

1 **Wnt/PCP controls spreading of Wnt/ β -catenin signals by cytonemes in**
2 **vertebrates**

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9 **Abstract**

10 Signaling filopodia, termed cytonemes, are dynamic actin-based membrane
11 structures that regulate the exchange of signaling molecules and their receptors
12 within tissues. However, how cytoneme formation is regulated remains unclear. Here,
13 we show that Wnt/PCP autocrine signaling controls the emergence of cytonemes,
14 and that cytonemes subsequently control paracrine Wnt/ β -catenin signal activation.
15 Upon binding of the Wnt family member Wnt8a, the receptor tyrosine kinase Ror2
16 gets activated. Ror2/PCP signaling leads to induction of cytonemes, which mediate
17 transport of Wnt8a to neighboring cells. In the Wnt receiving cells, Wnt8a on
18 cytonemes triggers Wnt/ β -catenin-dependent gene transcription and proliferation. We
19 show that cytoneme-based Wnt transport operates in diverse processes, including
20 zebrafish development, the murine intestinal crypt, and human cancer organoids,
21 demonstrating that Wnt transport by cytonemes and its control via the Ror2 pathway
22 is highly conserved in vertebrates.

23 Introduction

24 Wnt signaling regulates development and tissue homeostasis in multicellular
25 organisms (Nusse and Clevers, 2017), including processes such as cell fate
26 specification, proliferation, morphogenesis, and maintaining tissue integrity.
27 Dysregulation of Wnt/ β -catenin signaling has been causally linked to multiple
28 diseases, with Wnt signaling being one of the most frequently dysregulated pathways
29 in several cancer types (Anastas and Moon, 2013), including colorectal cancers,
30 pancreatic cancer, and gastric cancers (Chiurillo, 2015; Madan and Virshup, 2015).

31 The Wnt signaling network consists of several branches that can be classified
32 according to the receptors involved, and the specific signaling cascades they
33 activate. Two major branches of this network are the β -catenin dependent pathway
34 and the β -catenin independent Wnt/planar cell polarity (PCP) pathway (Niehrs,
35 2012). The β -catenin dependent pathway is triggered by the interaction of Wnt with
36 Frizzled (Fzd) receptors and the co-receptor Lrp6 (Logan and Nusse, 2004). Wnt/ β -
37 catenin signaling regulates expression of target genes such as *axin2* and *lef1*, and
38 tissue specific genes, and subsequently controls cell proliferation, as well as tissue
39 patterning. In the β -catenin-independent Wnt/PCP pathway (Yang and Mlodzik,
40 2015), Wnt proteins bind to Frizzled and co-receptors such as the receptor-tyrosine
41 kinase-like orphan receptor 2 (Ror2) to regulate cytoskeleton organization by actin
42 polymerization and cell polarity (Grumolato et al., 2010; Ho et al., 2012; Oishi et al.,
43 2003). To this end, small GTPases Rho, Rac1, and Cdc42 are regulated to control
44 formation of filopodia and lamellipodia, cell motility and morphogenetic movements in
45 vertebrates. Although the PCP pathway and β -catenin signaling generally act in a
46 mutually repressive fashion, by competing for similar hub proteins, such as the
47 effector protein Dishevelled (Dvl) (van Amerongen and Nusse, 2009), recent
48 evidence suggests that PCP signaling can act – dependent on the context – either in
49 opposition to, in concert with, or independently of β -catenin signaling.

50 The production and secretion of Wnt ligands requires lipid modification by the
51 acyltransferase Porcupine (Porcn) followed by binding to Evi/Wls, which serves as a
52 Wnt chaperone and facilitates its transport from the endoplasmic reticulum to the
53 plasma membrane (Bartscherer and Boutros, 2008; Bänziger et al., 2006; Yu et al.,
54 2014). From there, lipophilic Wnt is transported through the neighboring tissue to
55 exert its long-range signaling activity. Extracellular binding proteins have been
56 suggested to increase the solubility of Wg/Wnt in the aqueous extracellular space

57 and facilitate this activity (Mii et al., 2009; Mulligan et al., 2012). However, other
58 studies point to membrane-associated mechanisms of Wg/Wnt delivery, without
59 compromising its signaling capability (McGough and Vincent, 2016; Port and Basler,
60 2010; Stanganello and Scholpp, 2016). These trafficking routes include Wg/Wnt
61 protein distribution on the plasma membrane of dividing source cells (Alexandre et
62 al., 2014; Farin et al., 2016) and on actively migrating cells (Serralbo and Marcelle,
63 2014), or the dissemination of Wg/Wnt proteins on exovesicles (Panáková et al.,
64 2005), or more specifically exosomes (Beckett et al., 2013; Gross et al., 2012; Korkut
65 et al., 2009). Wg/Wnt proteins and their receptors are also transported on cell
66 protrusions in various tissues. Lipid-modified Wnt proteins were found at the cell
67 membrane on signaling filopodia – so-called cytonemes – in *Xenopus* and zebrafish
68 (Holzer et al., 2012; Luz et al., 2014; Stanganello et al., 2015), whereas Fzd receptor
69 proteins can be localized to filopodia in *Drosophila* and chicken (Huang and
70 Kornberg, 2016; Sagar et al., 2015). In zebrafish, an analysis of cytonemes
71 demonstrates that these are specialized filopodia, with stabilizing actin bundles at
72 their cores, which serve as a main transport device for the β -catenin ligand Wnt8a
73 during neural plate patterning (Stanganello et al., 2015). Wnt8a is loaded on
74 cytoneme tips and transferred to the neighboring cells by direct cell-cell contact. At
75 the contact sites, Wnt8a cytonemes induce Lrp6/Fzd receptor clustering into the Lrp6
76 signalosome to activate β -catenin signaling. Although the lengths and number of
77 cytonemes is crucial for the β -catenin signaling range during embryogenesis
78 (Stanganello et al., 2015), it is yet unclear what mechanism controls the formation of
79 Wnt cytonemes in a tissue.

80 Here we show that Wnt8a can activate both the PCP pathway by interaction with
81 Ror2 and the β -catenin pathway by interaction with Lrp6. This dual function allows
82 Wnt8a to control its own route of dissemination: In the source cells, Wnt8a binds and
83 activates the Ror2 co-receptor followed by activation of the PCP pathway. Wnt8a-
84 PCP influences convergent extension (CE) movement and activates the small
85 GTPase Cdc42, which leads to the outgrowth of signaling filopodia. Wnt8a is loaded
86 onto these cytonemes, and is transported through the tissue to bind to the β -catenin
87 specific co-receptor Lrp6 in the responding cells to activate the β -catenin pathway in
88 a paracrine fashion in both PAC2 fish fibroblasts and HEK293T human embryonic
89 kidney cells. Activation of the β -catenin pathway by Wnt cytonemes leads to target
90 gene induction and regulates zebrafish neural plate patterning. Ror2-mediated Wnt

91 cytonemes also regulate the proliferation of human gastric cancer cells. Furthermore,
92 we show that Wnt cytonemes induced by Ror2/PCP signaling are required for the
93 maintenance of murine intestinal crypt organoids. We conclude that Ror2-regulated
94 cytonemes are a critical transport route for Wnt proteins in vertebrates. The
95 molecular mechanism for Wnt cytoneme formation illustrates the co-dependent
96 interactions of the different branches of the Wnt signaling pathways.

97 **Results**

98 **Tyrosine kinase Ror2 regulates filopodia emergence *in vitro***

99 Cumulative evidence indicates that Wnt signal molecules are lipidated and remain
100 associated with membranes during secretion, action and degradation (Nusse and
101 Clevers, 2017). Our previous work demonstrated that Wnt molecules can be
102 distributed over 100 μm through a tissue via cytonemes (Stanganello et al., 2015).
103 Manipulation of the length or number of Wnt cytonemes led to alterations in Wnt
104 mediated tissue patterning and malformations of the zebrafish embryo. Therefore, we
105 hypothesized that formation, emergence, and maintenance of cytonemes are tightly
106 controlled. To identify potential cytoneme regulators, we performed a cell-culture-
107 based genetic screen (Fig. 1A, Suppl. Fig. 4A-J) by co-expressing Wnt8a-GFP and
108 GPI-anchored, membrane-bound mCherry (memCherry) in PAC2 cells together with
109 arrayed cDNA clones from a Medaka cDNA library consisting of 229 kinases (Chen
110 et al., 2014; Souren et al., 2009). 24 h post transfection, we quantified the length and
111 number of signaling filopodia of ten fibroblasts per cDNA using automated filopodia
112 detection software (Fig. 1B). The tyrosine-protein kinase transmembrane receptor
113 Ror2 was found to stimulate both filopodia number per cell as well as average
114 filopodia length above the 85th percentile (Fig. 1C,D).

115 To validate the screening results we co-transfected PAC2 fibroblasts with a
116 zebrafish full-length Ror2 expression construct and GPI-memCherry as a membrane
117 marker. The number and length of filopodia were measured in living cells (Fig. 1E).
118 Ror2 expression significantly increased the average number and length of filopodia
119 per cell (Fig.1F, Suppl. Fig. 1A,B). Ror2 requires homodimerization for
120 transautophosphorylation and subsequent downstream signaling (Liu et al., 2007),
121 which can be inhibited by over-expressing a kinase-dead construct. Transfection of
122 the dominant-negative mutant Ror2³¹ (Hikasa et al., 2002), caused a reduction in the
123 number of protrusions as well as their average length, consistent with an essential

124 role of the Ror2 kinase activity in filopodia induction in PAC2 fibroblasts. The Rho
125 GTPase Cdc42 is crucial for organizing the actin cytoskeleton to stabilize Wnt
126 cytonemes (Stanganello et al., 2015) and is thought to be a downstream target of the
127 Wnt/Ror2 pathway regulating filopodia (Schambony and Wedlich, 2007). To
128 determine if Ror2-induced filopodia require Cdc42 function for assembling an actin
129 scaffold we co-transfected Ror2 stimulated fibroblasts with dominant-negative
130 Cdc42^{T17N} (Nalbant et al., 2004). Blockage of Cdc42 function reduced filopodia
131 formation significantly (Fig. 1E,F).

132 BAR-domain containing proteins mold membranes into tube-like filopodia. Insulin
133 receptor tyrosine kinase substrate p53, IRSp53, is a BAR protein, as well as a Cdc42
134 effector, which connects filopodia initiation and maintenance by assembling the actin
135 scaffold (Yeh et al., 1996). IRSp53^{4K} contains four lysine residues mutated to
136 glutamic acid in the actin-binding sites, inhibiting Cdc42-mediated filopodia formation
137 (Disanza et al., 2013; Kast et al., 2014). IRSp53^{4K} expression, like Cdc42^{T17N}
138 transfection, blocks Ror2-induced filopodia formation (Fig. 1E,F). Treatment of Ror2
139 expressing cells with ML141, a GTPase inhibitor for Cdc42/Rac1 (Surviladze et al.,
140 2010), also caused a substantial reduction in both number and length of filopodia.
141 Thus, Ror2 is a crucial regulator of filopodia in PAC2 fibroblasts, and filopodia
142 depend on a Cdc42-mediated actin scaffold for their outgrowth and maintenance.

143 **Cluster formation of Wnt8a and Ror2 is dependent on the CRD domain**

144 Ror2 is a tyrosine kinase receptor that binds Wnt5a via its extracellular cysteine-
145 rich domain (CRD) (Hikasa et al., 2002) and serves as a β -catenin independent Wnt
146 co-receptor activating the PCP signaling pathway (Schambony and Wedlich, 2007).
147 To investigate the interaction between Ror2 and the β -catenin ligand Wnt8a, we
148 expressed fluorescently tagged Wnt8a and Ror2 proteins in the zebrafish embryo
149 during gastrulation. The mRNA concentration of the injected fluorescent constructs
150 was chosen in such a way that it did not induce phenotypic alteration at 24h (Suppl.
151 Fig. 3A). Zebrafish *ror2* is expressed ubiquitously in early development, with its
152 expression peaking during gastrulation between 2 and 9 hpf (Bai et al., 2014).
153 Expression of the β -catenin Wnt ligand *wnt8a* is confined to the embryonic margin
154 during zebrafish gastrulation, orchestrating patterning of the prospective neural plate
155 (Kelly et al., 1995; Rhinn et al., 2005). Confocal microscopy on live specimens
156 revealed that Wnt8a-GFP displays a punctate pattern in the cytoplasm and at the
157 membrane, including cytoneme tips, whereas Ror2-mCherry without Wnt present is

158 uniformly distributed in the cell membrane (Fig. 2A, Suppl. Fig. 2A). When Wnt8a-
159 GFP and Ror2-mCherry are co-expressed in the same cell, Ror2-mCherry
160 accumulates in punctae along the membrane (Fig. 2A, Suppl. Movie 1). Correlated
161 fluorescence intensity analysis of Wnt8a-GFP and Ror2-mCherry suggest both
162 proteins predominantly co-localize in membrane associated clusters (Fig. 2B). This is
163 supported by a Pearson-based correlation analysis of Wnt8a-GFP and Ror2-mCherry
164 in embryonic tissue of a volume of $40 \times 40 \times 60 \mu\text{m}^3$ (N=8, Suppl Fig. 2G). We found
165 a similar intensity correlation between Wnt5b and Ror2 (Suppl. Fig. 2B,C). The CRD
166 of Ror2 is crucial for interaction with Wnt ligands (Hikasa et al., 2002; Mikels et al.,
167 2009). To exclude non-specific clustering of fluorescent fusion proteins, we used a
168 Ror2 construct with a deletion in the Fzd-like CRD. We observed that Ror2- Δ CRD-
169 GFP still localizes to the cell membrane and with Wnt8a-mCherry forming clusters
170 therein. However, image profile analysis showed a marked reduction of the intensity
171 peaks at the cluster site, indicating that Ror2-CRD is required for the interaction with
172 Wnt8a (Fig. 2B).

173 **Wnt8a and Ror2 co-migration and protein binding in signaling clusters**

174 We further characterized Wnt8a/Ror2 protein-protein interactions *in vivo* using
175 line-scanning fluorescence correlation spectroscopy (lsFCS; Suppl. Fig. 2D), which
176 measures concentrations and diffusion coefficients of ligands and receptors in the
177 presence of a membrane (Dörlich et al., 2015). We performed lsFCS analysis in two
178 different spots, at a Ror2 positive membrane domain (spot 1) or at a Wnt8a/Ror2
179 membrane cluster (spot 2; Fig. 2C). A focused laser spot was scanned across the
180 membrane for 390 s while the intensity was measured as a function of time. After
181 compensation for membrane fluctuations, the intensity time traces were time-
182 correlated. In spot 1, we found intensity fluctuation from Ror2-mCherry emission (Fig.
183 2D). A fit of the autocorrelation function revealed a receptor concentration (area
184 density) $C_r = (37 \pm 3) \mu\text{m}^{-2}$. The diffusion coefficient, $D = (0.28 \pm 0.03) \mu\text{m}^2 \text{s}^{-1}$, is
185 similar to values found for LRP6 receptors in the plasma membrane (Dörlich et al.,
186 2015). There was no clear emission from Wnt8a-GFP molecules in spot 1. By
187 contrast, lsFCS on spot 2 revealed clear autocorrelations in both color channels (Fig.
188 2E), indicating the presence of both Wnt8a-GFP and Ror2-mCherry at this site. We
189 found a dual-color cross-correlation between Wnt8a-GFP and Ror2-mCherry (Fig.
190 2E), indicating concerted intensity fluctuations in both color channels, which arise
191 from their co-diffusion in the plasma membrane due to binding. Therefore, the cross-

192 correlation IsFCS data provide clear evidence of complex formation between Wnt8a
193 and Ror2. Furthermore, the low diffusion coefficient of the bound species, $D = (0.02 \pm$
194 $0.01) \mu\text{m}^2 \text{s}^{-1}$, indicates that the complexes diffuse as large clusters.

195 We have previously shown that Fzd7 also interacts with Ror2 and enhances
196 Ror2-mediated signaling during *Xenopus* gastrulation (Brinkmann et al., 2016),
197 suggesting Fzd7a could be a part of the Wnt8a/Ror2 cluster in zebrafish. To test this,
198 we overexpressed Ror2-mCherry, Fzd7a-CFP and Wnt8a-GFP and found co-
199 localization of all three proteins in the cell membrane (Suppl. Fig. 2E,F).

200 From our data, we conclude that Wnt8a interacts with Ror2 by binding to its CRD.
201 Wnt8a and Ror2 co-migrate and form dense protein clusters. The data suggest that
202 the Wnt8a/Ror2 clusters are in close steric contact with other components of the Wnt
203 signaling complex including Fzd7a.

204 **Wnt8a/Ror2 signaling activates the PCP pathway**

205 The interaction of Wnt8a with Ror2 could trigger non-canonical PCP signaling via
206 the Ror2 pathway. PCP signaling plays a role in regulating tissue migration during
207 gastrulation (Tada and Heisenberg, 2012). PCP signaling via Ror2 activation
208 regulates collective cell migration towards the embryonic midline, which is most
209 pronounced in the mesodermal germ layer in zebrafish (Bai et al., 2014). We utilized
210 this classical PCP controlled process to observe the involvement of Wnt8/Ror2 in
211 non-canonical signaling. CE can be visualized by condensation of the *no tail* (*ntl*)
212 positive notochordal plate at the embryonic midline at 11 hpf (Fig. 3A). To this end,
213 we over-expressed the Ror2 receptor, which alone had a very small effect on the
214 establishment of the *ntl* expression domain (for classification see Suppl. Fig. 3B).
215 However, over-expression of Wnt8a leads to a broadening and shortening of the *ntl*
216 expression domain. This phenotype is reminiscent of Wnt5b activation. A similar
217 phenotype was observed when Wnt8a and Ror2 were co-expressed. Categorization
218 of the phenotypes suggests that the co-activation of Ror2/Wnt8a or Ror2/Wnt5b have
219 similar effects (Fig. 3B). Inhibition of Ror2 function by either Ror2^{3l} expression or a
220 Morpholino-based Ror2 knock-down also led to CE defects. Our data suggest that
221 Fzd7a may be a member of the Ror2-Wnt8a signaling complex (Suppl. Fig. 2E,F).
222 We found an enhanced broadening of the embryonic midline if Wnt8/Fzd7a and
223 Wnt8a/Fzd7a/Ror2 were overexpressed (Suppl. Fig. 3C). This suggests that
224 endogenous Ror2 is expressed at high levels already, and the available Wnt ligand

225 concentration is the key quantity controlling step for PCP signaling during zebrafish
226 CE.

227 During CE, cells intercalate in the notochordal plate (convergence), push
228 previously adjacent cells apart, and lengthen the field along the AP axis (extension)
229 (Glickman et al., 2003). We investigated the shape of the notochord cells in embryos
230 with ectopically expressing Wnt8/Ror2 signaling. We found that the cells had a less
231 bipolar shape and displayed a more circular form in embryos with Wnt8/Ror2
232 signaling, reminiscent of Ror2 activation by Wnt5a (Figure 3C,D), suggesting that
233 mediolateral narrowing of axial mesoderm is reduced.

