

1 Dual functions of Discoidin domain receptor coordinate cell-matrix adhesion and
2 collective polarity in migratory cardiopharyngeal progenitors

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11

12 **ABSTRACT**

13

14 Integrated analyses of regulated effector genes, cellular processes, and extrinsic signals
15 are required to understand how transcriptional networks coordinate fate specification
16 and cell behavior during embryogenesis. Migratory pairs of cardiac progenitors in the
17 tunicate *Ciona* provide the simplest model of collective migration in chordate embryos.
18 *Ciona* cardiopharyngeal progenitors (aka trunk ventral cells, TVCs) polarize as leader
19 and trailer cells, and migrate between the ventral epidermis and trunk endoderm, which
20 influences collective polarity. Using functional perturbations and quantitative analyses,
21 we show that the TVC-specific and collagen-binding Discoidin-domain receptor (Ddr)
22 cooperates with Integrin- β 1 to promote cell-matrix adhesion to the epidermis. We found
23 that endoderm cells secrete a collagen, Col9-a1, that is deposited in the basal epidermal
24 matrix and activates Ddr at the ventral membrane of migrating TVCs. A functional
25 antagonism between Ddr/Int β 1-mediated cell-matrix adhesion and Vegfr signaling
26 appears to modulate the position of cardiopharyngeal progenitors between the
27 endoderm and epidermis. Finally, we show that Ddr activity promotes leader-trailer-
28 polarized BMP-Smad signaling independently of its role in cell-matrix adhesion. We
29 propose that dual functions of Ddr act downstream of cardiopharyngeal-specific
30 transcriptional inputs to coordinate subcellular processes underlying collective polarity
31 and directed migration.

32 During embryonic development, complex tissue-scale movements emerge from
33 collective behaviors of individual cells. Morphogenesis is tissue-specific, indicating that
34 the gene regulatory networks (GRNs) that control cell identity also determine cell
35 behavior. How GRNs control and coordinate cell fate and behavior has been illustrated
36 using as diverse models as mesoderm invagination and migration in *Drosophila* and sea
37 urchins, gastrulation in *Xenopus*, and neural crest cell migration in amniotes¹⁻⁸. Among
38 other essential morphogenetic processes, collective migration is observed in various
39 physiological and pathological conditions such as neural crest cell migration,
40 gastrulation, wound healing, and cancer metastasis^{9, 10}. During collective movements,
41 cells can be of the same identity but adopt different leader and follower states, with
42 distinctive morphologies¹¹. Collective polarity must be established, maintained and
43 coordinated with the direction of movement, and both polarity and directionality arise
44 from anisotropic exposure to extrinsic cues, such as the free edge of leading cells,
45 gradients of secreted molecules from surrounding tissues, or asymmetric distribution of
46 the adjacent extracellular matrix^{12, 13}. Integrative mechanisms must therefore connect
47 regulatory inputs with the production of state-specific cell behavior in response to
48 extrinsic cues.

49
50 The cardiac lineage in the tunicate *Ciona* provides the simplest example of collective
51 cell migration¹³⁻¹⁶. In *Ciona*, multipotent cardiopharyngeal progenitors derive from the
52 B7.5 blastomeres in 110-cell embryos, and later produce fate-restricted heart and
53 pharyngeal muscle precursors¹⁷⁻²⁰. On either side of tailbud embryos, pairs of
54 cardiopharyngeal progenitor cells (aka trunk ventral cells, TVCs) collectively polarize
55 and migrate between the ventral epidermis and the trunk endoderm, until they stop and
56 produce distinct fate-restricted progenitors^{13, 14, 16, 19}. During migration, the leader TVC
57 extends dynamic protrusions and generates a broad leading edge, while the trailer
58 terminates in a tapered retraction end^{13, 16}. Before migration, the surrounding trunk
59 endoderm preferentially contacts the prospective leader cell, and experimental
60 perturbation of secretion in endoderm cells disrupts collective leader-trailer (LT)
61 polarity¹³, suggesting that anisotropic exposure to biochemical cues secreted by the
62 endoderm contributes to collective TVC polarity. Moreover, B7.5-lineage-specific
63 transcriptional inputs from *Mesp*, FGF/MAPK signaling and *Foxf* control and coordinate
64 cardiopharyngeal fate specification and TVC migration^{15, 16, 18, 21}, and the transcriptome of
65 migratory TVCs have been extensively profiled^{16, 22-24}. Therefore, TVC migration provides

66 an attractive model to functionally connect fate-specific transcriptional inputs with
67 cellular effectors and extrinsic cues governing collective polarity and directed migration.

68

69 Among regulated cellular effectors possibly shaping cell responses to extrinsic cues,
70 we identified several receptor tyrosine kinases (RTKs)¹⁶. Other developmental cell
71 behaviors have been shown to involve RTK signaling, including for collective polarization
72 of migratory cell groups in vertebrates and *Drosophila*^{10, 25-30}. We thus selected a small
73 group of candidate RTKs, and analyzed their roles in TVC migration. Among these
74 candidates, the sole *Ciona* homolog of *Discoidin domain receptor (Ddr)* appeared to be
75 upregulated specifically in newborn TVCs, downstream of both FGF/MAPK signaling
76 and *Foxf* inputs¹⁶. DDRs are single pass transmembrane receptors that bind extracellular
77 collagen^{31, 32}, they can mediate weak adhesion to collagen and regulate cadherins,
78 integrins and ECM interacting proteins to modulate cell-matrix adhesion³³⁻³⁵.

