a glass-
654 based dish (3911-035, Iwaki) and were imaged by LSM710 confocal microscopy (Carl
655 Zeiss). 40× and 25× water-immersion objective lenses (LD C-Apochromat 40x/1.1 W
656 Korr M27 and LD LCI Plan-Apochromat 25x/0.8 Imm Corr DIC M27, Carl Zeiss) and
657 a 5× objective lens (EC Plan-Neofluar 5x/0.16) were used. The contrast adjustment and

658 cropping of captured images were performed by Fiji (version 1.53c; Schindelin et al.,
659 2012).

660 For immunostaining of cross sections in 3 dpf larvae of *charon:EGFP*, fixed
661 specimens were embedded in 4% LMP agarose/PBS, and 100-200 μ m sections were
662 prepared using Vibratome 3000 Tissue Sectioning System. Free-floating sections were
663 stained with appropriate antibodies and mounted with 50% Glycerol/PBS on a slide
664 glass.

665 For DAB staining of EGFP-expressing cells, fixed *charon:EGFP* embryos
666 were blocked with 0.3% H₂O₂ for 30 min and with 2% BSA in PBST for 2 hours,
667 stained with anti-GFP antibody (1/800, 632592, Clontech) and biotinylated anti-rabbit
668 IgG (1/200, BA-1000, Vector Laboratories). VECTASTAIN® Elite® ABC Kit (Vector
669 Laboratories) and DAB tablets (D5905, Sigma-Aldrich) were used for DAB
670 chromogenic reaction. Stained embryos were embedded in Technovit 7100 resin (Kulzer
671 Technique) and 5 μ m sections were obtained using an RM2245 microtome (Leica).
672 Sections were counterstained with Mayer's hematoxylin solution and images were
673 captured by BX61 microscope (Olympus) and AxioCam MRc 5 camera (Carl Zeiss)
674 using a 40 $\times$ objective lens.

675

676 Fluorescence *in situ* hybridization and immunofluorescence (FISH-IF)

677 *charon:EGFP* embryos at stages of interest were processed according to He et al.,

678 (2020) with minor modifications. Briefly, fixed embryos were permeabilized with 1%

679 Triton X-100 in PBS for 1 hour and were hybridized with DIG-labelled probes at 60°C.

680 Chromogenic reaction was performed with either TSA Plus Cyanine 3 Kit (Akoya

681 Biosciences) for *nkx3.1* or ImmPACT Vector Red (Vector Laboratories) for the other

682 genes. Afterward, embryos were subjected to immunostaining against EGFP using anti-

683 GFP antibody (1/500, A11122, Invitrogen).

684

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Figure 1

Figure 4

Figure 7

1 AATTCTCGTAGTCTCTTTTTGTGTTCTCTTGTAAGCACCATGAATTTTTCTCACCATATGTGTCCCTGAGCCACAAAACATCTTAGGTTACATATTTG 100
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TGTTTTCTAAACCCTTATATTAACCTTGTTGATGAATGTGCAATAGAAAAATCTCTTGCTGTGTCAATTTTCATTAAATCTGACATCAGCGTTTCCC
TAAAAATCCAGCTCAAGTGTTAGTTTCTTAACGACATAAATTTGTTCTCGAGGGTCTCCAAGTAACCCATTTTATATATATAAAGAGTCACTGAAGCTG
AAAGAAAAACAGAAAGGTCTCATGTCTACTAAATGTCAATTTAACCC TGCCGGAATACTAACACCACAAATGTAACCTTAAAGGATAATGCTCTAAAAA
ACCAGGTTGGCTTTCAGAACTTACTTTTTCTATGT CAGGAATTGCAAACTTGTA AAAAAATTTGTTTACCATATTTGACATTTTACAAGGACAATAAAGGCA
CGAGGATAGCTGCATCCAGAAATGTTTTCATGACGTTTACTGCTTGGAAAAAATAGAAGCATTTTCTTGTTTCCACTTCCAAGTTCAAAGTGCAGGTT
AGGAGCATGACTGGTGTTATTTTATTTTATTTTATTTTATTTGATCTGATGTACTATTAAGTAAGTGGGACTCTTGATTGCATTATCAAAGTGACCTAAATC
CAGTATAACCATTTCTCTGTTCTTTAAAAATGTTGCCAACATTTATGTTTTTAAAAATTTCTCAAAACAGTTAATAATTTAAGGGTTTT CATTAGAAGATACACT
TGTATAAAAAAACA CAATACACCTAATTTCTAAGAGTACATTTTCTAATTGTAATCTCTAGAAAAATTTCTGTTACTGATCAGTTTTTCTCATTTTAGA
TATTTATTTGATGTAATTTCTACATGT CAGCTGGGCTGTTTAATAATAAGGTTATACAGAAAGCTCAACAGTCTGTGTGATCTTAATAAATTAATGAA
ATAAAGAAATGAACGTGTGTATGACTATCACAATGTGTTAGTGTTCATCGTTTGTA AAAAAAGTGCCCCCTTGTGGAGACAAAAAAGCCTTGCTTCAGAG
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TTTAAATCTGTGCTGCAAAAAGAAATACAGTTCACTAAATAGATGTATCCACTAACAGCTCATAGTTGATGGTTTGCACACACACTTATATGGTAATTCCT
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GTCAGGCTCAAGTCCCCCATTTTAAATTTATAGTTTAA CAGTTTAAATCTAATTTTACATTTTAAATAAATGAAGTTTAAATTTGACCATATCCAAGGG
CTTTGACACATTAACATTTGGCTGGTTTAAATAATGTTGTTTTCATGCTTTTCTTTGAAACAATCACCAAAAATATATCTTCAGTGAAATAATTTTCCTC
AGATTCAAAGTAAATCTTGATCTTTCTACAGCTAACTTTAACAAAGCAGTAAGTCTGGGAAACAGAGGAGTGTAACAAGAAAAAAGAGTCTTTGAA
CACTTTTTGGGTTACACGAGAGTCAAACACTAGTCCCCTTGCAGCATTTGTGAAGCCCGTGTGTGATCATGTGCGGGTGATGGGGCAGAGTGAAGCTCAA
CGGCATGGTGGCCGGGACTTGGAAGCTGCTTGTGGGAATAAATGAACAGGACGCAGCTTGTTTACATAGGAAACATAAACTCATCATTTGCTTTACAATAA
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GCTCTCAGTTTGTCTTCTGCTCTTTTAAACATGGCTTAAAAAACTATTTATAGTAGCTGATCTGTATTAAAGAGGCAATTAAACAGGAAAAACACCAAT
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GGAAGTTTGTAGAAACAAAGAAATAGATTTACCTTT CAGGTATTTTTTTTACGACTGTGTTTACGACACTTACCTGAGACGCACTCTCATCCACACCTA
AACTTTGAAGTTTGGTGATGCTGATCCGGTGTGGGAAATGTGACACTTGTTTGCATTTAAACGCGGGTGAGAACTGACAGCTTGGGCATAAAAAG
GATGTCCTCGGGACAACGCTCAAAGCACTGACAGCAAGGTTGCCGTTTGGAGCCTTGACCACAAC CAGCAGAGAGTTTCAGGATTTCTCATTAGTTTCT
GCATTTATTTCTCATCAAGAC

Supplementary Figure 8