234 Activation and inhibition of the PCP signaling pathway leads to a similar
235 phenotype (Tada and Heisenberg, 2012). Therefore, we were unable to distinguish
236 how Wnt8a/Ror2 alters PCP signaling. During *Xenopus* gastrulation, Wnt5A activates
237 Ror2 downstream signaling, leading to Cdc42 activation, JNK phosphorylation, and,
238 ultimately, the enhancement of ATF2 transcription (Hikasa et al., 2002; Schambony
239 and Wedlich, 2007). To test if zebrafish Wnt8a is able to activate Ror2 signaling, we
240 used a reporter assay with an ATF2 responsive element driving luciferase expression
241 in *Xenopus* embryos (Brinkmann et al., 2016; Ohkawara and Niehrs, 2011). Wnt8a
242 co-expressed with Ror2 produced a greater than five-fold induction of the ATF2
243 reporter in *Xenopus* (Fig. 3E). Co-expression of Wnt5A/Ror2 leads to a similar
244 activation of reporter expression, whereas expressed Ror2 without a co-expressed
245 ligand did not alter expression of the ATF2 reporter. We determined if the kinase
246 domain of Ror2 is required for Wnt8a dependent activation of the PCP pathway by
247 overexpressing Wnt8a together with dominant-negative Ror2³¹ and observed a
248 reduction of ATF2 reporter activation compared to activation of Wnt8a/Ror2.

249 Taken together, our data indicate that Wnt8a serves as a ligand for the receptor
250 Ror2 and induces PCP signaling upon binding. Thus, ectopic over-expression of
251 Wnt8a modulates cell movements and cell morphology in zebrafish and gene
252 transcription in *Xenopus*.

253 **Ror2 induces Wnt-cytosomes during zebrafish neural patterning.**

254 To study the dynamics of filopodia formation in the Wnt8a positive germ ring
255 during normal development in zebrafish (Suppl. Movie 2), we quantified signaling
256 filopodia during gastrulation in live embryos. In confocal image stacks, filopodia were
257 traced using a semi-automatic live wire approach (Barrett and Mortensen, 1997). A

258 precise measurement of filopodia protrusion lengths in 3D was obtained using
259 manually selected nucleation start points and filopodia end points. We found that the
260 number of filopodia significantly increase from 4 – 6hpf, which comprises the neural
261 plate patterning phase (Fig. 4A,B). This coincides with increasing Ror2 expression
262 levels during zebrafish development (Bai et al., 2014). We determined if formation of
263 these filopodia was dependent on Ror2 function by manipulating Ror2 signaling and
264 measured germ ring cell filopodia number at 6 hpf. We found only a modest increase
265 of filopodia number if Ror2 was activated, suggesting Ror2 itself is not rate-limiting
266 (Fig. 4C,D). However, when Ror2 function was reduced by over-expression of Ror2^{3l},
267 we observed a significant reduction in filopodia number. We conclude that Ror2
268 signaling is required for filopodia induction of embryonic marginal cells during
269 zebrafish development.

270 We speculated that Ror2 might influence formation of filopodia carrying Wnt8a
271 protein - Wnt8a-cytonemes - during zebrafish gastrulation. To visualize these
272 cytonemes we generated cell clones at the embryonic margin expressing Wnt8a-
273 GFP and memCherry. Wnt8a-GFP clusters were seen in the cell membrane and
274 cytoneme tips of germ ring cells (Fig. 4E). Statistically more filopodia carrying Wnt8a-
275 GFP clusters on their tips were detected upon Ror2 overexpression within cells (Fig.
276 4E,F). Conversely, we found a significant reduction in the number of cytonemes in
277 Ror2 deficient marginal cells. Wnt8a-GFP is still present at the plasma membranes of
278 cells with compromised Ror2 function, suggesting that intracellular routing of Wnt8a
279 from the producing organelles to the cell membrane is independent of Ror2-
280 dependent cytonemal transport. Filopodia without detectable Wnt8a seemed to be
281 unaffected by Ror2 signaling in zebrafish (Fig. 4F). This suggests a function of Ror2
282 in regulating a specific subset of filopodia, those carrying Wnt8a - the Wnt cytonemes
283 - in the zebrafish embryo *in vivo*.

284 Based on these findings, we hypothesized that Ror2 function in the Wnt source
285 cells is crucial for Wnt dissemination via cytonemes. To test the consequences of
286 altered Ror2 signaling quantitatively, we used a simulation of morphogen distribution
287 via cytonemes (Stanganello et al., 2015). The simulation takes into account ligand
288 transport by cytonemes, ligand decay and migration of epiblast cells using a Monte
289 Carlo based direct event simulation approach. Employing cytonemes as the
290 exclusive transport mechanism from the producing cell group to the target cell group,
291 and assuming an unlimited source of the ligand, we found that cytonemes can

292 distribute Wnt8a in a graded manner in the dynamically evolving target tissue (Fig.
293 4G). We tested two scenarios with varying cytoneme number, based on our *in vivo*
294 measurements after alteration of Ror2 function (Fig. 4F). We found that ligand
295 concentration within the morphogenetic field depends on the appearance of the
296 cytonemes (Stanganello et al., 2015). We found that increasing the number of
297 cytonemes per cell (by the experimentally determined factor of 1.61) after expression
298 of Ror2 and Wnt8a in the source cells, leads to an enhanced ejection (187%) of the
299 ligand into the target tissue compared to the Wnt8a producing cells (Fig. 4G).
300 Consistently, by using the measured cytoneme parameters after blockage of Ror2
301 function (number scaled by 0.388), we found a decrease to 36% of the Wnt8a
302 concentration compared to the WT situation and a smaller range of the morphogen
303 gradient within the tissue. These data suggest that Ror2 signaling can specifically
304 regulates the number of Wnt positive cytonemes *in vivo* and thus represents a
305 cytoneme specific regulator. Furthermore, based on the simulations, we predicted a
306 strong decrease in the range of Wnt signal activation in the neighboring tissue if Ror2
307 function is compromised in the Wnt8a source cells.

308 **Ror2 presents Wnt8a to the target cell to induce ligand-receptor cluster.**

309 Next we asked how Wnt8a and Ror2 interact to facilitated cytoneme-mediated
310 transport. Therefore, we performed a high-resolution imaging approach in the
311 developing zebrafish embryo by over-expressing tagged constructs (Fig. 5). The
312 high-sensitivity of the image-based approach allowed us to reduce the expression
313 levels of the tagged construct significantly and morphological alterations of the
314 embryonic phenotype was not observed at 24hpf (Suppl. Fig. 3A). By a time-lapse
315 analysis, we observe the formation of Wnt8a-GFP positive clusters, which transit in
316 the plasma membrane of the secreting cell (Fig. 5B). Then, Wnt8a co-localizes with
317 Ror2 at the plasma membrane suggesting Wnt8a-Ror2 cluster induction (Fig. 5C) as
318 previously described (Fig. 2A,B). These clusters initiate cytoneme formation and,
319 consequently, they decorate the tip of the outgrowing cytoneme. The cytoneme
320 contact the target cell and Wnt8a-Ror2 forms a cluster at the receiving cell (Fig. 5C,
321 Suppl. Movie 3). Within minutes, this clusters is endocytosed into the target cell. We
322 were wondering if Wnt8a-Ror2 induces the Wnt signaling cascade in the target cell.
323 Therefore, we analyzed Lrp6-signalosome formation at the plasma membrane of the
324 target cell by induction of a Wnt8a secreting cell clone and aLrp6-expressing
325 receiving cell clone (Fig. 5D). We find that cytonemal Wnt8a-mCherry induces Lrp6-

326 GFP cluster formation at the membrane of the target (Fig. 5E,F). We hypothesized
327 that the source cell presents Wnt8a by clustering the ligand on Ror2 positive
328 cytonemes. Indeed, we observe a Lrp6-GFP cluster that at the contact points of
329 Ror2-positive cytonemes (Fig. 5E). The following dynamics of Lrp6-signalosome has
330 been described recently in zebrafish development (Hagemann et al., 2014).

331 Therefore, we conclude that Ror2 clusters on cytoneme tips to act as a platform
332 to present Wnt8a to the target cell and induce the Wnt signaling cascade therein.

333 **Ror2 regulates PCP signaling in the Wnt source cells and β -catenin signaling** 334 **in the Wnt receiving cells**

335 Based on our simulations, we speculated that Ror2 signaling may have a function
336 in Wnt ligand trafficking and, consequently, in paracrine β -catenin signaling during
337 zebrafish gastrulation. To test this, we over-expressed Ror2 and analyzed its effect
338 on CE processes (via PCP signaling) and, simultaneously, on neural plate patterning
339 (via β -catenin signaling; Suppl. Fig. 6A) during embryogenesis. Over-expression of
340 Ror2 by injection of low levels of mRNA did not induce gross morphological changes
341 in zebrafish embryos, consistent with our findings that Ror2 without a suitable ligand
342 only mildly impacts on PCP mediated processes (Fig. 3A,C,E). Over-expression of
343 Wnt8a resulted in a substantial alteration in neural plate patterning as described
344 above, with β -catenin signaling being activated in the entire neural plate, marked by
345 ubiquitous *axin2* expression at 6 hpf (Fig. 6A). As a consequence, we observed
346 posteriorization of the developing nervous system, observed as an anterior shift of
347 the *fgf8a* positive midbrain-hindbrain boundary (MHB) at 9 hpf and a loss of the
348 anterior *pax6a* positive forebrain at 26 hpf. In embryos co-expressing Wnt8a together
349 with Ror2, we still observed the posteriorization phenotype in the neural plate and, in
350 addition, we found that CE is compromised, as the neural plate does not converge to
351 the midline and, consequently, the expression domains of *fgf8a* at the MHB showed
352 a pronounced gap.

353 We compared these observation to embryos expressing the β -catenin
354 independent ligand Wnt5a, and Wnt5a together with Ror2. Ror2 mediated Wnt5a
355 signaling induces CE in *Xenopus* (Hikasa et al., 2002) and represses β -catenin
356 signaling in mouse embryos (Mikels et al., 2009). In both settings, we observed a
357 strong effect on CE movement in the zebrafish embryo. In addition, Wnt5a/Ror2
358 over-expression led to reduced β -catenin signaling, causing a reduction in target
359 gene expression (*axin2*) and anteriorization of the neural plate, leading to a posterior

360 shift of *pax6a* expression in the forebrain. We conclude that Wnt8a can activate β -
361 catenin signaling and PCP signaling via the Ror2 receptor during zebrafish
362 development. We showed that Wnt5b/Ror2-mediated PCP signaling represses
363 Wnt/ β -catenin signaling. We hypothesized that Wnt8a function depends on the route
364 of secretion. However, global over-expression did not differentiate between autocrine
365 and paracrine Wnt8a signaling mechanisms and on subsequent downstream
366 activation.

367 To separate Wnt-producing from Wnt-receiving cells, we performed a co-
368 cultivation assay using HEK293T cells, which are typically Wnt-Off due to low
369 endogenous expression of Wnt ligands (Voloshanenko et al., 2017). Cytoneme
370 regulators were transfected into HEK293T source cells (Wnt-producing cells) and co-
371 cultivated with HEK293T cells expressing the SuperTOPFlash TCF/Wnt reporter,
372 with seven TCF responsive elements hooked up to nuclear mCherry (7xTRE-NLS-
373 mCherry (Moro et al., 2012), Suppl. Fig. 6B). Ror2 transfection into source cells did
374 not alter the induction of 7xTRE-nucRFP in the receiving cells (Fig. 6B,C). However,
375 Wnt8a-producing cells lead to activation of signaling activity in the HEK293T reporter
376 cells. Reporter expression was further enhanced (147.3% compared to Wnt8a
377 transfected source cells) when Wnt-producing cells co-expressed Wnt8a and Ror2,
378 indicating a synergistic interaction between Wnt8a and Ror2 (Fig. 6D). Co-
379 transfection of Wnt8a with dominant-negative Ror2³¹ resulted in a 34.6% decrease in
380 reporter activation, compared to Wnt8a transfected source cells. These findings
381 support our simulations regarding the available Wnt8a concentration in the target
382 tissue after alteration of Ror2-dependent cytoneme appearance (Fig. 4G). We
383 conclude that Wnt8a can be transmitted via Ror2 dependent cytonemes in HEK293T
384 cells, while Wnt5b and Wnt5b/Ror2 transfections were unable to activate the β -
385 catenin signaling reporter in neighboring cells (Fig. 6D).

386 To test whether Ror2-mediated Wnt cytonemes affect β -catenin dependent target
387 gene activation in neighboring cells *in vivo*, we generated small-source clones by
388 microinjecting cytoneme regulator mRNAs at the 16-cell stage (Suppl. Fig. 6C). By
389 mid-gastrulation, the source cells were distributed over an area of the embryo and
390 intermingled with WT host cells, generating many responding cells around a few
391 source cells. At 6 hpf, we analyzed the transcriptional profile of the embryos for the β -
392 catenin target genes *axin2* and *lef1*. Embryos with cells over-expressing Ror2 or
393 Ror2³¹ showed no alteration in *axin2* or *lef1* expression (Fig. 6D). However, source

394 cells over-expressing Wnt8a resulted in a significant increase in β -catenin dependent
395 target gene expression, which was not further enhanced by Ror2 addition. However,
396 blockage of cytoneme formation in the Wnt8a source cells by co-expression of Ror2^{3l}
397 led to a significant reduction of β -catenin target gene induction in neighboring cells.
398 Blockage of filopodia *per se* by over-expression of the dominant-negative form of
399 IRSp53^{4K} caused a similar reduction of activation of *axin2* and *lef1* expression in
400 embryos. This suggests that, during zebrafish gastrulation, the majority of Wnt8a
401 protein is transmitted via cytonemes and that the formation of these Wnt cytonemes
402 is Ror2 dependent.

403 **Ror2-dependent cytonemes in cancer cell lines and intestinal organoids**

404 Over-activation of canonical β -catenin signaling can be identified in one-third of
405 gastric cancers (Chiurillo, 2015). β -catenin signaling is essential for self-renewal of
406 gastric cancer stem cells, leading to Wnt-mediated resistance to apoptosis, which
407 may be responsible for recurrences in these tumors. The β -catenin-independent
408 branch plays a similarly important role in cancer progression: The key ligands Wnt5a
409 and Ror2 are upregulated in various gastric cancers, regardless of the histological
410 phenotype. To determine if Wnt ligands are transported on cytonemes between
411 gastric cancer cells, we used the gastric cancer (GC) cells lines MKN7, MKN28 and
412 AGS. Transfected Wnt8a-mCherry localized on filopodia in GC cell lines (Fig. 7A,
413 Suppl. Fig. 7). Forced expression of Ror2 led to a strong increase of the number of
414 filopodia in GC cells (Fig. 7B,C), whereas, there was a significant reduction of the
415 cumulative filopodia length in cells expressing dominant-negative Ror2^{3l} (Fig. 7C),
416 indicating that Ror2 also control filopodia formation in GC cells.

417 We focused on AGS cells as they show highly dynamic formation and retraction
418 of filopodia, are particularly receptive to Ror2 manipulation, express Wnt1 at constant
419 high levels, and, thus, have high endogenous β -catenin activity, which has been
420 linked to the increased proliferation rate of this cell line (Mao et al., 2014). To assess
421 whether cytoneme-mediated Wnt transport influences AGS cell behavior, and
422 specifically proliferation, we co-cultivated Ror2 transfected cells with cells carrying
423 the nuclear marker nucRFP. Cells over-expressing Ror2 significantly increase cell
424 proliferation in neighboring AGS cells (Fig. 7D,E). Co-expression of the specific
425 filopodia inhibitor IRSp53^{4K} dampened the increased proliferation rate induced by
426 Ror2 expression. Inhibition of Wnt signaling by the tankyrase inhibitor IWR1
427 abrogated the stimulatory effect of Ror2 expression, confirming that the Ror2 effect is

428 due to increased Wnt signaling. We conclude that Wnt is moved on cytonemes
429 between GC cells to stimulate Wnt/ β -catenin signaling and proliferation in
430 neighboring cells. Abrogation of this transport route has a similar consequence as
431 inhibition of Wnt signaling *per se* – it leads to reduced proliferation.

432 As the intestinal crypt requires a constant supply of Wnt signaling for tissue
433 homeostasis (Beumer and Clevers, 2016; Kuhnert et al., 2004; Pinto et al., 2003;
434 Sailaja et al., 2016) we asked if Wnt cytonemes operate in the mouse intestinal crypt.
435 *In vivo*, subepithelial myofibroblasts provide the major source of physiologically
436 relevant Wnts, which maintain the crypt *in vivo* (Kabiri et al., 2014; Valenta et al.,
437 2016). It has been further demonstrated that these Pdgfr α positive myofibroblasts
438 regulate the intestinal stem-cell niche by Wnts and RSPO3 (Greicius et al., 2018).
439 The intestinal myofibroblasts form a large amount of filopodia (Fig. 7F). The
440 formation of filopodia is inhibited by siRNA-mediated knock-down of Ror2. We used
441 an organoid formation assay to analyze the requirement of Wnt signaling filopodia in
442 the intestinal crypt. We used Wnt deficient Porcn^{-/-} crypt cells, co-cultivating them
443 with Wnt3a-secreting L cells to grow Wnt-deficient crypt organoids. Co-culture of WT
444 myofibroblasts with Wnt-deficient crypt cells leads to induction and maintenance of
445 crypt organoids (Kabiri et al., 2014). These myofibroblasts extend filopodia to engulf
446 crypt organoids (Suppl. Fig. 7C). If Ror2 is knocked down in the Wnt-producing
447 myofibroblasts we observed a substantial decrease in the number of organoids (Fig.
448 7G). This suggests that Wnt signaling transport on Ror2-dependent cytonemes from
449 the myofibroblasts is crucial for induction and maintenance of the intestinal crypt. We
450 conclude that cytonemes are vital for Wnt protein dissemination in vertebrates and
451 their appearance is regulated by Ror2-mediated PCP signaling.

452 **Discussion**

453 **Cytonemes transport signaling molecules**

454 Cytonemes are actin-based filopodia that transport an array of signaling
455 molecules and their receptors, facilitating juxtacrine signaling. Their appearance is
456 highly dynamic during development (Gradilla and Guerrero, 2013; Kornberg and Roy,
457 2014; Stanganello and Scholpp, 2016). A continuous adjustment of the number,
458 length, rate of formation, direction, and retraction of cytonemes is crucial to regulate
459 exchange of signaling proteins within a tissue. However, how cytoneme formation is
460 controlled remains unclear. Cytonemes change in response to signaling protein

461 levels in the source cell (Sato and Kornberg, 2002; Stanganello et al., 2015)
462 suggesting that cytoneme formation is linked to signal production. A signaling
463 pathway influencing cytoneme emergence is the Wnt signaling network. We have
464 found that cytoneme initiation is linked to Wnt8a production. Wnt8a activates the
465 PCP signaling pathway in ligand-producing cells and, in turn, PCP induces
466 cytonemes, which can be loaded with Wnt protein. Data from *Drosophila* show that
467 cytonemes require the PCP components Prickle and Vangl, which are essential for
468 Fgf and Dpp positive cytonemes in the air sac primordium (Huang and Kornberg,
469 2016). However, Prickle/Vangl modulate heparin proteoglycan content in the
470 extracellular matrix to allow cytoneme outgrowth. In contrast, we found that Wnt8a
471 activates the PCP pathway via interaction with the Ror2 receptor to initiate cytoneme
472 formation. Our *in vivo* analysis suggests that activation of Ror2 in the zebrafish
473 embryonic margin is necessary and sufficient for formation of Wnt cytonemes, as
474 other filopodia remain unperturbed. We conclude that Ror2 is a specific cytoneme
475 regulator.