79 Here we describe the functions of *Ddr* and its interactions with integrin, *Vegfr* and
80 BMP-Smad signaling in regulating cell-matrix adhesion and collective polarity during
81 TVC migration. We developed methods to quantify TVC morphology and movements,
82 and defined an experimental and analytical framework to study morphogenetic
83 determinants. We found that the endoderm secretes a type IX collagen, *Col9-a1*, that is
84 deposited onto the basal epidermal matrix and activates *Ddr* at the ventral surface of the
85 migrating TVCs, thus promoting integrin-based cell-matrix adhesion. We dissected a
86 signaling antagonism between *Ddr*/Integrin-mediated cell-matrix adhesion and *Vegfr*
87 signaling. Finally, we show that *Ddr* promotes polarized BMP-Smad signaling, thus
88 contributing to establishing distinct leader and trailer states. The latter function appears
89 independent from integrin-mediated cell-matrix adhesion, suggesting that dual
90 functions of *Ddr* coordinate collective polarity and cell-matrix adhesion during TVC
91 migration.

92

93 **RESULTS**

94

95 **Cardiopharyngeal progenitors express Receptor Tyrosine Kinases**

96 Transcriptome profiling identified candidate signaling molecules potentially involved
97 in guiding the movement of Ciona cardiopharyngeal progenitors, the TVCs. We used
98 whole mount *in situ* hybridization to confirm and better characterize the expression of
99 four candidate RTK-coding genes, *Discoidin domain receptor (Ddr)*, *Vascular*
100 *endothelium growth factor receptor (Vegfr)*, *Fibroblast growth factor receptor (Fgfr)*,
101 and epidermal growth factor receptor (*Egfr*). We did not detect *Egfr* transcripts prior to
102 or during TVC migration, neither did it show signs of being functional in overexpression
103 of truncated dominant negative forms (Figure S1, Movie 5), we thus excluded *Egfr* from
104 further analysis (Figure S1). *Ddr*, *Vegfr*, and *Fgfr* transcripts were detected in migrating
105 TVCs (Figures 1B, S1). *Vegfr* and *Fgfr* were expressed in B7.5 lineage founder cells, while
106 newborn TVCs upregulated *Ddr* before the onset of collective migration (Figure S1).

107

108 The transcription factor *Foxf* was proposed to act as a key transcriptional regulator of
109 TVC migration¹⁵, and transcription profiling identified candidate target genes, including
110 *Ddr*¹⁶. Using B7.5-lineage-specific CRISPR/Cas9-mediated mutagenesis with *Foxf*
111 targeting single guide RNAs (sgRNAs;³⁶), we confirmed that loss of *Foxf* function
112 inhibited *Ddr* expression in the TVCs (Figure 1B,C). By contrast, *Foxf* function appeared
113 dispensable for *Vegfr* or *Fgfr* expression, a finding also consistent with previous
114 microarray analyses¹⁶. Parallel studies indicated that *Fgfr* is primarily required for
115 MAPK-dependent transcriptional regulation in migrating TVCs and beyond³⁷. Taken
116 together, these data led us to focus on *Ddr* and *Vegfr* as candidate cell migration
117 effectors, which we sought to further characterize.

118

119 **Ddr and Vegfr functions are required for proper TVC migration**

120 Wild type TVC migration is a highly stereotypical process. Soon after birth, pairs of
121 cousin TVCs collectively polarize to assume leader and trailer positions, potentially
122 through differential contacts with the mesenchyme and the trunk endoderm¹³, and these
123 relative positions are maintained throughout migration. The cells move anteriorly,
124 between the ventral epidermis and trunk endoderm^{13,16}, until they reach a final position
125 adjacent to the midline, where they divide asymmetrically to generate the first and
126 second heart precursors, and atrial siphon muscle precursors^{19,20}. To better characterize

127 TVC migration in control and experimental conditions, we developed quantitative
128 parameters describing cell movements in live embryos. We used an epidermal transgene,
129 *EfnB>hCD4::mCherry*¹³, and a B7.5-lineage-specific nuclear marker, *Mesp>H2B::GFP*,
130 to label live embryos and migrating cardiopharyngeal progenitors and imaged them
131 using time lapse confocal microscopy for the duration of TVC migration (Figure 1D,
132 movies S1-S5). To quantify TVC movements in four dimensions, we used morphological
133 landmarks in the epidermis to define sagittal and frontal planes. We used GFP+ TVC
134 nuclei to define the leader-trailer (LT) axis, and calculated the angles it formed with
135 sagittal and frontal planes at each time point (Figures 1D, S2A, Methods). We used cell
136 division times to align temporal axes and combine time-lapse recordings from multiple
137 embryos (n=7). In this way, we found that the TVC migration path is highly constrained
138 in control embryos, with LT angles with frontal and sagittal planes changing within
139 defined ranges as cells migrate (Figures 1D,G, S2).