476 **Ror2 in filopodia formation**

477 Ror2 is a member of the family of orphan receptor tyrosine kinases, possessing
478 an extracellular Fzd-like CRD, a cytoplasmic tyrosine kinase domain, and a proline-
479 rich domain (PRD) (Yoda et al., 2003). Ror2 plays crucial roles in developmental
480 morphogenesis in vertebrates. Ror2-deficient mice exhibit skeletal, genital, and
481 cardiovascular abnormalities caused by disrupted CE movements during gastrulation
482 (Oishi et al., 2003; Takeuchi et al., 2000). Ror2 is required for filopodia formation and
483 XWnt5a-induced cell migration in *Xenopus* (Nishita et al., 2006). Irrespective of
484 stimulation by Wnt proteins, ectopic expression of Ror2 can induce filopodia
485 formation by actin polymerization via coupling of the Ror2-PRD to the actin-binding
486 protein Filamin A. Our *in vitro* data show that Ror2 induces filopodia formation in
487 PAC2 fibroblasts, myofibroblasts and gastric cancer cells. Moreover, we show that
488 Ror2 is crucial for Wnt cytoneme formation *in vivo*, and it is known to contribute to
489 cytoskeleton remodeling and JNK activation in migrating cells (Oishi et al., 2003). We
490 have previously shown that Wnt cytonemes require Cdc42 function to stabilize the
491 intracytonemal actin skeleton (Stanganello et al., 2015). Here we demonstrate that
492 Ror2-dependent cytonemes also require Cdc42 function for formation and
493 maintenance of the actin scaffold. Our data are supported by an analysis of CE
494 movement in *Xenopus*: Co-expression of Cdc42^{T17N} rescues XRor2-induced CE

495 alterations (Hikasa et al., 2002), suggesting that Ror2 regulates Cdc42-dependent
496 processes.

497 **Ror2 and Wnt ligands**

498 Ror2 acts as a receptor for Wnt5A in mice (Mikels et al., 2006). In *Xenopus*,
499 Wnt5A/Ror2 activates the PCP signaling pathway, including transcription of ATF2
500 and PAPC (Schambony and Wedlich, 2007). Although there is *in vitro* evidence that
501 multiple Wnts can associate with the CRD domain of Ror2, only a few Wnts have
502 been demonstrated to elicit Ror2 activation *in vivo*. For example, Wnt1 and Wnt3a
503 bind to the CRD domain of Ror2 (Billiard et al., 2005) yet neither altered receptor
504 activity as assessed by levels of Ror2 autophosphorylation. *Xenopus* Wnt8 binds to
505 the ectodomain of Ror2 (Hikasa et al., 2002) but it is not known if XWnt8 induces
506 Ror2 signaling. In zebrafish, evidence suggest Wnt11 is a potential binding and
507 signaling partner for Ror2 (Bai et al., 2014). Wnt11 activates Ror2 to modulate CE
508 during zebrafish gastrulation. We have observed that Wnt8a co-localizes with Ror2 in
509 cytonemes. Although tagged constructs have been examined at very low
510 concentration, this does not necessarily imply correct subcellular localization.
511 However, our results are in line with studies on endogenous Wnt8a localization on
512 cytonemes (Stanganello et al., 2015) and with studies on Ror2 localization on
513 filopodia in mouse (Paganoni and Ferreira, 2003; Laird, et al., 2011) and in *Xenopus*
514 (Nishita, et al. 2006; Brinkmann et al., 2014). We have further observed that Wnt8a
515 binds and activates Ror2 signaling *in vitro* and *in vivo*. It is possible that other β -
516 catenin independent Wnts, such as Wnt5a and Wnt11, might induce Ror2 cytonemes
517 to regulate β -catenin Wnt ligand trafficking and thus β -catenin signaling. Wnt5A and
518 Wnt11 are known to regulate dorso-ventral patterning of the neural tube and somites
519 in mice (Andre et al., 2015). However, the observed patterning defect and AP axis
520 shortening in Wnt5A/Wnt11 double knockout mice was explained by migration
521 alteration of the axial mesoderm.

522 **Interaction of the Wnt signaling branches**

523 Binding of Wnt proteins to their cognate Frizzled receptors activates several
524 distinct signaling pathways, including the canonical Lrp6/ β -catenin-dependent and
525 the non-canonical Ror2/ β -catenin-independent PCP pathways (Niehrs, 2012).
526 According to the conventional classification, Wnt1, Wnt3a, and Wnt8a belong to the
527 β -catenin dependent Wnt signaling proteins, whereas Wnt5a and Wnt11 are
528 representatives of the β -catenin independent Wnt signaling proteins (Kikuchi et al.,

529 2011). Activation of the Ror2/PCP signaling branch is assumed to repress the β -
530 catenin signaling branch (Niehrs, 2012). Both Wnt signaling branches use the same
531 hub proteins as intracellular effectors, which may lead to competition and mutual
532 repression. For example, Wnt5a, which preferentially activates PCP signaling,
533 competes with Wnt3a for Fzd2 and thus inhibits the β -catenin-dependent pathway
534 (Sato et al., 2010). Furthermore, in *C. elegans*, Ror2 is thought to act by
535 sequestering β -catenin Wnt ligands, a function independent of its intracellular domain
536 (Green et al., 2007). In tissue culture, intracellular Ror2 signaling represses β -catenin
537 signaling via its tyrosine kinase activity (Mikels et al., 2006) and interaction with Dvl
538 (Witte et al., 2010). Furthermore, intracellular binding partners such as Tak1 interact
539 with the C-terminal region of Ror2 to further inhibit Wnt/ β -Catenin signaling (Winkel et
540 al., 2008). However, recent work reported that Ror2 signaling is able to enhance β -
541 catenin mediated signaling and proliferation in several tumor types (Rasmussen et
542 al., 2013; Roarty et al., 2017; Yan and Lin, 2008). These apparently contradictory
543 results could be explained by cross-regulation of Ror2 and the β -catenin signaling
544 being influenced by tissue heterogeneity, e.g. during tumor progression (Roarty et al.,
545 2017), where Ror2-expressing tumor cells regulate the spatial distribution, duration,
546 and amplitude of Wnt/ β -catenin signaling within a tumor, depending on the repertoire
547 of Wnt signaling components present. Our data provide a mechanistic explanation of
548 this effect. Although we cannot rule out the possibility of Ror2 activation within the
549 Wnt source cells, we hypothesize that the Ror2/PCP signaling pathway is activated in
550 an autocrine fashion based on our observations of the behavior of individual cells in
551 cell culture and in the embryo. We further provide evidence that Ror2/PCP-
552 dependent signaling is crucial for cytoneme emergence in Wnt source cells. Then,
553 cytonemes transmit Wnt ligands to neighboring cells to activate juxtacrine β -catenin
554 signaling. We demonstrate the importance of separating Wnt-producing from Wnt-
555 receiving cells by showing that Ror2-mediated Wnt transport is required for
556 controlling cell proliferation in AGS gastric cells. AGS cells express Wnt ligands such
557 as Wnt1, display a high endogenous β -catenin level, and show enhanced
558 proliferation (Mao et al., 2014). The anti-cancer drug salinomycin inhibits β -catenin
559 signaling by inducing degradation of the Wnt co-receptor Lrp6, resulting in reduced
560 proliferation. Autonomous activation of PCP signaling via Ror2 represses β -catenin
561 signaling and reduces proliferation in AGS cells (Yan et al., 2016), suggesting that
562 activation of PCP signaling could be used as a further strategy to inhibit uncontrolled

563 proliferation of gastric tumors. However, gastric tumors display a high degree of
564 tissue heterogeneity and we find that Ror2 can also enhance β -catenin signaling and
565 consequently cell proliferation. Our experimental approach is fundamentally different
566 compared to the former analysis: Wnt producing cells and Wnt receiving cells are
567 treated separately. We find that Ror2 activation in the Wnt producing cells increases
568 proliferation in the Wnt receiving cells. This hypothesis is supported by recent
569 findings showing that Wnt-Ror2 positive mesenchymal cells promote gastric cancer
570 cell proliferation if co-cultivated (Takiguchi et al., 2016).

571 **Cytonemes are a general carrier for Wnt protein dissemination**

572 Here, we describe Ror2/PCP-induced cytonemes as transport carrier for
573 Wnt8a in zebrafish. In cell culture experiments, we use PAC2 fibroblasts and
574 HEK293T cells to provide further evidence for the importance of Ror2-dependent
575 cytonemes in Wnt trafficking. In addition, we show that human gastric cancer cells
576 AGS, which primarily express the Wnt ligand Wnt1, process paracrine Wnt signaling
577 via cytonemes, which are influenced by Ror2 signaling. Finally, we use murine
578 intestinal stroma cells, which express Wnt2b to maintain the Wnt gradient operating
579 in the intestinal crypt (Aoki et al., 2016; Kabiri et al., 2014). We provide further
580 evidence that Wnts from intestinal stroma utilize Ror2-dependent cytonemes for their
581 transport. In summary, we show that autocrine PCP pathway activation via Ror2
582 induces Wnt cytonemes in the Wnt source cell to transmit Wnt to the neighboring cell
583 to activate paracrine Wnt/ β -catenin signaling. We propose cytonemes as an general
584 mechanism for mobilization of Wnt ligands in tissue homeostasis as well as in
585 development in vertebrates.

586

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602

603 **Author Contributions**

604 BM and SS designed, performed and analyzed all experiments except where
605 noted and wrote the manuscript. YD screened the kinase cDNA library, GG and DMV
606 performed the intestinal organoid studies, LTK and SÖ performed the ATF2 reporter
607 assay, BP and GUN contributed the FCS data, JR and AS designed and performed
608 the simulations, JS and RM developed the cytoneme analysis software.

609

610 **Declaration of Interests**

611 The authors declare no competing interests.

612 **Figure legends**

613 **Figure 1: Kinase Screen and automated image-analysis identifies the receptor**
614 **tyrosine kinase Ror2 as a potential cytoneme regulator upstream of Cdc42.** (A)

615 Schematic workflow of the cDNA kinase screen. Wnt8a-GFP and a membrane bound
616 mCherry was co-transfected with kinase library genes in a 96-well plate. Images
617 were acquired automatically and analyzed for filopodia length and numbers by a
618 filopodia detection software. (B) Automated Image analysis software detects and
619 counts filopodia of single cells using the memCherry signal and quantifies their length
620 by automatically tracing the tips back to the cell body. Scale bar represents 15 μ m.
621 (C, D) Diagram illustrating transfected kinase genes on x-axis and their relative
622 filopodia number/cell and length. Each bar represents one of 229 kinases, sorted
623 ascending by its value. The blue line indicates the 85th percentile (number: 1.20,
624 length: 1.25). The position of Ror2 in the diagram is highlighted by the red line
625 (number: 1,20, length: 1.47). (E) Quantification of filopodia in PAC2 cells 24h after
626 transfection or inhibitor treatment. memCherry was transfected together with
627 indicated constructs. Scale bar represents 10 μ m. (F) Boxplot for cumulative filopodia
628 length of cells transfected with indicated constructs measured by ImageJ. Centre
629 lines show the median; box limits indicate the 25th and 75th percentiles as
630 determined by R software; whiskers extend 1.5 times the interquartile range from the
631 25th and 75th percentiles, outliers are represented by dots. Significance level as
632 indicated: ***=P<0.001, **=P<0.01, =P<0.05. Data meet the assumption that the
633 length of filopodia is significantly different in the analyzed groups by one-way ANOVA
634 with a P=5.79*10⁻⁹⁹, confidence interval 95%, F value=118, and df=1047.

635 **Figure 2: Image-based protein interaction analysis highlights Ror2s implication**
636 **in Wnt8a binding and cluster formation.** (A) At the 8–16-cell stage, single

637 blastomeres of zebrafish embryos were microinjected with 25ng/ μ l mRNA of
638 indicated constructs to generate cell clones at 50% epiboly for confocal microscopy
639 analysis. Imaging visualizes co-localization of proteins at a single confocal plane
640 including high-magnification of clusters. (B) Co-localization channel by fire LUT and
641 the intensity plot profile were generated in ImageJ. Intensity plots show one individual
642 measurement along the membrane (left plot, orange arrows) and the average of
643 measurements across the membrane incl. SEM (right plot, light blue arrows). Graph
644 represents 10 membrane clusters acquired from 5 different cells of 2 different
645 embryos per treatment. (C) Close-up of a 6 – 8 hpf live zebrafish embryo showing

646 schematically how two-color two-foci line-scanning fluorescence correlation
647 spectroscopy (2c2f IsFCS) data were taken by laser scanning perpendicular to the
648 membrane (white lines) to collect data in the absence (spot 1) or presence (spot 2) of
649 Wnt8a-GFP. Embryos were mounted in agarose and injected with Wnt8a-GFP and
650 Ror2-mCherry. (D) Autocorrelation functions of Ror2-mCherry (red) and Wnt8a-GFP
651 (green) (symbols) and model fit (line) measured in spot 1. The total time of the
652 measurements was 390s. Importantly, there is no Wnt8a-GFP at this position as
653 shown by the lacking autocorrelation in the green channel. Error bars represent
654 standard deviations from two measurements. (E) Autocorrelation functions of Ror2-
655 mCherry (red) and Wnt8a-GFP (green) and the dual-color cross-correlation (blue)
656 (symbols) and model fits (lines) measured in spot 2. Here, Wnt8a-GFP is present,
657 and a high cross-correlation amplitude indicates co-diffusion of bound Ror2-mCherry
658 and Wnt8a-GFP. Error bars indicate standard deviations from two measurements.

659 **Figure 3: Synergistic Wnt PCP activation by Wnt8a and Ror2 in zebrafish and**
660 **Xenopus embryos.** (A) Embryos at 11 hpf were fixed and subjected to *in situ*
661 hybridization against *hgg/ntl*. Scale bar represents 200 μ m. (B) Embryos were sorted
662 according to their phenotypes into groups (see Supplementary Figure 2 for details).
663 (C) *gsc:memGFP* embryos were microinjected with indicated constructs, fixed at 11
664 hpf and a defined z-stack was imaged by confocal microscopy. Confocal stack shows
665 the notochord marked by *gsc:memGFP* from a dorsal view with animal site to the top.
666 Magnified inset highlights the shape of notochord cells. Scale bar represents 20 μ m.
667 (D) Analysis of cell roundness. Boxplot shows the width/length ratio of 25 notochordal
668 cells. Circularity ranges from 0 (infinitely elongated polygon) to 1 (perfect circle).
669 ANOVA confirmed that cell roundness is significantly different in the analyzed groups
670 with a $P= 2.91 \times 10^{-24}$, confidence interval 95%, F value=37, and df=149. (E) ATF
671 luciferase reporter assay of pooled *Xenopus* gastrulae injected with indicated
672 constructs and the ATF2 firefly and Renilla luciferase reporter. E shows the mean
673 with S.D. of three independent experiments. ***= $P < 0.001$, **= $P < 0.01$, *= $P < 0.05$.

674 **Figure 4: In-depth analysis and simulation of Wnt-positive cytonemes in**
675 **zebrafish embryos *in vivo*** (A) Live confocal microscopy analysis for filopodia
676 dynamics over time. Mosaic expression of memCherry was utilized to quantify the
677 protrusions of single cells at positions as indicated. The image shows the same cells
678 at different times during zebrafish gastrulation. (B) Filopodia of cells in A were
679 measured by a semi-quantitative segmentation software (see Supplementary Figure

680 4 for details). Quantification illustrates the mean filopodia length and number per cell
681 with SEM at different time points. (C) Effect of Ror2 on filopodia length/number. Live
682 confocal microscopy analysis for filopodia of embryos injected with Ror2 or Ror2^{3l}
683 mRNA at one cell stage. (D) Diagram of mean filopodia length and number per cell
684 with SEM. (E) Analysis of Wnt cytonemes during live imaging. Embryos were
685 microinjected at 16 cell stage to generate a cell clone expressing Wnt8a-GFP and
686 memCherry to visualize cytonemes and Ror2, Ror2^{3l} or Ror2-MO oligomer. Confocal
687 images were taken of single cells and subjected to filopodia length/number
688 measurement. See Suppl. Fig. 4 for stacked diagrams with chi-squared test analysis.
689 (F) Bar diagram shows number of filopodia without Wnt8a-GFP (i.e. GFP signal
690 below detection limit) or filopodia carrying Wnt8a-GFP (cytonemes) (i.e. GFP signal
691 above detection limit) per cell. Statistical analysis ***=P<0.001, **=P<0.01, =P<0.05.
692 Data meet the assumption (B) that filopodia length and number are significantly
693 different at the analyzed time-points (One-way ANOVA, P=1.35*10⁻⁹, confidence
694 interval 95%, F value=27.6, and df=73) and (D) that the filopodia number and length
695 are significantly different across treatments (P=1.61542*10⁻⁷, confidence interval
696 95%, F value=24.6, and df=39). (G) Simulation of Wnt dissemination in a growing
697 tissue over 180min. Wnt8a is distributed in a graded manner in the target tissue.
698 Modeling the dynamically expanding tissue with single cell resolution and discrete
699 implementation of cytoneme-based transport establishes a morphogen gradient over
700 the length of the developing tissue. Based on the cytoneme appearance measured in
701 (D & F), Ror2 activity in the Wnt producing cells alters the input of Wnt in the target
702 field which is directly incorporated into the simulation by altering the formation
703 frequency of cytonemes and thus ligand concentration in the neighboring tissue.
704 Simulated time in minutes after the onset of Wnt production at 4hpf.

705 **Figure 5: Visualization of Ror2-Wnt8a cytonemal transport and Lrp6 receptor**
706 **clustering in the living zebrafish .** (A) Illustration of the injection procedure to
707 generate single cell clones. (B-C) Time series of a single confocal plane of Ror2-
708 mCherry/Wnt8a-GFP expressing cells to (B) observe Wnt8a recruitment to the
709 membrane and cytoneme initiation and (C) cytonemal target finding and Ror2/Wnt8a
710 cluster endocytosis into the receiving cell. Optimal microscopical settings were used
711 to allow an improved axial resolution of ca. 250nm. Single channels in Suppl. Fig 5A
712 and B and movie in Suppl. Movie 3. White arrows indicate the Wnt8a/Ror2 cluster
713 while the yellow arrow highlights pruning of the cytoneme tip after successful

714 cytonemal delivery. Yellow arrows mark the Wnt8a/Ror2 cluster. (D) Wnt8a-mCherry
715 cytoneme leads to Lrp6-GFP accumulation and co-localization at the receiving cell.
716 (E) Illustration of the injection procedure to generate clones to visualize paracrine
717 signal transport. (E) 3D-stack of a Wnt8a-mCherry cytoneme leading to Lrp6-GFP
718 accumulation and co-localization at the receiving cell. Close-up shows a single plane
719 including orthogonal YZ and XZ views of the cytoneme contact point. (F) Single plane
720 image of a Ror2-mCherry/Wnt8a cell leading to Lrp6-GFP clustering on cytoneme
721 contact site. Scale bars = 10 μm ; in E = 20 μm .

722 **Figure 6: Ror2 enhances paracrine Wnt/ β -catenin signaling in zebrafish**
723 **embryos and HEK293T co-culture.** (A) Microinjected embryos at indicated stages
724 were fixed and subjected to *in situ* hybridization against the Wnt target gene (*axin2*)
725 or markers for brain patterning (*fgf8a*, *pax6a*). Brackets indicate expansion of Wnt
726 target gene expression (*axin2*), distance of *fgf8* expression domains or forebrain
727 territory (*pax6a*), while the asterisk indicates a lack of forebrain tissue. (B) Co-culture
728 reporter gene assay in HEK293T cells. TCF/Wnt reporter population was transfected
729 with 7xTRE Super TOPFlash-NLS-mCherry and Lrp6 and co-cultured with a second
730 population as indicated. Co-culture principle can be seen in Supplementary Figure 6.
731 (C) Relative reporter activation by measuring the mCherry signal. Bar diagram
732 represents the mean value with SEM of 3 independent experiments. Scale bar
733 represents 200 μm . (D) RT-qPCR-based expression assay in zebrafish embryos.
734 Embryos were injected at 16 cell stage to generate a distinct signaling center at 75%
735 epiboly (see Supplementary Figure 6 for details) and were subjected for RTqPCR.
736 Relative $\Delta\Delta\text{Ct}$ expression values of Wnt- β -catenin target genes *axin2* and *lef1* is
737 acquired by normalization to *actb1* and in comparison to a control-injected sample
738 (relative ctrl expression level shown as a dotted line). Each condition represents the
739 mean Rt-qPCR result of 4 x 50 embryos acquired from 2 independent experiments.
740 ***= $P < 0.001$, *= $P < 0.05$. ANOVA confirmed hypothesis that expression levels are
741 different between treatments with a $P = 0.00025$, confidence interval 95%, F value
742 33.9, df 11.

743 **Figure 7: Importance of ror2 dependent cytonemes in gastric cancer cell**
744 **proliferation and intestinal crypt cells organoid formation.** (A) Confocal Z-
745 projection of live AGS cells transfected with memGFP and Wnt8a-mCherry. Yellow
746 arrow marks a cytoneme and yellow outlined arrow highlights circular Wnt8a-
747 mCherry spots around the source cell. (B) Confocal Z-projection of fixed AGS cells

748 transfected with Ror2-mCherry and stained with LifeAct. Yellow arrow marks filopodia
749 connections to adjacent cells and yellow outlined arrow highlights Ror2-mCherry
750 clusters in a non-transfected adjacent cell. (C) Boxplot of cumulative filopodia lengths
751 for AGS cells transfected with an empty plasmid, Ror2 or Ror2^{3l}. (D, E) Proliferation
752 assay of nucRFP transfected AGS cells after a 48 hrs co-cultivation with cells
753 transfected with indicated constructs. D shows fluorescent images which were
754 subjected for cell counting. Average nucRFP cells per image are shown in Boxplot E.
755 (F) Fluorescent images of purified intestinal myofibroblasts transfected with control
756 siRNA (n=31) or Ror2 siRNA (n=29) were quantified for cumulative filopodia length
757 per cell. (G) Boxplot of number of formed organoids of PORCN deficient intestinal
758 crypt cells co-cultured with indicated cell population for 3-4 days. Organoid numbers
759 were normalized to crypt cells transfected with ctrl siRNA. ***=P<0.001, **=P<0.01,
760 *=P<0.05. One-way ANOVA confirmed significant differences between treatments
761 with a confidence interval 95% in (C) P= 3.6151×10^{-7} , F value 16.8, df 122; in (D) P =
762 6.67274×10^{-5} , F value 9.4, df 46; (in F) P = 3.32078×10^{-06} , F value 26.5, df 59; and
763 in (G) P = 1.42034×10^{-18} , F value 106.9, df 41.

764 **Material and Methods**

765

766 **Plasmids.** The following plasmids have been used: pCS2+zfWnt8aORF1 (Addgene
767 17048), pCS2+zfWnt8aORF1-GFP , pCS2+zfWnt8aORF1-mCherry (Stanganello et
768 al., 2015), pCS2+xWnt5a-GFP, pCS2+zfWnt5b (Wallkamm et al., 2014), xRor2-
769 mCherry (Feike et al., 2010), xRor2^{3l} (Casella et al., 1981), mRor2-dCRD-GFP,
770 pCS2+Fz7a-CFP , pcDNA3-EGFP-Cdc42T17N (Addgene 12976), 7xTRE Super
771 TOPFlash-NLS-mCherry (Moro et al., 2012), pmKate2-f-mem (Evrogen), GPI-
772 anchored mCherry in pCS2+ (Scholpp et al., 2009), IRSp53^{4K} (Casella et al., 1981).
773 To generate the pCS2+xRor2 construct, the open reading frame of xRor2-mCherry
774 was inserted in the ClaI/XhoI site of pCS2+.

775

776 **Maintenance of fish.** Breeding zebrafish (*Danio rerio*) were maintained at 28°C on a
777 14 h light/10 h dark cycle (Brand et al., 2002). To prevent pigment formation,
778 embryos were raised in 0.2 mM 1-phenyl-2-thiourea (PTU, Sigma, St Louis, MO
779 63103 USA) after 24 hpf. The data we present in this study were acquired with wild-
780 type zebrafish (AB2O2) as well as with the transgenic zebrafish line tg(-6gsc:EGFP-
781 CAAX) (Smutny et al., 2017). All animal work (zebrafish husbandry and experimental
782 procedures) were undertaken under project and personnel licences granted by the
783 UK Home Office under the United Kingdom Animals (Scientific Procedures) Act, in
784 accordance with The University of Exeter's ethical policies and approved by the
785 University of Exeter's Animal Welfare and Ethical Review Body, and in accordance
786 with the German law on Animal Protection approved by Local Animal-Protection
787 Committee (Regierungspräsidium Karlsruhe, Az.35-9185.64) and the Karlsruhe
788 Institute of Technology (KIT).

789

790 **Cell culture experiments.** Experiments were performed in zebrafish PAC2
791 fibroblasts derived from 24 h old zebrafish embryos cultivated at 28°C without
792 additional CO₂ supply, primary gastric adenocarcinoma cells (AGS), Gastric tubular
793 adenocarcinoma liver metastasis cells (MKN28, MKN7 (Motoyama et al., 1986)) and
794 human embryonic kidney cells (HEK293T; CRL-1573) cultivated at 37°C with 5%
795 additional CO₂ supply. PAC2 were maintained in Leibowitz-15 media, HEK in DMEM,
796 AGS and MKN28 in RPMI 1640, all supplemented with 10% fetal bovine serum, 1%

797 L-Glutamine (2 mM) and 1% Penicillin/Streptomycin. All the material used for cell
798 culture was purchased from Life Technologies, Gibco.

799 For transfection experiments FuGENE HD Transfection Reagent (Promega) was
800 used and cells were imaged after 48 hrs. For co-culture experiments, transfected
801 cells were incubated for 24 hrs, detached by Trypsin-EDTA (0.05%) and incubated in
802 a mixed population for another 48 hrs before image acquisition.

803 Assays for SuperTOPFlash (STF) TCF/Wnt reporter expression and proliferation
804 required initial co-cultivation of two distinct cell populations. Cells were transfected
805 with pDest7xTCF-NLS-mCherry or pCS2+nucRFP plasmids, respectively, and
806 incubated for 24 hrs, detached by Trypsin-EDTA (0.05%) and further incubated in a
807 mixed population for another 72 hrs before image acquisition. For one replication
808 seven 10x magnification images were taken per sample with identical laser settings.
809 Image locations were saved by the Mark&Find microscope feature to reproduce
810 similar scanning setups. For measuring TCF/Wnt reporter activation, fluorescent
811 nuclei were processed using the Dot-Plugin in Imaris and the average grey value of
812 the nuclei was determined or fluorescent nuclei were counted to measure cell
813 proliferation.

814 For the chemical treatment, cell cultures were treated with GTPase Inhibitor
815 ML141 10 mM (Merck Millipore) or 50 μ M IWR-1 (Sigma) to antagonise the Wnt
816 signaling pathway. For staining, cells were fixed with 4% PFA at RT, washed with
817 PBS. Cells were incubated with 50 μ g/ml phalloidin (P1951, Sigma) and 10 μ g/ml
818 DAPI (D9542, Sigma).

819

820 **Organoid formation of intestinal crypt cells.** Myofibroblasts were prepared from
821 C57BL/6-*Tg(Pdgfra-cre)1Clc/J* /*Rosa^{mTmG}* mice and cultured as previously
822 described (Greicius et al., 2018). As confluence of cultured cells was reaching 80%,
823 they were transfected with respective siRNA (Dharmacon mouse ROR2 siRNA pool
824 Cat#LQ-041074-00-0002, four siRNAs combined in equal parts at 10nM) using
825 siRNAmix reagent (Invitrogen Cat#13778-030). On day 2 post-transfection,
826 myofibroblasts were mixed with *Porcn* deficient intestinal epithelial cells and cultured
827 using RSPO1 supplemented medium. Organoid counting was performed at the time
828 point when group containing no stromal cells had no surviving organoids left (end of
829 day 3- beginning of day 4 of co-culture). siRNA transfected cells were imaged using
830 OlympusLive Imaging system IX83. Acquired 3D image stacks were de-convoluted

831 using cellSens Dimension (Olympus) and are presented as maximum intensity
832 projections.

833

834 **Automated filopodia analysis software.** Cells and their attached filopodia were
835 automatically detected in the RFP channel (mem-mCherry) of the acquired images
836 (Suppl. Fig. 4A). The images were initially filtered using a Gaussian low-pass filter
837 ($\sigma^2 = 1$) and subsequently used detect the cell body as well as the cell's filopodia
838 (Suppl. Fig. 4B). For the filopodia detection we used an objectness filter ($\sigma = 1, \alpha =$
839 $1, \beta = 1, \gamma = 0.003, N = 2$) that emphasized line-like structures based on the
840 eigenvalues of the Hessian matrix at each pixel location (Suppl. Fig. 4C (Antiga,
841 2007)). The obtained edge-enhanced image was then binarized (Suppl. Fig. 4D) using
842 a local adaptive threshold filter that set pixels to foreground if their intensity value
843 was larger than a regional mean intensity minus a multiple of the regional intensity
844 standard deviation and otherwise to background (window radius = 200, std. dev.
845 multiplier = 1). To segment the cell body, we applied the local adaptive threshold
846 (window radius = 200, std. dev. multiplier = 1) on the smoothed input image (Suppl.
847 Fig. 4E) and subsequently used a morphological opening operation (kernel radius =
848 2) to get rid of noise and remaining filopodia parts (Suppl. Fig. 4F (Soille et al.,
849 2011)). The cell body was given by the largest connected component in the opened
850 binary image. The segmentation mask of the cell body including filopodia was then
851 constructed by combining the binarized edge-enhanced image with the binary cell
852 body image (Suppl. Fig. 4G). The combined cell image was subsequently
853 skeletonized to identify potential filopodia tips at the end points of the skeleton
854 (Suppl. Fig. 4H). All above-mentioned preprocessing steps were implemented in the
855 open-source software tool XPIWIT (Bartschat et al., 2016).

856 The preprocessing results were then imported to a dedicated MATLAB tool that was
857 developed to validate, correct and analyze this kind of images (Suppl. Fig. 4I, J). In
858 order to automatically trace filopodia from the identified tips to the cell body, we used
859 an adapted livewire algorithm (Barrett and Mortensen, 1997). The output of the
860 objectness filter was used as an edge map (Suppl. Fig. 4C) on which the livewire
861 algorithm tried to find a maximally scoring path from the tip of the filopodium to the
862 center of the cell body. Based on the segmentation of the cell body (Suppl. Fig. 4F),
863 the automatic tracing was stopped as soon as the cell body was reached. The
864 interactive user interface was then used to add, remove and correct both

865 segmentation masks and detected filopodia on a per-cell basis. For each cell's
866 filopodia, we calculated the Euclidean distance along the the path from the tip to the
867 cell body. The same image preprocessing and tracing was applied to semi-
868 automatically extract filopodia in 3D confocal images (Suppl. Fig. 4K). However, for
869 the 3D case, start and end points of the filopodia were provided by the user via a
870 graphical user interface and the livewire approach was applied twice. First on an
871 axial maximum intensity projection (z) to obtain the lateral path (xy). Subsequently,
872 the axial positioning of the filopodium was obtained by searching for the highest
873 scoring path between the provided start and end points solely in the z-direction
874 (Suppl. Fig. 4K). Multiple automatically traced filopodia can then be exported and
875 used to obtain average statistics of all filopodia of interest.

876

877 **IsFCS.** For the FCS measurements, a home-built confocal microscope was used as
878 previously described (Dörlich et al., 2015), with slight modifications. We used a water
879 immersion objective (HCX PL APO CS 63x /1.2, Leica, Wetzlar, Germany) instead of
880 an oil immersion objective; the multimode fiber, which acts as a confocal pinhole,
881 was modified accordingly to ensure a pinhole size of 1 AU. Data were collected for
882 390 s by continuously scanning the focus perpendicularly through the membrane.
883 Each scan line consisted of 100 pixels, with a step size of 100 nm. eGFP was excited
884 with a 488 nm continuous wave (cw) laser and mCherry with a 561 nm cw laser. After
885 splitting the fluorescence signal into two color channels by using a 555 nm dichroic
886 filter, 525/50 (eGFP) and 600/37 (mCherry) band pass filters were used for detection.
887 To avoid artefacts in the correlation curves caused by scanner flyback and
888 wavelength switching, the membrane was always kept in the center of the field of
889 view. The intensity data were arranged as an $x-t$ pseudo image, and the intensities of
890 those pixels containing membrane fluorescence were integrated to obtain an intensity
891 time trace for correlation analysis, as described earlier (Hunter, 2007).

892

893 **Functional analysis.** The injection of mRNAs and Morpholino oligomers were
894 performed according to the description in the text and in (Mattes et al., 2012). Ror2
895 MO was used in a 0.5mM concentration (5'-CAGTGTAACAACCTCCAAACTCTCC -
896 3') (Gene Tools, Philomath, OR 97370 USA). Capped and *in vitro* transcribed mRNA,
897 (mMessage Machine Kit, Ambion) was microinjected into one cell or into the yolk for
898 ubiquitous expression or in one of 16 blastomeres to generate cell clones. Embryos

899 were incubated at 28°C until subjected for image acquisition or fixed for whole-mount
900 mRNA *in situ* hybridization (ISH).

901 For *in situ* hybridization, *hgg*, *ntl*, *axin2*, *fgf8a* and *pax6a* digoxigenin- and
902 fluorescein-labeled probes were prepared from linearized templates using an RNA
903 labelling and detection kit (Roche) as described in (Scholpp and Brand, 2003).
904 Images were taken on an Olympus SZX16 microscope equipped with a DP71 digital
905 camera by using Cell D imaging software.

906 For real-time quantitative PCR (RT-qPCR), 50 embryos each were lysed in 1 ml
907 TriZol (Sigma), and total RNA was prepared using Direct-zol RNA Mini Prep Kit from
908 Zymo Research. cDNA was prepared using MMLV reverse transcriptase from
909 Promega and analysed in a Real-Time PCR system from LifeTechnologies (ABI
910 StepOnePlus). Primers with the following sequence were used: *beta-actin* (5'-
911 CCTTCCTTCCTGGGTATGG-3'; 5'-GGTCCTTACGGATGTCCAC-3'), *axin2* (5'-
912 CAATGGACGAAAGGAAAGATCC-3'; 5'-AGAAGTACGTGACTACCGTC-3'), *lef1* (
913 5'-CAGACATTCCCAATTTCTATCC-3'; 5'-TGTGATGTGAGAACCA ACC-3').
914 Results were analysed using the $\Delta\Delta$ CT method.

915
916 Simulations. Computer simulations have been implemented in Python using the
917 libraries numpy, scipy and matplotlib (Hunter, 2007) and is based on a simulation of
918 cytoneme-based ligand distribution described in detail in (Stanganello et al.,
919 2015). The modelling of the tissue is split up into two subproblems (1) the correct
920 modelling of the dynamically forming tissue and (2) the morphogen transport through
921 that tissue. The tissue dynamics are modelled by a two-dimensional non-periodic
922 plane of discrete cells. The simulated area is 1000 by 1000 μ m in size and each cell
923 occupies a circle of 8 μ m radius. Possible cell positions are precomputed and kept
924 fixed during the course of the simulation. Cells can change positions by moving along
925 those precomputed positions. In every simulation step ($\Delta t=1$ s) each individual cell
926 has the possibility to perform some actions with predetermined probabilities which
927 are directly derived from experimental measurements.

928 Possible actions are (i) Signaling from the producing tissue, (ii) Cell insertion by
929 division or intercalation of the receiving tissue, (iii) Cell migration - directed or non-
930 directed nearest neighbor swapping of the receiving tissue, and (iv) Morphogen
931 decay in the receiving tissue. Two different cell types are distinguished. The
932 marginal, Wnt active cells are producing morphogen and are able to deposit

933 morphogen via cytonemes to cells of the receiving tissue. Once a signaling event is
 934 accepted for a marginal cell an angle is randomly chosen from the respective
 935 distribution determined in (Stanganello et al., 2015). The length is randomly chosen
 936 from a Gaussian distribution with the peak value l_{filo} . A virtual filopodium is formed
 937 with those values originating from the marginal cell. If the tip of the filopodium ends
 938 up in vicinity of the surface of a receiving cell ($\pm 2\mu\text{m}$) the Wnt content of that cell is
 939 increased, otherwise the filopodium is deleted. Probabilities used in the simulation
 940 were:

	Signaling probability p_{filo}
WT	$\frac{1}{30}$
Ror2	$1.61 \cdot \frac{1}{30} = 0.0537$
Ror2 ^{3l}	$0.338 \cdot \frac{1}{30} = 0.0113$

941

942 Tissue expansion during neural plate patterning is driven by the intercalation of cell
 943 layers during the thinning out of the cell sheet as well as cell division. Since we are
 944 only modelling one cell layer both of these processes can be incorporated by a single
 945 action, namely cell insertions. If a cell insertion event is accepted in a receiving cell a
 946 path to the nearest empty grid spot is obtained and all cells are subsequently moved
 947 along that path. The emerging empty grid spot is then filled with a randomly chosen
 948 cell with the same distance $\pm 6\mu\text{m}$ from the marginal cells. To include the highly
 949 dynamic intermingling of the cells during that process an additional action, cell
 950 migration, is added. This action allows for a cell to swap places with a randomly
 951 chosen nearest neighbor cell. Morphogen decay inside the receiving tissue is
 952 implemented by an action that decreases the Wnt content of the cell once accepted.

953 **Luciferase reporter assay in Xenopus embryos.** For the ATF2 luciferase reporter
 954 assay, 4-cell stage Xenopus embryos were injected into both animal ventral
 955 blastomeres with the 100 pg ATF2-Luciferase reporter plasmid in combination with
 956 10 pg TK-Renilla-Luciferase reporter plasmid. The reporter plasmids were injected
 957 alone or together with 500 pg of the respective synthetic mRNAs. Luciferase reporter
 958 assays were carried out from triplicates of five gastrula stage (st.12) embryos lysed
 959 to measure Luciferase activity using the Dual luciferase system (Promega).