140 To study the functions of selected RTKs during TVC migration, we generated passive
141 dominant negative versions of Ddr and Vegfr (henceforth referred to as dnDdr and
142 dnVegfr), by deleting the cytoplasmic kinase domain of each receptor. We expressed
143 these constructs in the newborn TVCs using a defined *Foxf* enhancer¹⁵, and assayed
144 migration quantitatively. TVCs expressing *Foxf*-driven dnDdr retained their ability to
145 initiate migration, but cells tumbled, failing to maintain relative leader/trailer positions
146 and followed more variable migration paths (Figure 1D-G, Movie S2). DnDdr
147 misexpression also caused a decrease in total displacement of TVCs and in path
148 straightness (Figure 1E,F) consistent with altered directionality (Supplemental Fig. 2).
149 These observations indicate that proper function of the *Foxf* target Ddr is required for
150 maintenance of collective polarity and directional migration of the TVCs.

151
152 TVCs expressing *Foxf*-driven dnVegfr were also able to initiate migration, and their
153 total displacement was comparable to control cells (Figure 1G). However, dnVegfr
154 expression increased track straightness, as if extrinsic constraints canalized migration
155 more efficiently than in control embryos¹³ (Figure 1F). To quantify the variability of cell
156 behaviors, we calculated the standard deviation of the angles between LT axes and the
157 sagittal and frontal planes at each time point during migration. As expected, dnDdr
158 increased the standard deviation of the LT angle relative to both the sagittal and the
159 frontal plane, reflecting the variable positions of tumbling TVCs. By contrast, dnVegfr

160 only increased the standard deviation of the LT angle relative to the sagittal plane, which
161 is consistent with cells remaining constrained in at least one dimension (Figure 1G).

162

163 **Ddr promotes integrin-based cell-matrix adhesion to the epidermis**

164 We first focused on understanding how Ddr regulates TVC polarity and migration,
165 and analyzed changes in cell morphology and contacts with surrounding tissues¹³. We
166 used defined transgenes to label TVCs' nuclei and membranes, as well as surrounding
167 tissues, and used confocal imaging and computational segmentation of individual cells to
168 quantify morphometric parameters, including cell sphericity and the percentage of total
169 cell surface contacting the underlying epidermis. By revealing quantitative differences
170 between leader and trailer cells, these measurements allowed us to characterize
171 collective polarity in control and experimental conditions.

172 Sphericity is calculated as the ratio between the surface area of a sphere of the same
173 volume as the object of interest and the surface area of that object. The maximum
174 sphericity would thus be 1 if the cell was a perfect sphere, and it decreases in migratory
175 cells that protrude and flatten on a 2D substrate. Our analysis finds that the leader TVC
176 is less spherical than the trailer, which is consistent with its greater protrusive activity¹⁶.
177 Approximately 20% of the leader cell surface engages in the cell-cell junction with the
178 trailer (Figure S3). At the same time, 45-50% of the leader surface contacts the
179 underlying epidermis compared to 40-45% for the trailer (Figure 2A,C). Expression of
180 dnDdr significantly increased the sphericity of the leader, abolishing leader-trailer
181 differences (Figure 2A,B). Concurrently, dnDdr significantly reduced the surface of TVC
182 contact with the underlying epidermis (Figure 2A,C). dnDdr-expressing TVCs contacted
183 the epidermis with about 30% of their surface on average with 35% of the cells
184 completely detaching from the epidermis. Complementary loss-of-function approaches
185 validated the specificity of the dnDdr construct, as lineage-specific RNAi using short
186 hairpin microRNA (shmiR) constructs synergized with suboptimal doses of dnDdr to
187 cause a penetrant "de-adhesion" phenotype (Figure S4). These data suggest that Ddr is
188 required for the TVCs to maintain contact with the epidermis during migration.

189

190 Phenotypes produced by dnDdr suggested that cells failed to maintain adhesion to
191 the extracellular matrix lining the ventral epidermis. For instance, Ddr homologs can
192 mediate weak adhesion to collagen, and increase cells' adhesion to collagens through
193 integrins^{34, 38}. We thus sought to test whether Ddr contributes to cell-matrix adhesion,

194 and identify its possible integrin partners in the migrating TVCs. Using whole mount
195 fluorescent *in situ* hybridization assays and TVC-specific transcriptome profiling data¹⁶,
196 we detected expression of Integrin- β 1 (Int β 1), among several other candidates
197 (Supplementary Figure 5). We generated a dominant negative form of Int β 1 (dnInt β 1) by
198 truncating the C-terminal cytoplasmic domain³⁹, and assayed cell sphericity and the
199 ability of dnInt β 1-expressing TVCs to contact the epidermis (Figure 2A-C). Altering Int β 1
200 function increased cells' sphericity to levels comparable to those observed with dnDdr
201 (Figure 2B). Furthermore, dnInt β 1-expressing TVCs contacted the epidermis with only
202 20 to 30% of their surface. Compared to control cells, this reduction is similar to that
203 observed with dnDdr. Finally, dnInt β 1 also increased the variability of TVC position
204 during migration, similar to dnDdr (Figure 2D). Taken together, these data indicate that
205 altering Integrin- β 1 and Ddr functions cause similar phenotypes characterized by a loss
206 of adhesion to the ventral epidermis, cell rounding and unstable collective polarity. We
207 thus conclude that Ddr, like Integrin- β 1, functions primarily to promote cell-matrix
208 adhesion at the contact with the ventral epidermis, and this adhesion stabilizes collective
209 polarity, permitting directed migration.