960 **Image acquisition.** For confocal analysis, live embryos were embedded in 0.7% low
961 melting agarose (Sigma-Aldrich) dissolved in 1x Ringer's solution. Images of cells
962 and embryos were obtained with a Leica TCS SP5 X or SP8X confocal laser-
963 scanning microscope using 20x or 63x dip-in objective or for the kinase library screen
964 a Leica DMI600SD with 20x objective was used. Image processing was performed
965 with Imaris 9.1 software (Bitplane AG, Switzerland). Filopodia and cytoneme
966 measurements from confocal z-Stacks of living embryos was performed via the semi-
967 automated filopodia analysis software described before. Cell culture quantifications
968 were carried out by using Fiji software. Roundness of notochordal embryo cells was
969 determined by calculating the width to length ratio per cell in Fiji.

970

971 **Statistical analysis.** All experiments were carried out at least in biological triplicates
972 if not indicated otherwise. Significance was calculated by Student's t-test while
973 asterisks represent indicated p-values. One-way ANOVA was used to analyse
974 groups of experimental data. All groups are random samples from the same
975 population. Variances are similar across treatments and residuals are normally
976 distributed. P-values, F values and degrees of freedom (df) are indicated. Box plots:
977 Centre lines show the medians; box limits indicate the 25th and 75th percentiles as
978 determined by R software; whiskers extend 1.5 times the interquartile range from the
979 25th and 75th percentiles, outliers are represented by dots.

980

981 **Supplementary figure legends**

982

983 **Supplementary Figure 1:** (A-B) Bar diagram shows the average lengths (A) or
984 numbers (B) of filopodia per PAC2 cell. Error bar represents the SEM.

985

986 **Supplementary Figure 2:** (A) 3D confocal image of a cell clone at 50% epiboly.
987 Embryo was injected with memCherry and Wnt8a-GFP. Cytosomes feature Wnt8a-
988 GFP clusters on their tip (arrows). (B) Single confocal planes of embryos injected
989 with Wnt5b-GFP and memCherry or Ror2-mCherry. (C) Intensity profiles along 15
990 μm of cell membrane containing Wnt5b-GFP spots, showing spatial intensity
991 correlations with Ror2-mCherry. (D) Schematic depiction of the principle of line
992 scanning FCS measurements, which allows us to remove fluorescence fluctuations
993 due to membrane motion. Red and green emitting laser foci are scanned
994 perpendicularly through the plasma membrane. The emission is recorded as a
995 function of time and corrected for membrane fluctuations. The autocorrelation
996 functions of Ror2-mCherry (red excitation) and Wnt8a-GFP (green excitation) are
997 computed from the intensity time traces and yield local concentrations (i.e., area
998 densities) and diffusional correlation times. The cross-correlation between red and
999 green is a measure of joint intensity fluctuations in the two channels caused by
1000 Wnt8a-GFP bound to Ror2-mCherry diffusing together through the excitation focus.
1001 (E) Diagram of confocal planes of embryos injected with Wnt8a-GFP, Ror2-mCherry
1002 and Fzd7a-CFP and the corresponding intensity correlation. (F, G) Pearson
1003 correlation coefficients of pairs of fluorescent proteins in a dual-color image (as
1004 indicated by the black boxes below the bar graph). The result is +1 for perfect
1005 correlation, 0 for no correlation. (F) shows the analysis of confocal images of
1006 embryos at 50% epiboly injected with the indicated constructs, as determined by
1007 Imaris software. (G) shows a Pearson co-localization analysis of indicated
1008 fluorescent constructs in a tissue volume of $40 \times 40 \times 60 \mu\text{m}^3$ of 8 different embryos.
1009 *** = $P < 0.001$.

1010

1011 **Supplementary Figure 3:** (A) Microinjection of Ror2-mCherry mRNA in rising
1012 concentrations and classification of phenotypes into groups showing developmental
1013 alterations at 24 hpf. Chi-squared test was performed to determine p-values. (B)
1014 Classification of embryos into 3 groups of phenotypes depending on their *ntl*

1015 expression. (C) Quantification of the ntl expression width. Location of measurement
1016 is shown in the left image (dorsal view, animal to the top) and the bar diagram
1017 represents average width with SEM. ***= $P < 0.001$, *= $P < 0.05$.

1018

1019 **Supplementary Figure 4:** Cytoneme quantification software to automatically extract
1020 filopodia using the memCherry channel of the acquired images. The raw image (A)
1021 was initially smoothed with a Gaussian low-pass filter (B) to reduce image noise for
1022 facilitated processing. To emphasize filopodia in the image, we used an objectness
1023 filter that emphasizes line-like structures based on the eigenvalues of the Hessian
1024 matrix (C) and binarized the resulting edge image (D) using local adaptive
1025 thresholding. For the detection of the cell body, we applied a local adaptive threshold
1026 to the Gaussian smoothed raw image (E) and performed a morphological opening
1027 operation to remove filopodia remains from the cell body (F). The two binarized
1028 images (D) and (F) were then combined to yield the final segmentation mask
1029 including cell body and filopodia (G). Finally, the combined binary image (G) was
1030 used to obtain a skeleton image (H), which in turn allows extracting potential filopodia
1031 tips at the end points of the skeleton. The identified cell body (cyan outline) and the
1032 potential filopodia tips (magenta dots) are shown in (I). A livewire approach was then
1033 used to automatically trace filopodia from the tips to the cell body (J, red lines). The
1034 white arrow indicates errors of the automatic tracing and a dedicated software can be
1035 used to manually correct such errors. (K) To measure analyse filopodia in acquired
1036 3D images, we developed custom-made MATLAB GUI that allows to scroll through
1037 the slices of the stack and to semi-automatically trace filopodia based on manually
1038 provided start and end points of a filopodium of interest (yellow arrows). The software
1039 automatically traces the filopodium in 3D using a livewire approach to obtain accurate
1040 length quantifications. The software keeps track of all segmented filopodia, provides
1041 filopodia counts, length measurements as well as average quantifications of all
1042 identified filopodia. In addition, in a window of the GFP channel we are able to
1043 distinguish between Wnt positive and Wnt negative filopodia for embryos expressing
1044 Wnt8a-GFP.

1045

1046 **Supplementary Figure 5: Visualization of Ror2-Wnt8a cytonemal transport in**
1047 **the living zebrafish.** Time series of still pictures of single channels of Ror2-
1048 mCherry/Wnt8a-GFP expressing cells to (A) observe Wnt8a recruitment to the

1049 membrane and cytoneme initiation and (B) cytonemal target finding and Ror2/Wnt8a
1050 cluster endocytosis into the receiving cell. White arrows indicate the Wnt8a/Ror2
1051 cluster while the yellow arrow highlights pruning of the cytoneme tip after successful
1052 cytonemal delivery. (B) includes also a series of merged pictures with the bright field
1053 channel. Scale bars = 10 μ m; in E = 20 μ m.

1054 **Supplementary Figure 6:** (A) Illustration of expression domains of landmark genes
1055 e.g. *axin2*, *fgf8* or *pax6a*. (B) Illustration of the experimental procedure for *in vitro* co-
1056 cultivation assays with HEK293T cells expressing cytoneme regulators and the
1057 TCF/Wnt reporter 7xTRE SuperTOPFlash-NLS-mCherry. Fluorescence image shows
1058 some activated cells expressing nuclear mCherry. All cells were fixed and stained
1059 with DAPI. (C) Workflow of clonal injection to induce a local Wnt source with following
1060 RTqPCR analysis of target genes in responding cells.

1061
1062 **Supplementary Figure 7:** (A) Confocal images of gastric cancer cells AGS, MKN7
1063 and MKN28. Cells were transfected with Wnt8a-mCherry (left) or Ror2mCherry
1064 (right), fixed and stained with LifeAct. Yellow Arrows highlight Wnt8a-mCherry
1065 cytonemes or Ror2-induced protrusions while yellow outlined arrows visualize the
1066 dissemination of Wnt8a-mCherry clusters to untransfected adjacent cells. (B)
1067 Illustration of the experimental procedure in co-cultivation assays to determine the
1068 proliferation of a second cell population. (C) Mouse crypt organoid supported by a
1069 purified intestinal myofibroblasts derived from a PdgfR α -Cre driven GFP reporter
1070 mouse. Image was taken 3 days after co-culture of myofibroblasts with *Porcn*
1071 deficient intestinal epithelial cells. Long cell protrusions surrounding the organoid are
1072 highlighted by yellow arrows.

1073
1074 **Supplementary movie legends**

1075
1076 **Supplementary Movie 1:** Time-lapse movie of localization of Ror2-mCherry and
1077 Wnt8a-GFP in a developing zebrafish embryo at gastrulation stages. Movie illustrates
1078 the co-localization of Ror2-mCherry and Wnt8a-GFP in clusters and their dynamics
1079 over time. Single confocal plane was imaged at 6 hpf for 20 minutes with a frame rate
1080 of 15 seconds. Still pictures of the movie in Fig. 2A.

1081

1082 **Supplementary Movie 2:** Time-lapse movie of clonal cells forming cytonemes in a
1083 developing zebrafish embryo at gastrulation stages. Z-projection of a clone of Wnt8a-
1084 GFP and Ror2-mCherry positive cells was imaged at 8 hpf for 20 minutes with a
1085 frame rate of 15 seconds.

1086

1087 **Supplementary Movie 3:** Time-lapse movie of clonal cells forming cytonemes in a
1088 developing zebrafish embryo at gastrulation stages. Single confocal plane of a clone
1089 of Wnt8a-GFP and Ror2-mCherry positive cells showing cytonemal target finding and
1090 subsequent Ror2/Wnt8a cluster endocytosis of the receiving cell. Movie was imaged
1091 at 8 hpf for 20 minutes with a frame rate of 15 seconds. Still pictures of the movie in
1092 Fig. 5C and single channels are presented in Suppl. Figure 5B.

1093

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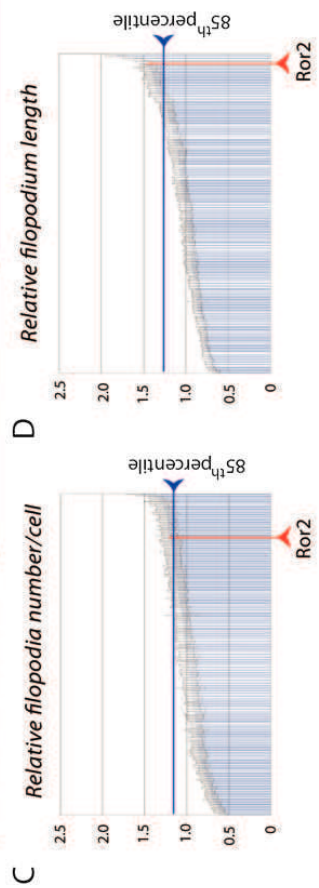
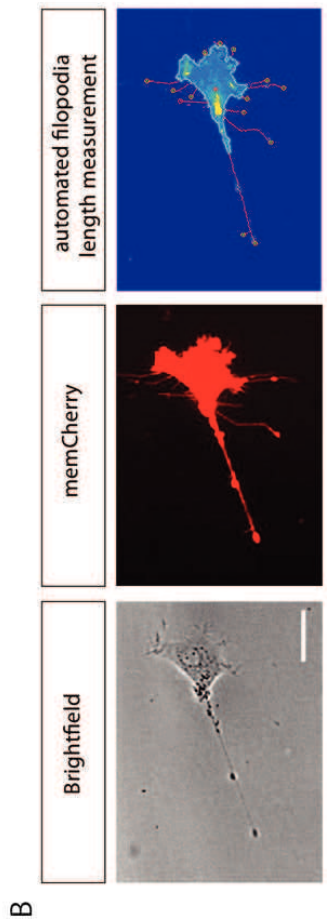
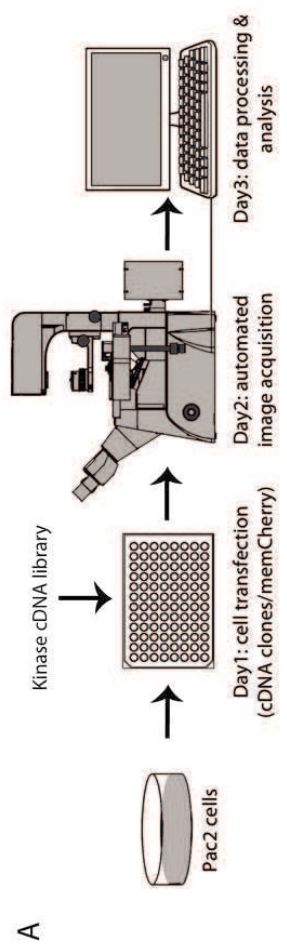
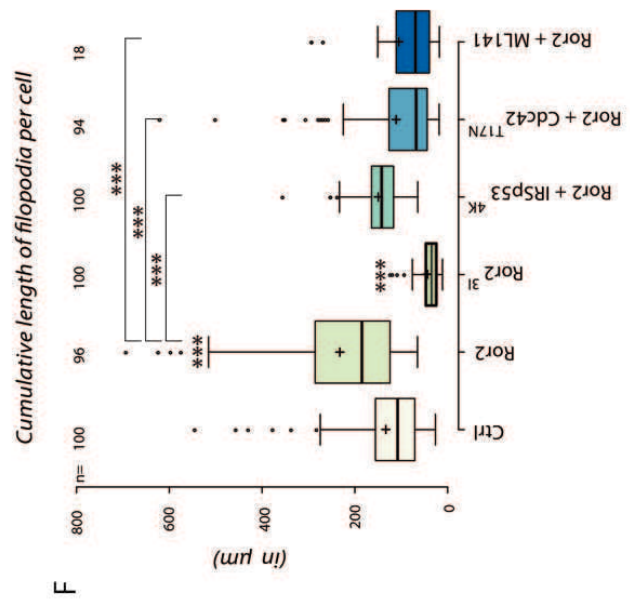
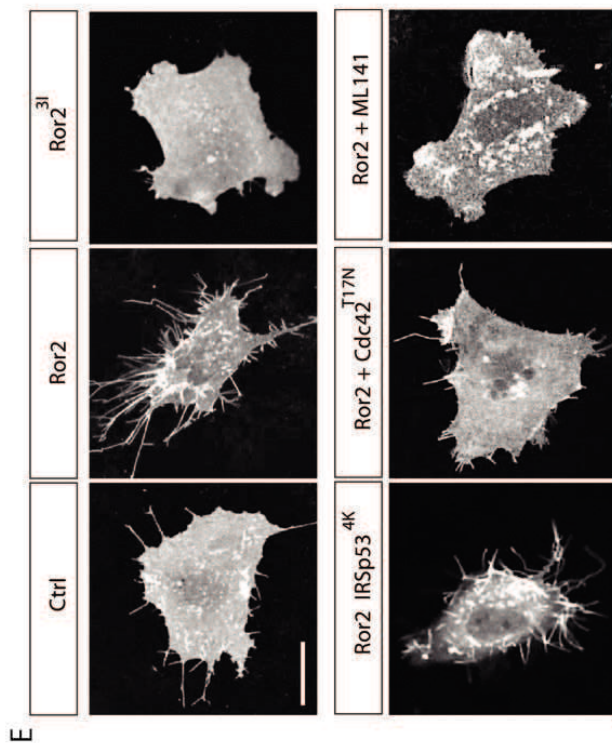


Figure 1; Mattes et al., 2018

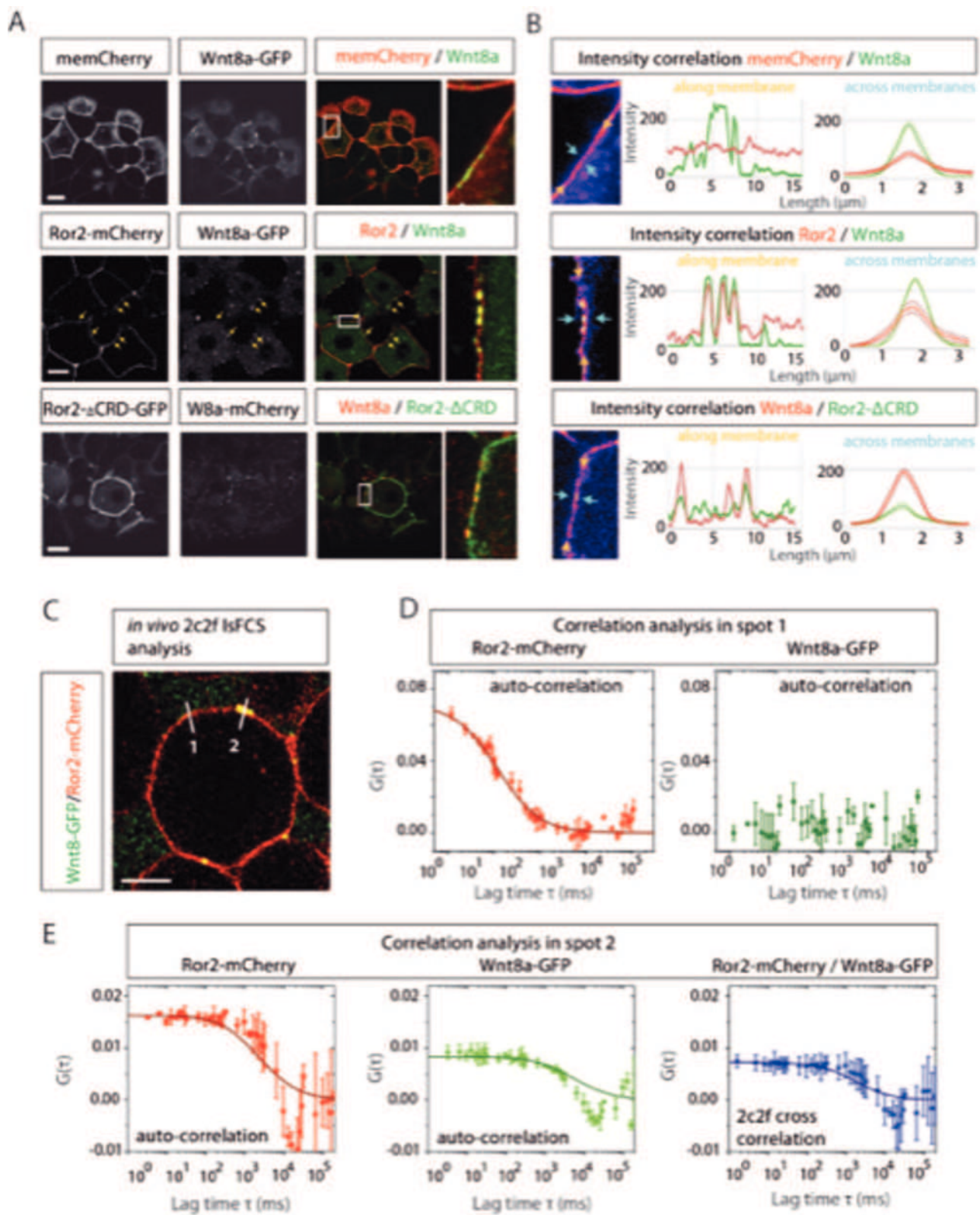


Figure 2; Mattes et al., 2018

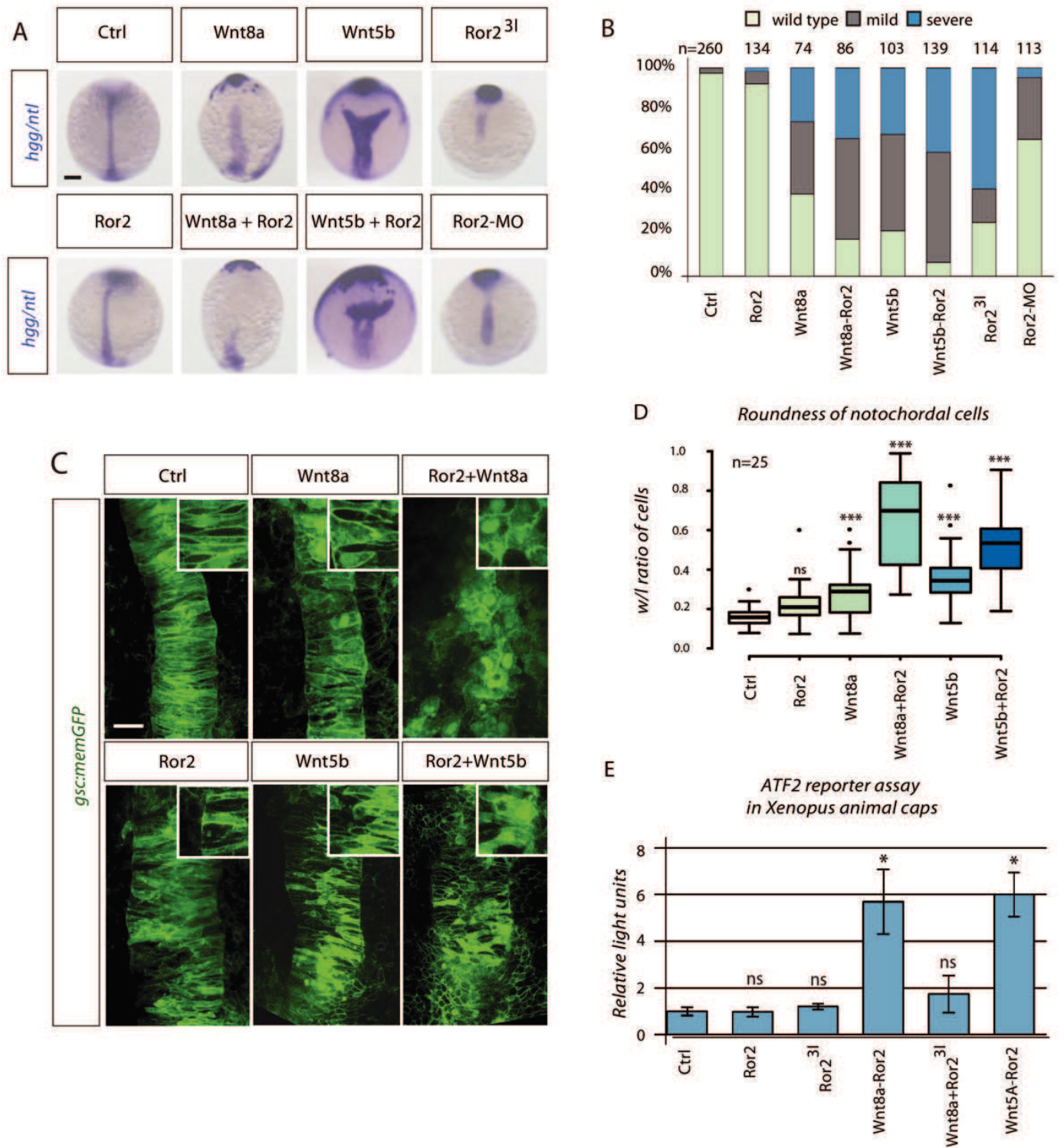


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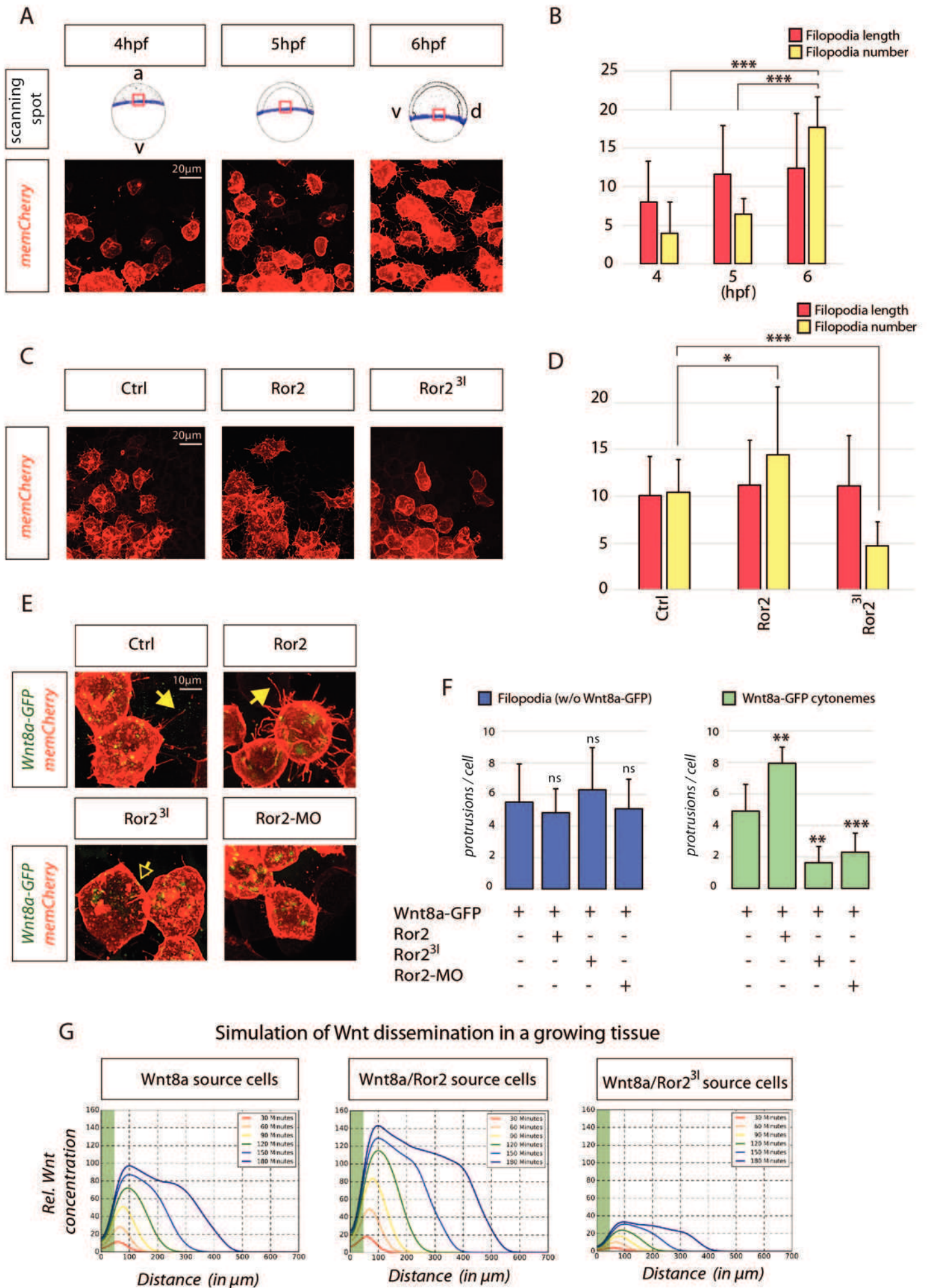


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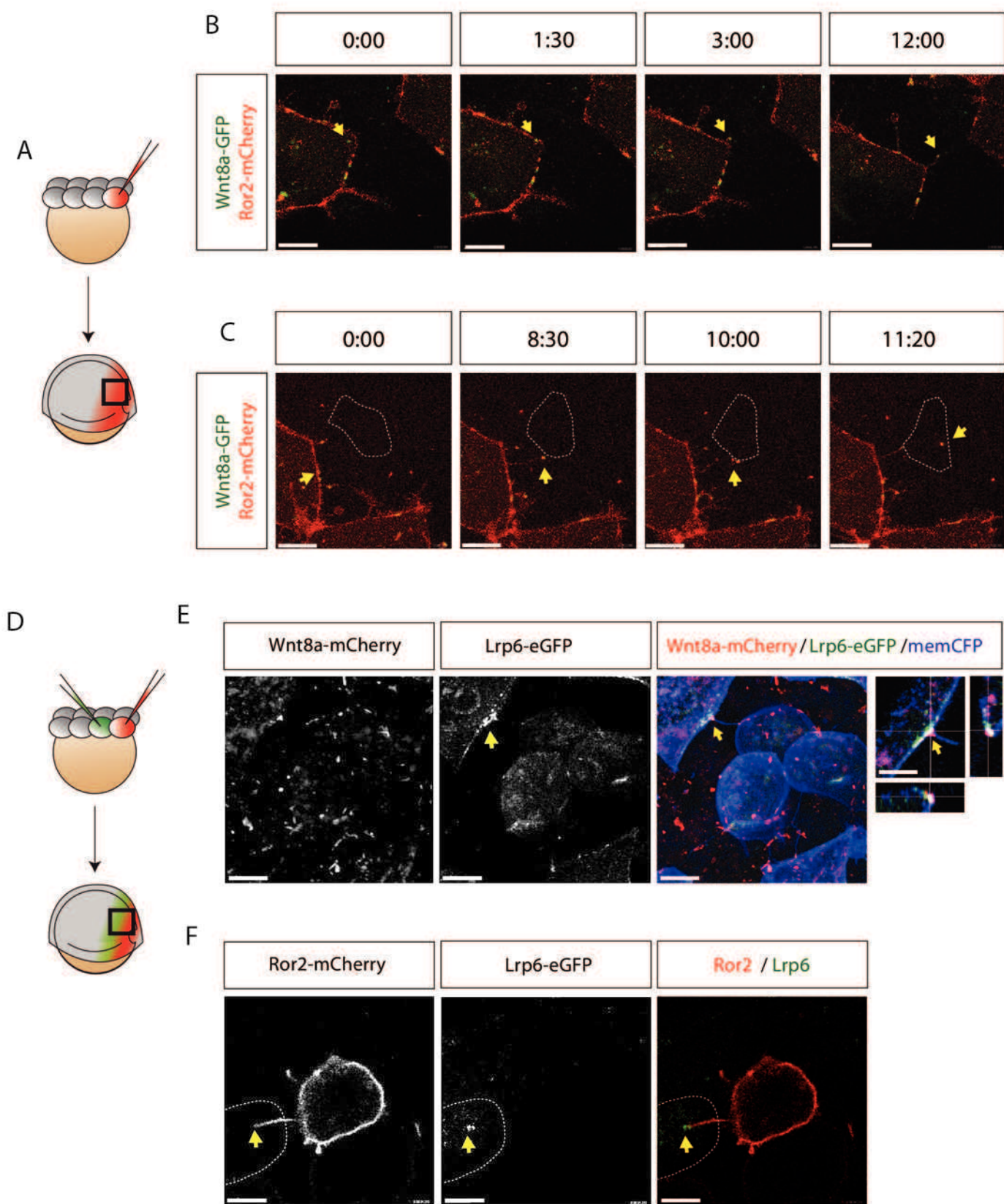


Figure 5; Mattes et al., 2018

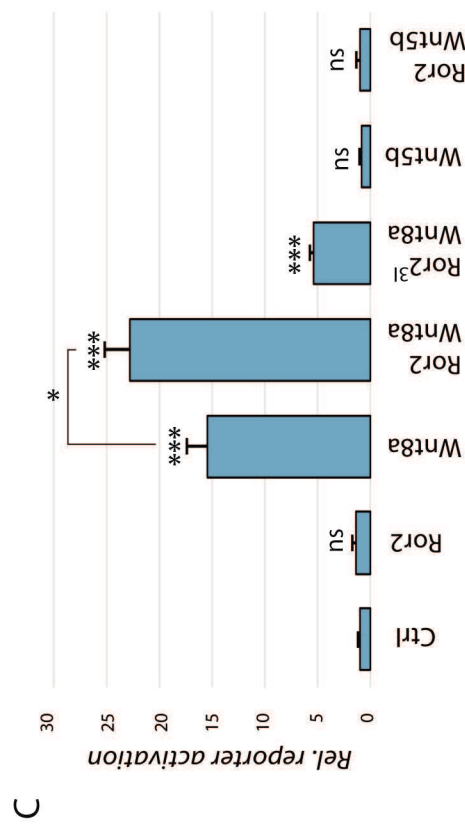
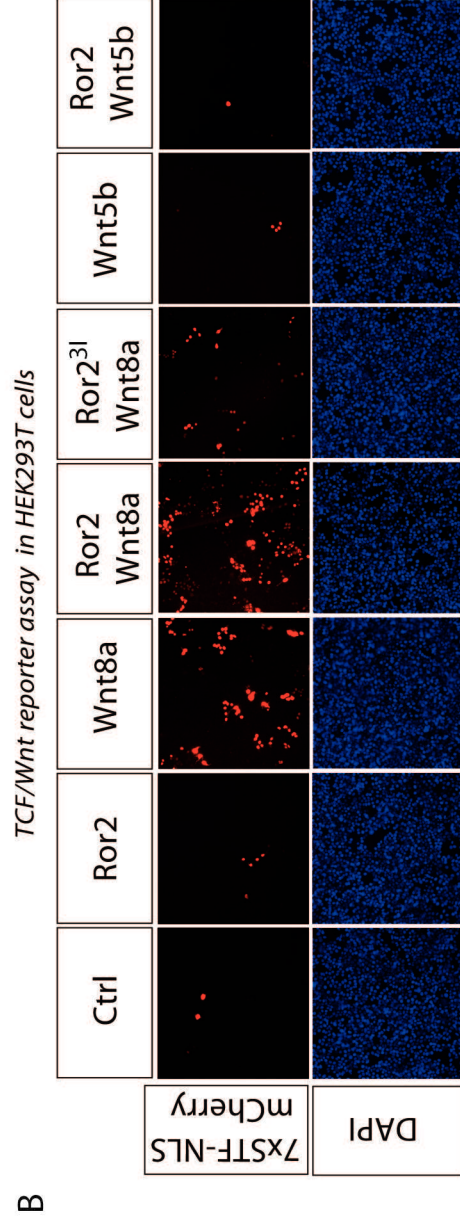
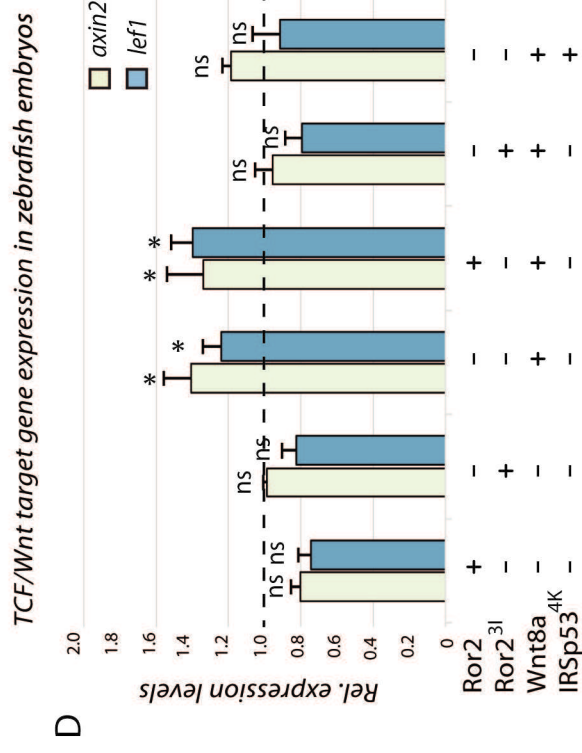
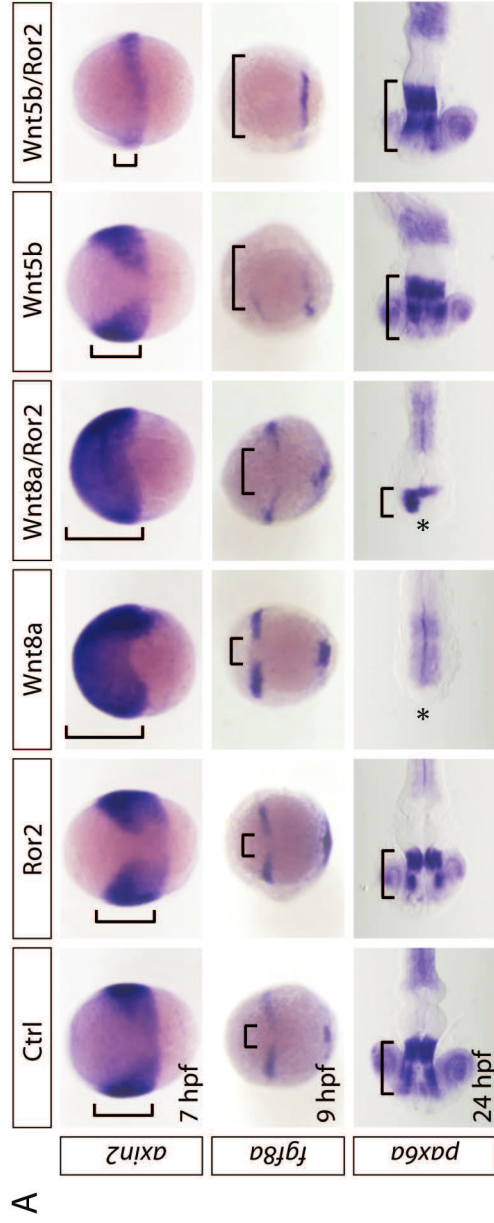