210

211 Since both Ddr and Int β 1 can function as collagen receptors^{31, 32}, we sought to test
212 whether they interact to regulate cell-matrix adhesion. We quantified TVCs' contacts
213 with the epidermis following dnDdr and/or dnInt β 1 expression. Co-expression of
214 suboptimal doses of dnDdr and dnInt β 1 aggravated the detachment phenotype, but to a
215 lesser extent than expected for additive effects (Figure 2A,C). This suggests that Ddr and
216 Int β 1 function primarily in overlapping pathways to regulate TVC/epidermis adhesion.
217 To further characterize the functional relationships between the two collagen-receptors,
218 we asked whether Int β 1 is required for Ddr activation. To assay localization and
219 activation of full-length Ddr, we used the minimal *Foxf* TVC enhancer and expressed a
220 3xHA-tagged version of Ddr at minimal detectable levels, to avoid non-specific
221 localization and over-expression phenotypes. We then performed immunohistochemistry
222 (IHC) combined with a proximity ligation assay (PLA) using anti-HA and anti-phospho-
223 Tyrosine antibodies to visualize the phosphorylated form of Ddr^{38, 40}. Full-length
224 Ddr::3xHA proteins were localized to both intracellular vesicles and the ventral plasma
225 membrane (Figures 2E). However, activated Ddr preferentially localized to the ventral
226 TVC surface, which contacts the epidermis (Figure 2E). Quantitative analysis showed

227 that, at any given time, 12 to 20% of tagged Ddr proteins were phosphorylated,
228 suggesting that there is a large pool of inactive Ddr proteins within the cells.

229 To test whether Ddr activation on the ventral/epidermal side of migrating TVCs
230 requires integrin-based cell-matrix adhesion, we repeated the PLA assays in embryos
231 expressing the B7.5 lineage-specific *Mesp>dnIntβ1* transgene. We found that dnIntβ1
232 misexpression reduced Ddr phosphorylation levels to approximately 6%, and the
233 enrichment of activated Ddr on the ventral cell surface of the TVCs was lost (Figure
234 2E,F), indicating that Intβ1 activity is required to localize and activate Ddr on the
235 ventral/epidermal side of migrating TVCs. Taken together, these observations reinforce
236 the notion that Ddr and Intβ1 interact to promote TVC adhesion to the extracellular
237 matrix on the epidermal side of the cells.

238

239 **The endoderm contributes to cell-matrix adhesion and Ddr activation**

240 Previous work showed that expressing a dominant negative form of the small GTPase
241 Sar1 (dnSar1) with the endoderm-specific *Nkx2-1* enhancer inhibited ER-to-Golgi
242 transport and secretion from the endoderm, and caused a tumbling phenotype
243 reminiscent of that observed with dnDdr and dnIntβ1¹³. To determine if the *Nkx2-*
244 *1>dnSar1* construct caused the TVCs to detach from the epidermal matrix during
245 migration, we quantified contacts between the TVCs and the epidermis. As was observed
246 with TVC-specific dnDdr/dnIntβ1 misexpression, *Nkx2-1>dnSar1*-mediated inhibition of
247 secretion in the endoderm caused a significant reduction of the TVC surface contact with
248 the epidermis (Figure 3B), suggesting secretion from the endoderm enables cell-matrix
249 adhesion between the migrating TVCs and the ventral epidermis.

250 To test whether endodermal cues potentiate Ddr function in the TVCs, we combined
251 secretion inhibition in the endoderm with TVC-specific dnDdr misexpression and
252 quantified contacts between the TVCs and the epidermis. We observed a significant
253 enhancement of the TVC detachment phenotype by combining *Nkx2-1>dnSar1* and
254 *Foxf>dnDdr* compared to the sum of either perturbation alone (Figure 3B,C), suggesting
255 that the secreted endodermal cue and Ddr function in partially redundant pathways. To
256 further probe the functional interaction between the endoderm and Ddr, we tested if
257 secretion from the endoderm is required for Ddr activation in migrating TVCs. PLA
258 analysis revealed that loss of endodermal secretion decreased the fraction of
259 phosphorylated Ddr on the ventral/epidermal side of migrating cells (Figure 3C,D),

260 suggesting a functional relationship between (a) secreted endodermal cue(s) and the
261 collagen receptor Ddr.

262

263 **Endodermal *Col9-a1* is required for Ddr activation and cell-matrix adhesion**
264 **at the TVC/epidermis interface**

265 As Ddr is a collagen receptor, we hypothesized that that potential endodermal
266 secreted cues could include collagens. *Col9-a1* expression was reported in the endoderm
267 of developing *Ciona* embryos where it contributed to the development of the intestine⁴¹.
268 We confirmed that *Col9-a1* is expressed in the endoderm adjacent to the ventral
269 epidermis prior to the onset of TVC migration (Figure 4A), thus potentially acting as a
270 source of extracellular collagen for subsequent TVC migration. Other *Col9-a1* isoforms
271 are also expressed strongly in the notochord and the endodermal strand that runs the
272 length of the tail (Figure 4A).