Figure 6; Mattes et al., 2018

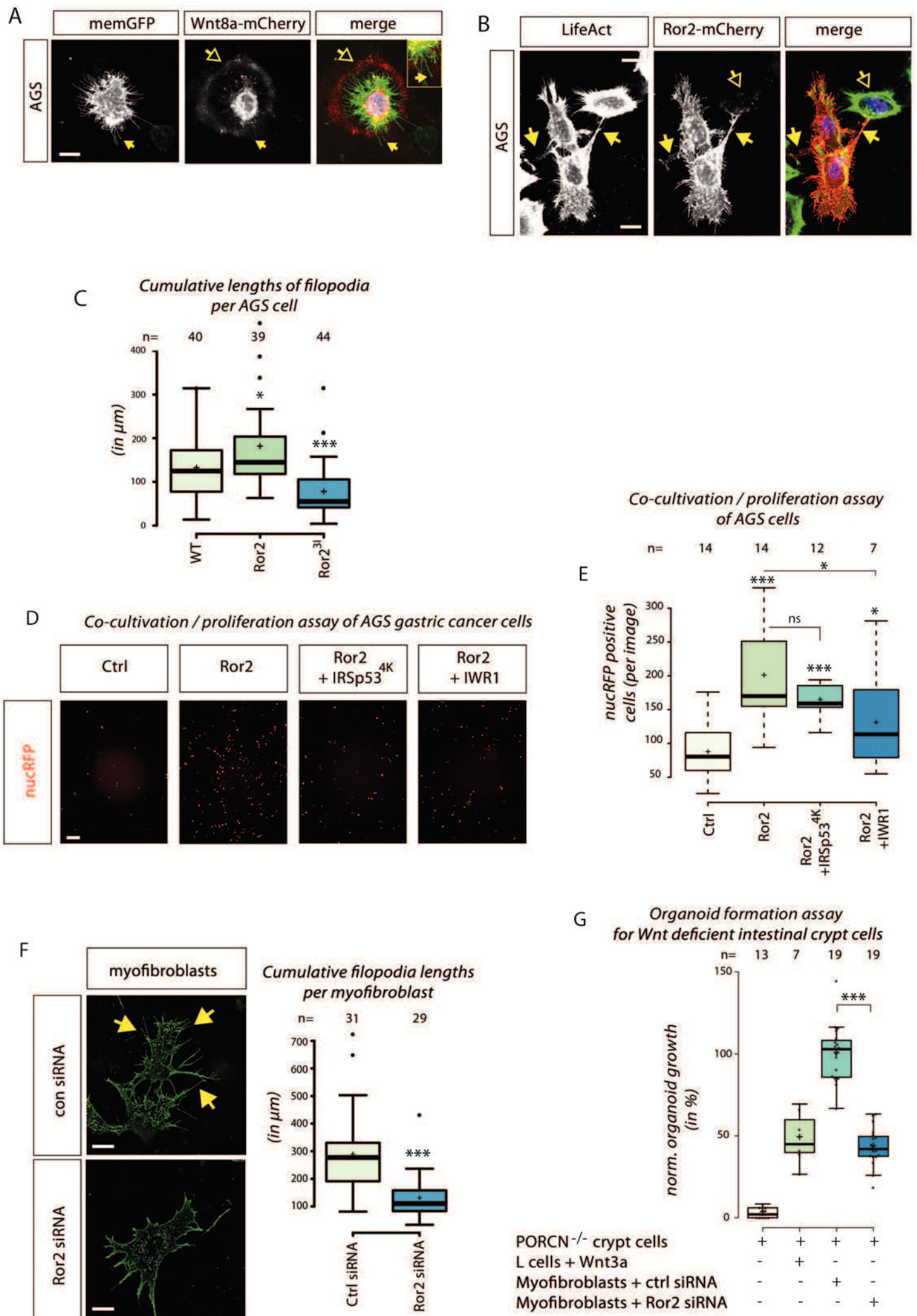
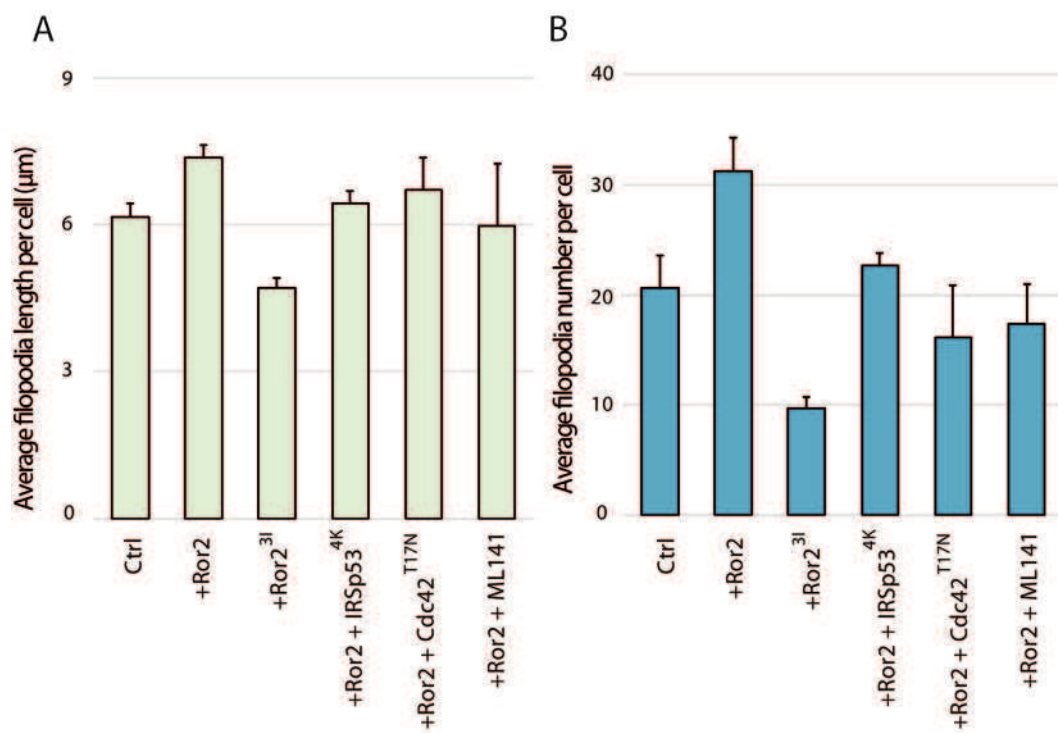
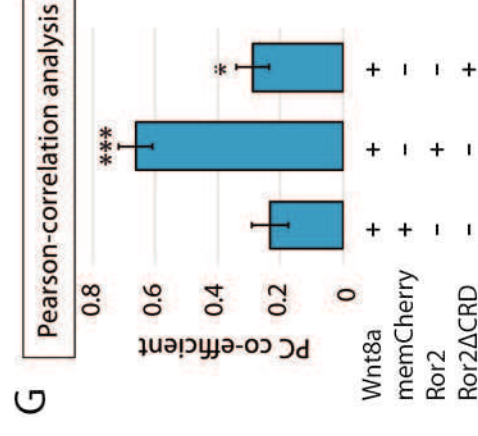
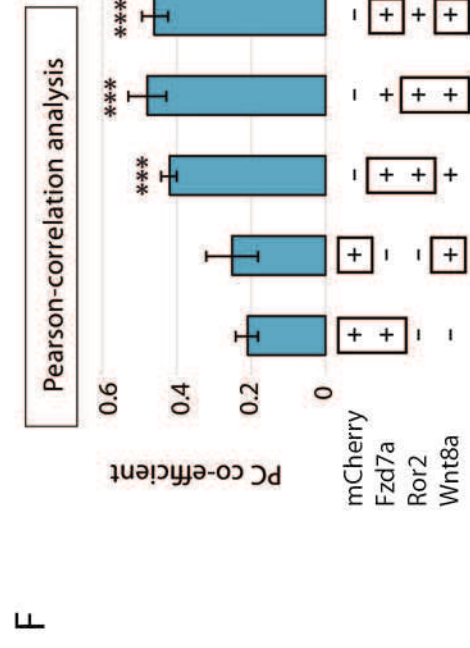
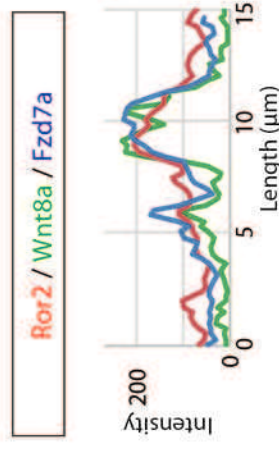
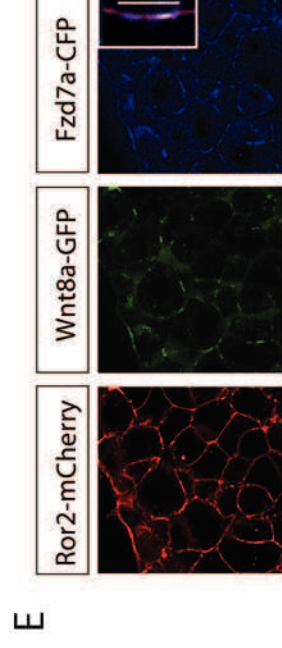
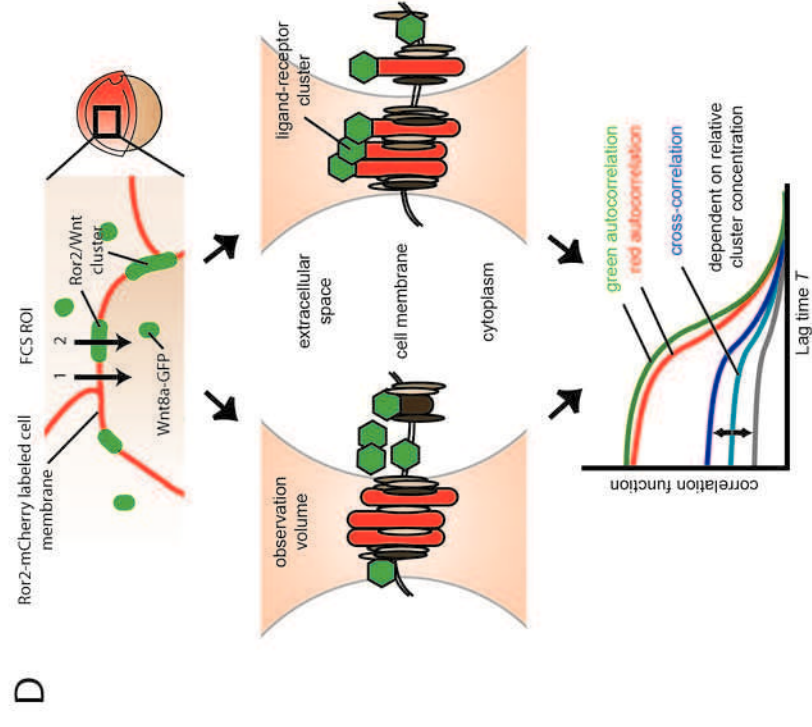
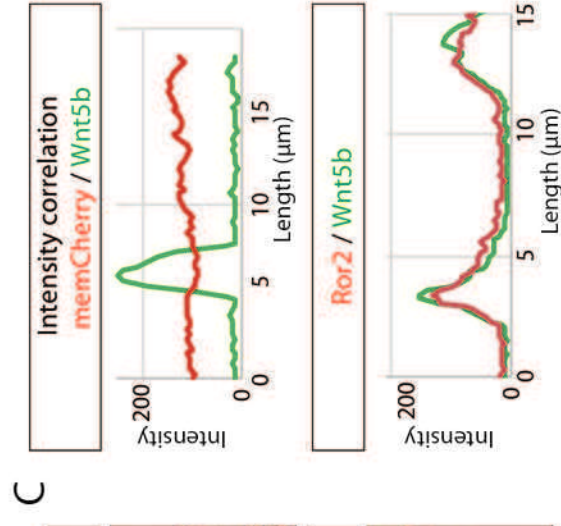
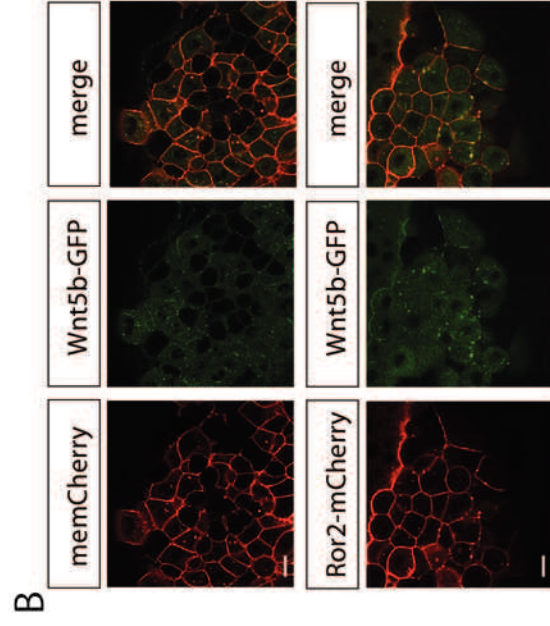
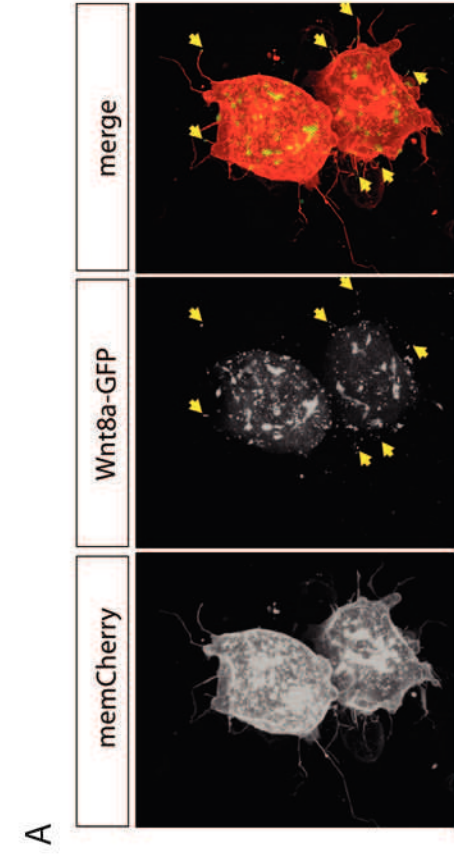
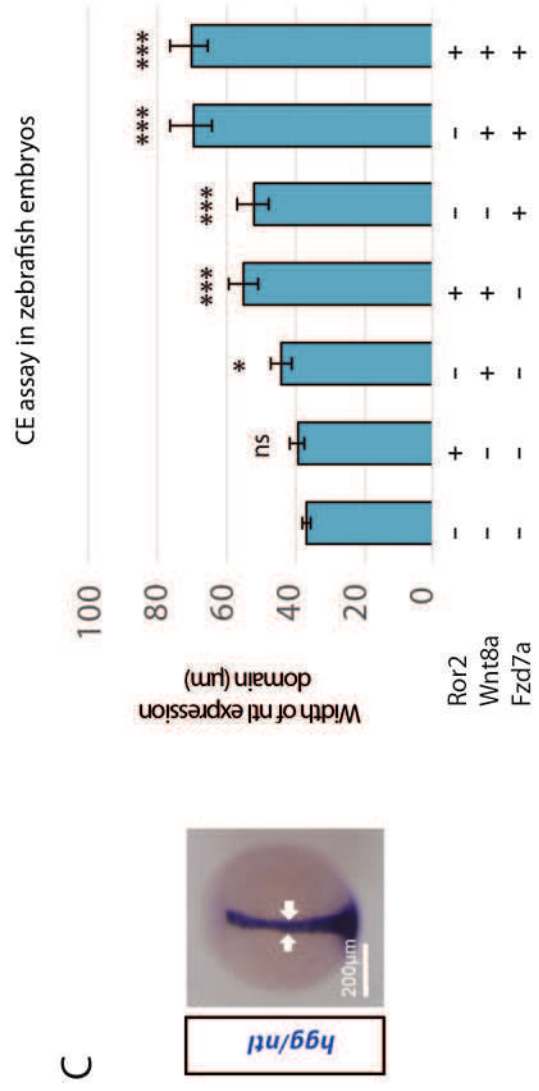
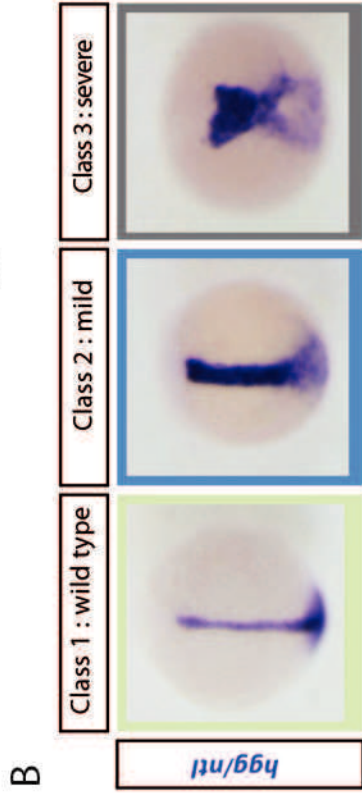
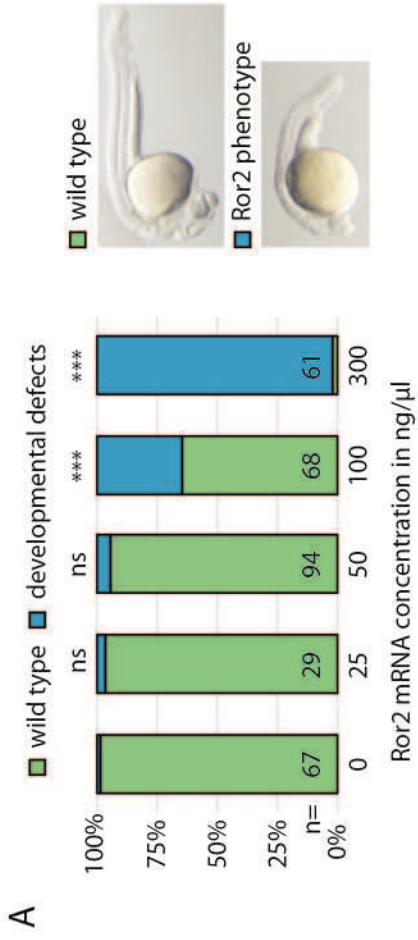


Figure 7; Mattes et al., 2018

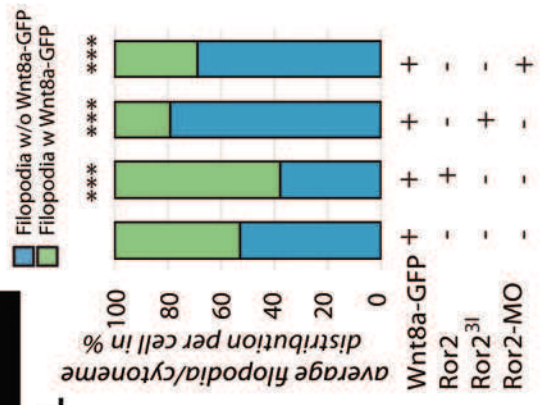
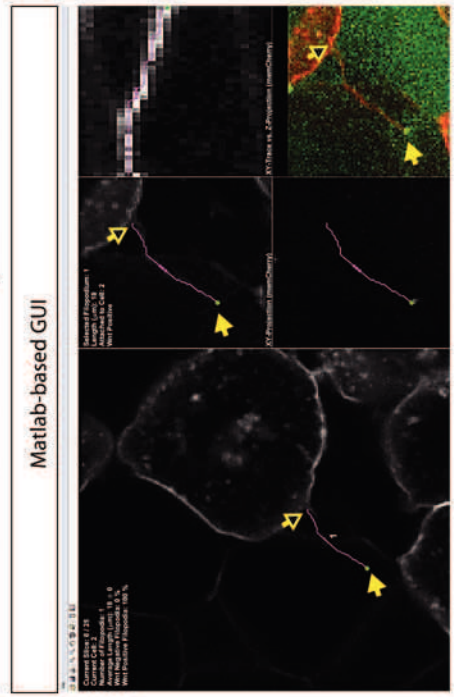
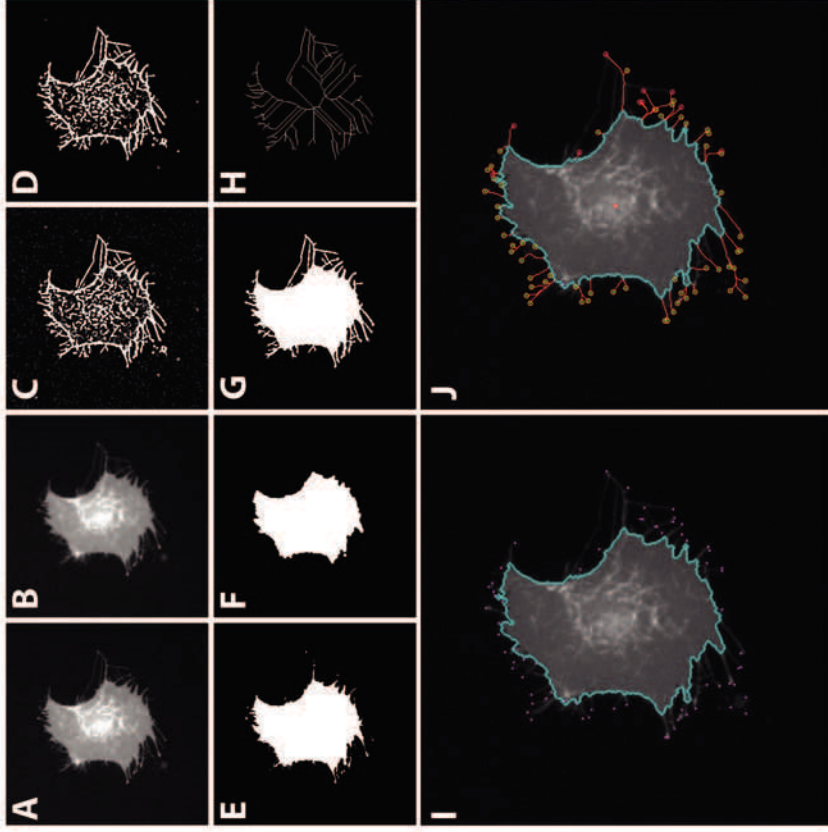