273

274 To test whether *Col9-a1* is the endoderm-derived extracellular cue that activates Ddr
275 and promotes cell-matrix adhesion to the ventral epidermis, we used CRISPR/Cas9 to
276 mutagenize *Col9-a1* in the endoderm by expressing Cas9 using an early vegetal enhancer
277 from the gene *FoxD*⁴²⁻⁴⁴. Two single guide RNAs (sgRNAs) targeting *Col9-a1* exons were
278 used to generate large deletions in the gene, and we assayed contacts between the TVCs
279 and ventral epidermis. *Col9-a1* gene inactivation in vegetal endoderm progenitors
280 produced a detachment phenotype that mimicked the dnDdr and dnSar1 phenotypes,
281 indicating that collagen *Col9-a1* secretion from the endoderm is required for cell-matrix
282 adhesion on the ventral epidermal side of migrating TVCs (Figure 4B).

283

284 Since loss of *Col9-a1* function phenocopied dnDdr misexpression, and Ddr
285 presumably bind collagens, we sought to test whether endodermal *Col9-a1* is required to
286 activate Ddr in the TVCs. We used CRISPR/Cas9 to mutagenize *Col9-a1* in the endoderm
287 and quantified Ddr activation by proximity ligation assay as described above. Loss of
288 *Col9-a1* in the endoderm reduced Ddr activation and virtually abolished its localization
289 at ventral membrane contacting the epidermis (Figure 4C), as previously shown upon
290 inhibition of endodermal secretion. Therefore, *Col9-a1* is likely the collagen ligand
291 responsible for Ddr activation in the TVCs.

292

293 As TVCs migrate into the trunk, they intercalate between the endoderm, which
294 deforms and envelops their dorsal surface, and the epidermis, which remains ventral and
295 seemingly provides the substrate for TVC migration^{13, 16}. Since Col9-a1 appears to
296 originate from the endoderm, but activate Ddr on the ventral side where TVCs contact
297 the epidermis, we sought to visualize Col9-a1 localization in the extracellular matrix. We
298 generated a Col9-a1::GFP fusion, which we expressed in the endoderm using the *Nkx2-1*
299 enhancer (*Nkx2-1>Col9-a1-1::GFP*). Col9-a1::GFP was readily secreted from the
300 endoderm and accumulated in the extracellular matrix (Figure 4C). We found that,
301 although the endoderm was the only source of Col9-a1::GFP in these embryos, the GFP
302 signal was detectable in-between the migrating TVCs and the ventral epidermis, whereas
303 the TVC/endoderm interface was devoid of Col9-a1::GFP. We verified that all Col9-
304 a1::GFP proteins accumulated in large vesicles inside endoderm cells upon
305 misexpression of *dnSar1* (Figure 4D). This result is consistent with the possibility that
306 endoderm-specific expression of *dnSar1* blocks Col9-a1 secretion, which could explain
307 the effects of *Nkx2-1>dnSar1* on Ddr activation and TVC-matrix adhesion.

308 We attempted to rescue the *Nkx2-1>dnSar1*-induced phenotypes by providing Col9-
309 a1::GFP from an alternative source. Expressing Col9-a1::GFP from the epidermis using
310 an *EfnB* driver¹³ restored the extracellular GFP signal in the matrix lining the ventral
311 epidermis (Figure 4D, gray scale panel). We then quantified contacts between TVCs and
312 the ventral epidermis following misexpression of *dnSar1* in the endoderm, together with
313 *EfnB>Col9-a1::GFP* or *EfnB>GFP* as control. Remarkably, resupplying Col9-a1 to the
314 epidermal ECM was sufficient to rescue the TVC adhesion phenotype induced by *Nkx2-1>dnSar1*
315 expression (Figure 4E). This demonstrates that a primary role for the
316 endoderm in TVC migration is to deposit Col9-a1 onto the ventral epidermal ECM, most
317 likely prior to the migration of the TVCs, which then rely on this cue to activate Ddr and
318 adhere to their epidermal substrate.

319

320 **An antagonism between Vegfr signaling and Col9-a1/Ddr/Intβ1 functions** 321 **modulate TVC adhesion to the epidermal matrix**

322 In principle, excessive cell-matrix adhesion would cause TVCs to flatten onto to the
323 ventral epidermis. We occasionally observed this phenotype following TVC-specific
324 misexpression of *dnVegfr*, although this effect was subtle and did not significantly
325 change cell sphericity (Figures 5A-C). Since *dnVegfr* expression altered TVC migration
326 (Figure 1), and VEGF receptors and integrin complexes interact functionally in various

327 contexts⁴⁵⁻⁴⁷, we sought to test whether Vegfr signaling modulates Ddr/Int β 1-mediated
328 TVC-matrix adhesion. Co-expression of dnVegfr partially suppressed the de-adhesion
329 phenotypes produced by either dnDdr or dnInt β 1 in the TVCs (Figure 5A,B), suggesting
330 that a steady-state antagonism between baseline levels of Vegfr and Ddr/Int β 1 signaling
331 control TVC adhesion to the epidermal matrix.

332

333 We next tested whether components of the Col9-a1/Ddr/Int β 1-mediated cell-matrix
334 adhesion system modulate Vegfr activity. We performed PLA assays to quantify Vegfr
335 phosphorylation states following CRISPR/Cas9-mediated mutagenesis of *Col9-a1* in
336 endoderm progenitors, and misexpression of either dnDdr, or dnInt β 1 in the TVCs. In
337 control migrating TVCs, we observed most Vegfr::HA signal in cytoplasmic punctae,
338 where phosphorylated Vegfr represented about 20% of the total pool (Figure 5D,E). Both
339 Col9-a1 inhibition and dnInt β 1 misexpression resulted in an increased of Vegfr activity to
340 ~30% of the total pool, with a preferential localization at the ventral membrane
341 contacting the epidermal matrix (Figure 5). On the other hand, dnDdr misexpression
342 decreased Vegfr activity (Figure 5G). Taken together, the results obtained with Col9a1
343 and Int β 1 are consistent with the hypothesis that cell-matrix adhesion antagonizes Vegfr
344 signaling, thus contributing to a mutual antagonism, possibly modulating cell-matrix
345 adhesion in migrating TVCs. By contrast, the unexpected effects of dnDdr suggest that
346 Ddr is also required independently of its effects on cell-matrix adhesion to promote
347 Vegfr signaling.

348

349 **Asymmetric exposure to Col9-a1, and Ddr signaling establish and maintain** 350 **collective TVC polarity**

351 Previous studies showed that asymmetric contacts between the TVCs and
352 surrounding tissues contribute to canalizing TVC behavior towards collective
353 leader/trailer polarity and directed migration¹³. Specifically, the prospective leader cell,
354 born more ventrally, initially contacts the endoderm, whereas the future trailer interacts
355 with the mesenchyme. This asymmetry opens the possibility that an endodermal
356 product, such as Col9-a1, contributes to establishing collective leader-trailer polarity
357 prior to migration. To determine if differential contact with the endoderm determines
358 asymmetrical exposure to Col9-a1, we imaged Col9-a1::GFP deposition in the ECM and
359 quantified colocalization with the TVC membranes at early stages, prior to and shortly
360 after the onset of TVC migration (Figure 6A). *Nkx2-1*-driven Col9-a1::GFP gradually

361 accumulated in the ECM between stage 17 and 19, and clearly labeled the TVC/epidermis
362 interface by stage 21, when TVCs have begun their migration. The prospective leader cell
363 membrane initially colocalized more extensively with Col9-a1::GFP foci, compared to the
364 presumptive trailer (Figure 6A). This suggests that Col9a1::GFP does not diffuse
365 extensively, and potentially provides a polarizing signal to the TVCs through asymmetric
366 exposure. As cells initiated migration, endoderm-derived Col9-a1::GFP colocalized
367 similarly with leader and trailer. This implies that Col9-a1 induced polarity must be
368 established prior to the onset of migration, and subsequently maintained through
369 migration.

370

371 Collective TVC polarity is evidenced by morphological differences between the leader
372 and trailer cells^{13, 16}, but no clear molecular marker has been identified. We previously
373 proposed that TVCs are exposed to varying levels of BMP-Smad signaling⁴⁸. To test
374 whether BMP-Smad signaling is leader-trailer polarized in *Ciona* TVCs, we developed a
375 biosensor by humanizing the C-terminus of *Ciona robusta* Smad1/5/8 and adding an HA
376 tag at the N-terminus. Using both an anti-HA and an anti-phospho-SMAD5 antibody,
377 which cross-reacts poorly with the endogenous *Ciona* protein, we quantified BMP-Smad
378 signaling in the B7.5 lineage as the pSmad/HA ratio, following expression of this
379 HA::Smad1/5/8^{Hs} sensor using the *Mesp* enhancer. This sensor responded to defined
380 perturbations of the BMP-Smad pathway (Figure S6), indicating that it can serve as a
381 reliable readout for endogenous signaling. In control embryos, the pSmad/HA levels
382 were ~1.75 times higher in the leader compared to the trailer cell (Figure 6B,D).
383 Therefore, we used changes in pSmad/HA levels to assay the roles of Col9-1, Ddr, Intβ1
384 and Vegfr signaling in establishing and maintaining collective leader/trailer polarity.

385 We first quantified changes in BMP-Smad signaling following perturbations of Ddr,
386 Intβ1 and Vegfr functions. TVC-specific expression of dnDdr reduced the absolute
387 pSmad/HA ratios, and abolished differences between the leader and trailer cells,
388 suggesting that Ddr promotes BMP-Smad signaling and contributes to establishing and
389 maintaining TVC polarity. By contrast, misexpression of neither dnIntβ1 nor dnVegfr did
390 alter pSmad/HA levels or asymmetry in the TVCs (Figure 6B,C). In an attempt to reveal
391 cryptic activity, and because Vegfr appeared to antagonize the functions of Ddr and Intβ1
392 in cell-matrix adhesion, we tested whether dnVegfr or dnIntegB3 could modulate the
393 effects of dnDdr on BMP-Smad signaling. In neither case did we observe significant
394 differences compared to dnDdr alone. Taken together, these results suggest that Intβ1

395 and Vegfr act primarily upon cell-matrix adhesion, whereas Ddr coordinates collective
396 polarization and adhesion, by independently regulating each pathway.