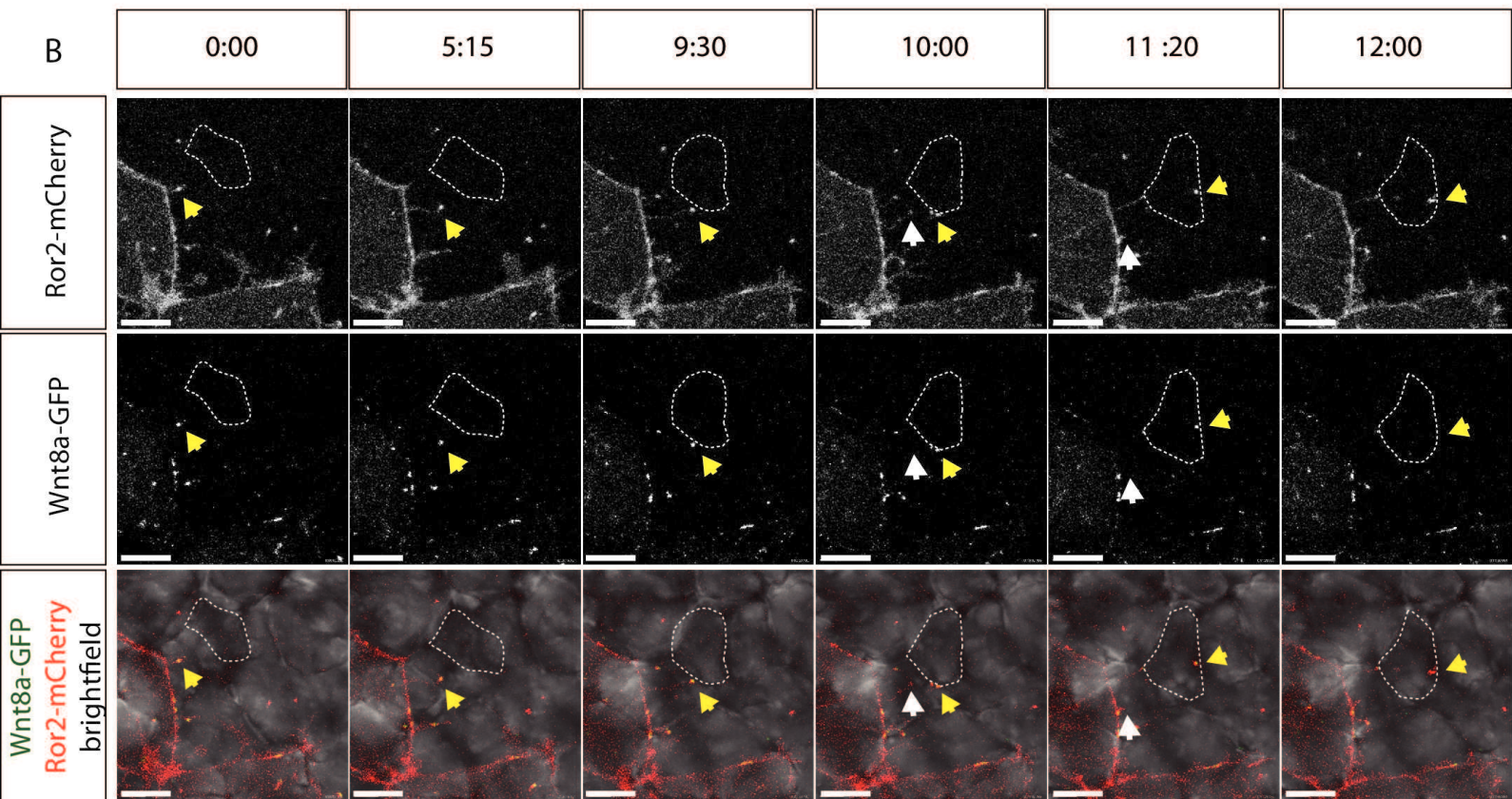
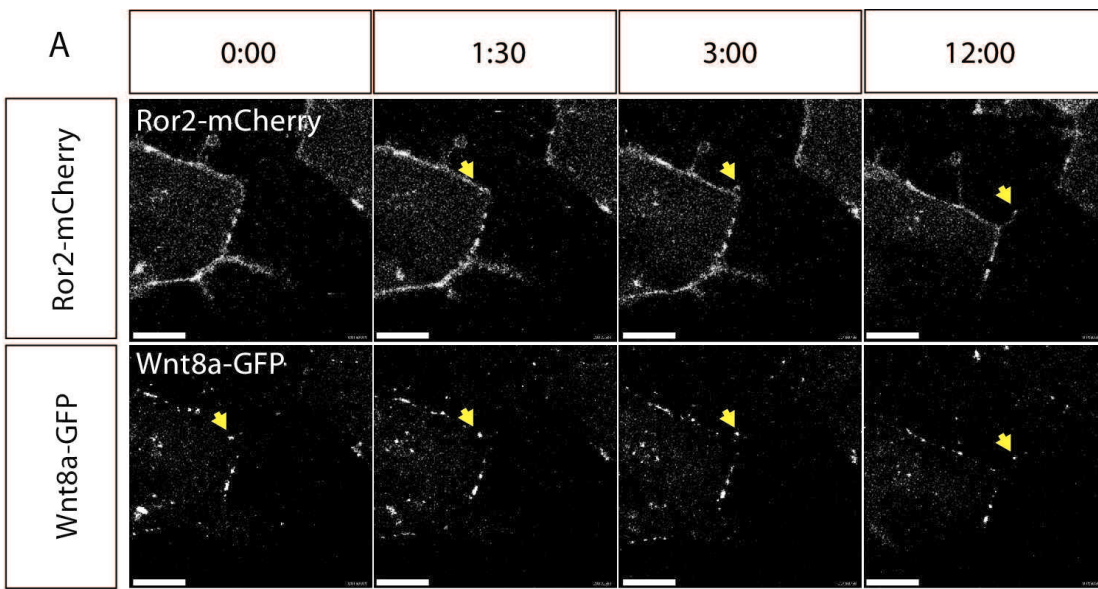
Supplementary Figure 1; Mattes et al., 2017





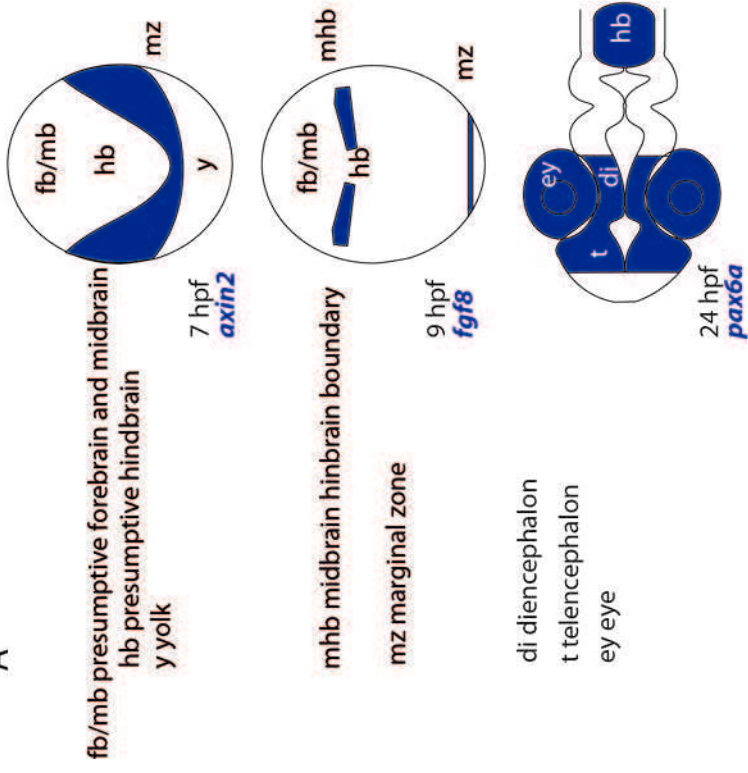
Workflow of the cytoneme quantification software



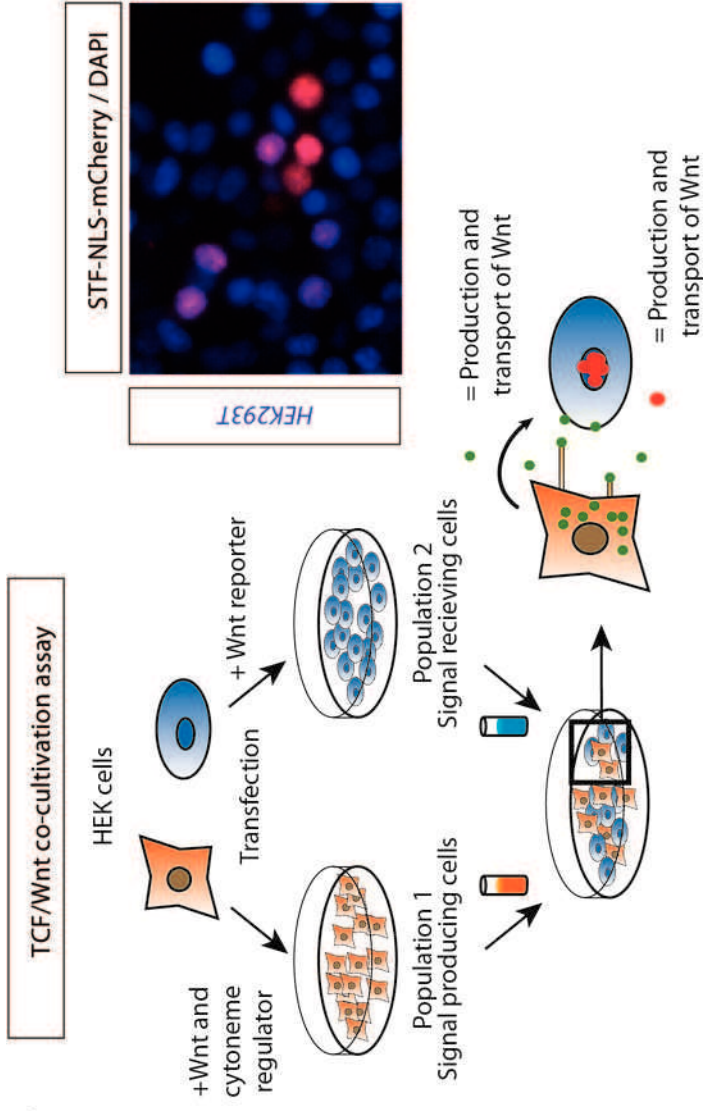


Supplementary Figure 5; Mattes et al., 2018

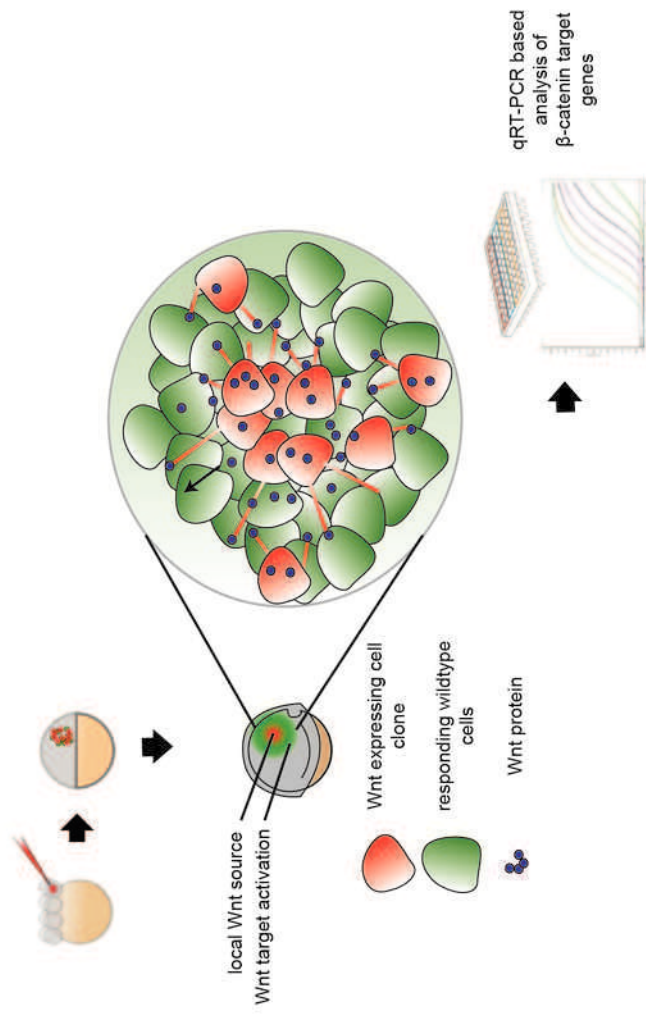
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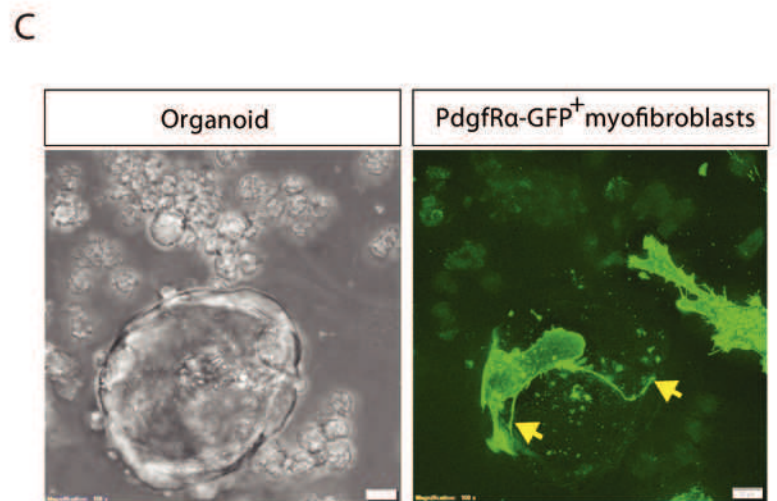
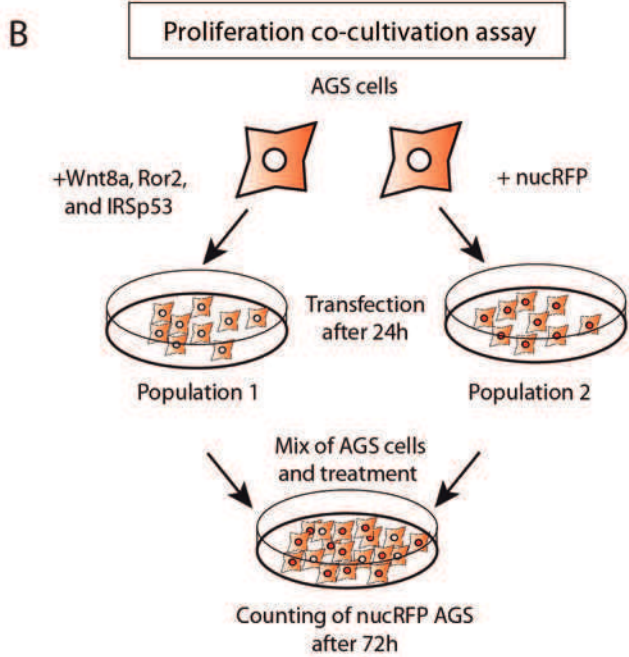
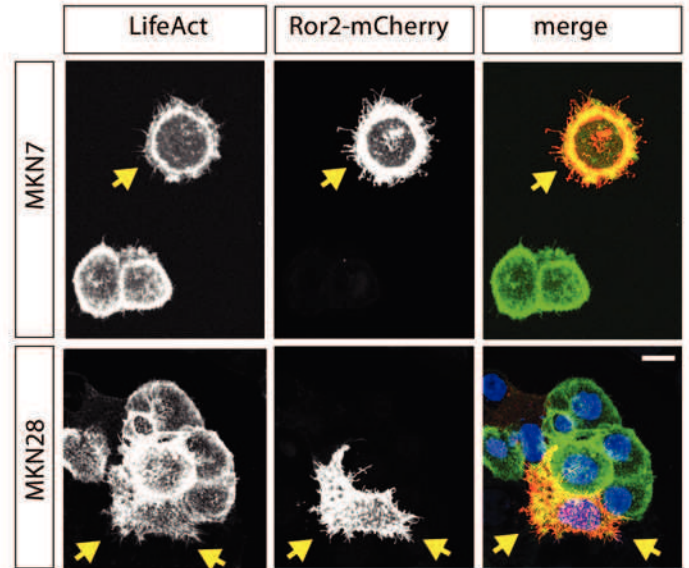
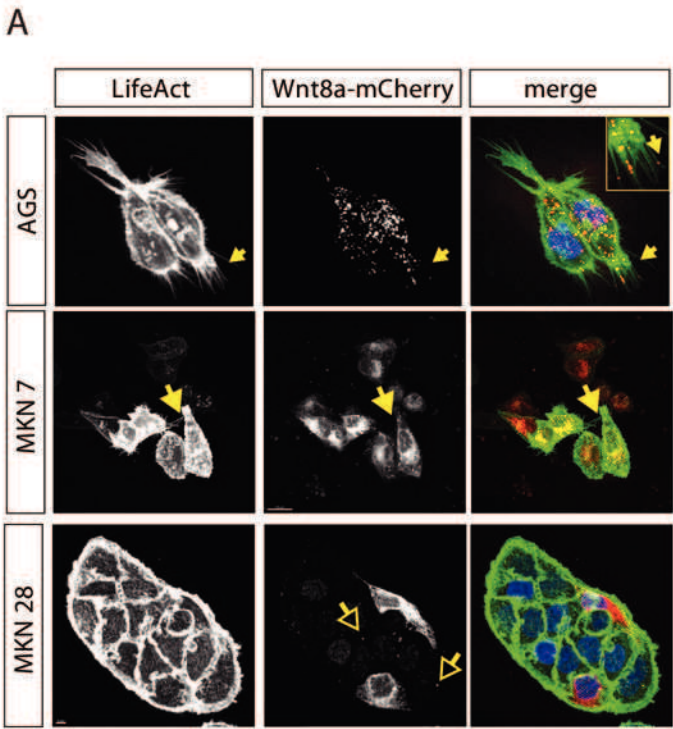


B



C





Supplementary Figure 7; Mattes et al., 2018