397

398 Finally, since endoderm-derived Col9-a1 is necessary to activate Ddr in the TVCs,
399 and newborn TVCs may be differentially exposed to extracellular Col9-a1, we probed
400 BMP-Smad signaling following CRISPR/Cas9-induced *Col9-a1* mutagenesis.
401 Unexpectedly, *Col9-a1* inactivation in the endoderm caused a general increase of
402 pSmad/HA levels in both leader and trailer cells, and a slight reduction in leader-trailer
403 ratio (Figure 6D). These observations are seemingly inconsistent with a role for Col9-a1-
404 activated Ddr in promoting BMP-Smad signaling in a polarized fashion. However,
405 because Col9-a1 acts in the extracellular milieu, we hypothesize that Col9-a1 may
406 contribute to the sequestration of BMP ligands, as shown for type IV collagens in
407 *Drosophila*⁴⁹, thus possibly reducing the availability of ligands for signaling via BMP
408 receptors at the TVC membrane. Although these hypotheses remain to be tested, our
409 results are consistent with a model whereby Col9-a1 regulates BMP-signaling via an
410 incoherent feedforward signaling circuit, which consists of an inhibition via ligand
411 sequestration that is partially compensated by activation via Ddr (Figure 7).

412

413

414 **DISCUSSION**

415 In embryos, the behaviors of progenitors cells emerge from the integration and
416 coordination of inputs from intrinsic factors, including transcriptional regulation of
417 cellular effector genes, and biochemical and mechanical extrinsic determinants¹. Here,
418 we showed that, in migratory cardiopharyngeal progenitors of the tunicate *Ciona*, the
419 transcription factor Foxf is necessary to upregulate the collagen receptor Ddr, which
420 senses the extracellular matrix and functions to promote both cell-matrix adhesion and
421 collective polarity through presumably independent pathways.

422 This study corroborates preceding transcriptional profiling results showing that Foxf,
423 an early component of the cardiopharyngeal regulatory network, which was previously
424 shown to impact protrusive activity by regulating the expression of the small GTPase
425 Rhod/f⁶, also connects the GRN to cellular effectors capable of sensing and responding
426 to the extracellular milieu. This suggests that Foxf regulates a cell-matrix adhesion
427 module, as previously proposed¹⁶. Notably, FoxF1 was shown to regulate Integrin- β 3
428 expression and cell-matrix adhesion in the mouse⁵⁰, suggesting an ancient and conserved
429 connection between Foxf homologs and the regulation of cell-matrix adhesion.

430 Previous studies used a dominant negative form of Foxf, with the WRPW repressor
431 motif fused to the DNA-binding domain, to propose that Foxf activity controls TVC
432 migration¹⁵. This reagent produced more severe migration phenotypes than our newly
433 developed CRISPR/Cas9 assay, which nevertheless strongly inhibits *Foxf* expression
434 (Gandhi et al., 2017³⁶ and data not shown). This discrepancy suggests that the repressor
435 form of Foxf caused non-specific defects, such as inhibiting the targets of other Forkhead
436 box family factors expressed in the B7.5 lineage¹⁶, which may not be affected by CRISPR-
437 induced Foxf loss-of-function. Future studies will clarify the functions of these other Fox
438 factors at the transcription-migration interface, and their possible interactions with Foxf.

439 While TVC-specific transcriptional inputs control the expression of cell-matrix
440 adhesion determinants, asymmetric adhesion to the extracellular matrix was previously
441 shown to polarize the founder cells and permit localized TVC induction, and thus *Foxf*
442 expression⁵¹. Precocious misexpression of dnDdr using the *Mesp* driver interfered with
443 this integrin-dependent TVC induction, thus supporting the notion that dnDdr interferes
444 with cell-matrix adhesion (Figure S7). This illustrates the notion that cell fate
445 specification and subcellular processes are dynamical and interdependent components of
446 the transcription-cell behavior interface, which is best understood as an integrated
447 system.

448

449 Our results indicate that at least two putative collagen receptors, Ddr and Int β 1,
450 contribute to cell-matrix adhesion during TVC migration. Discoidin-domain receptors
451 and integrins have been showed to interact during cell adhesion to collagen matrices^{34, 52,}
452 ⁵³. Whereas the function of integrins as cell-matrix adhesion receptors mechanically
453 coupled to the cytoskeleton is well described^{54, 55}, Ddr homologs bind collagens
454 independently of integrins^{38, 53, 56}, and they appear to function as weak adhesion molecule
455 and/or an ECM sensor, which can promote cell-matrix adhesion through other
456 pathways, including integrins^{34, 57}.

457 A prominent feature of our model is the functional antagonism between Vegfr
458 signaling and Ddr/Int β 1-dependent cell-matrix adhesion. Although ligands and signal
459 transduction pathways remain to be characterized, the antagonism appears to occur in
460 part through local inhibition of Vegfr at the ventral membrane, where Ddr and Int β 1
461 bind the ECM (Fig. 5). While functional interactions between VEGF receptors and
462 integrin complexes are largely context-dependent⁴⁵, VEGFR2 and α v β 3 integrins were
463 found to directly interact through their cytoplasmic tails⁵⁸ and integrin activity can
464 promote VEGFR2 degradation^{46, 47}. Conversely, VEGFR2 activity has been linked to
465 turnover of integrin-based focal adhesion⁵⁹. It is thus possible that Vegfr can induce
466 endocytosis of integrin complexes, thus weakening cells' adhesion to the basal lamina.
467 Remarkably, in *Ciona* cardiopharyngeal progenitors, the Ddr/Int β 1-Vegfr antagonism
468 contributes to positioning cells between the endoderm and the epidermis, a mesodermal
469 hallmark in triploblastic animals.

470

471 Besides their role in cell-matrix adhesion, the ECM-sensing properties of Ddr
472 contribute to the establishment of distinct leader and trailer states, as a result of
473 differential exposure of to extracellular collagen. This is reminiscent of the roles of ECM
474 components in establishing of leader cell states in wound scratch assays *in vitro*⁶⁰, and in
475 guiding endomesoderm migration during gastrulation⁶¹. Moreover, DDR1 can interact
476 with the PAR complex to polarize the actin cytoskeleton in individual cells⁶². Future
477 studies will uncover the molecular pathways by which Ddr control collective polarity,
478 and the elusive cell-cell communication mechanisms that we must invoke, by analogy
479 with other systems^{63, 64}, to explain maintenance of leader-trailer polarity throughout
480 migration.

481 We reported differential BMP-Smad signaling as a reliable read-out of polarized
482 leader/trailer states. Ddr and Col9-a1 are required for this anisotropic BMP signal, which
483 appeared largely independent of Int β 1-mediated cell-matrix adhesion and Vegfr
484 signaling. This indicates that Ddr acts as a dual function receptor, promoting cell-
485 adhesion and transducing polarity information through independent pathways.
486 Although Ddr is activated by exposure to Col9-a1 and promotes BMP-Smad signaling,
487 loss of Col9-a1 function increased BMP-Smad signaling. To reconcile these seemingly
488 inconsistent results, we hypothesize that BMP ligands are sequestered by extracellular
489 collagen and inaccessible for signaling, as reported in the *Drosophila* ovary⁴⁹. In this
490 model, extracellular Col9-a1 would thus control BMP-Smad signaling through an
491 incoherent feedforward signaling circuit, whereby activation of Ddr-mediated signaling
492 would compensate ligand sequestration in the extracellular matrix. Future studies will
493 test these possibilities and explore the signal transduction pathways connecting Ddr to
494 BMP-Smad signaling.

495

496 **Acknowledgements**

497 We thank Justin Le Lorier for cloning the HA::Smad1/5/8^{HS} sensor. This work was
498 supported by NIH F32 GM108369-01A1 post-doctoral fellowship to Y.B., NIH F32
499 GM105216-01A1 post-doctoral fellowship to S.G., and NIH/NIGMS R01 GM096032
500 award to L.C.

501

502 **Authors' contributions**

503 Y.B. and L.C. designed the experiments, Y.B., S.G., S.B., and W.W. performed the
504 experiments. Y.B. and L.C. wrote the paper.

505

506

507 **Materials and Methods**

508 *Electroporation and transgene expression*

509 *Ciona robusta* (formerly known as *Ciona intestinalis* type A⁶⁵) adults were purchased
510 from M-Rep, San Diego, Ca. Gamete isolation, fertilization, dechoriation, and
511 electroporation were performed as described⁶⁶⁻⁶⁸. The amount of DNA electroporated
512 varied from 10µg to 80µg. Animals were reared at 16 to 20°C. For proximity ligation
513 assays, embryos were fixed in cold 100% methanol for 10 minutes. For *in situ*
514 hybridization experiments, embryos were fixed for 2 hours in 4% MEM-PFA, dehydrated
515 in an ethanol series and stored in 75% ethanol at -20°C as described^{66, 69}. Embryos used
516 for direct visualization of fusions were fixed in 4% MEM-FA for 30 minutes, cleared with
517 an NH₄Cl solution, and imaged using a Leica SP8 X Confocal microscope.

518

519 *Molecular Cloning*

520 Coding sequences of Ddr (KH.C9.371) Vegfr (KH.C14.345), Fgfr (KH.S742.2), and (Egfr
521 (KH.L22.45) were amplified from mid-tailbud *Ciona* cDNA libraries. To generate
522 dominant negative constructs, cytoplasmic kinase domains were removed by truncating
523 the sequence after the transmembrane and extracellular domains. Primer lists are given
524 in Supplementary Table 1. To subclone larger protein-coding fragments we used a multi-
525 fragment assembly approach using the NEB InFusion homologous recombination kits.
526 Coding regions that were greater than 2kb in length were subdivided into overlapping
527 kernels and ligations of up to 4 kernels were performed.

528

529 *Live imaging and TVC tracking*

530 To generate 4D datasets, 4.5 hpf old embryos were mounted on glass bottom microwell
531 petri dishes (MatTek, part# P35G-1.5-20-C) in artificial seawater. Plates were sealed by
532 piping a border of a mix of Vaseline and 5% mineral oil by volume (Sigma, item #M841-
533 100 ml) and covering with a 22x22 Fisherbrand Cover Glass (item # 12-541-B). Embryos
534 were imaged on a Leica inverted SP8 X Confocal microscope every 3.5 minutes for 4 to 5
535 hours. B7.5 lineage nuclei and epidermal cell membranes were visualized using
536 *Mesp*>H2B::GFP and *EfnB*>hCD4::mCherry¹³, respectively, and TVC migration was
537 tracked using Imaris Software.

538 To express TVC position during migration in 3D we subdivide the embryo into quadrants
539 using two conceptual orthogonal planes that bisect the developing embryo. The position
540 of the midsagittal plane is determined by the midline of the epidermis visible using the
541 *EfnB*>hCD4::mCherry transgene. The frontal plane is orthogonal to the mid-sagittal
542 plane and passes through the nucleus of the anterior ATM and the future position of the
543 palps, the most anterior point of the embryo. Imaris Bitplane is used to calculate a vector
544 line based on position of GFP+ TVC nuclei.

545

546 *Proximity Ligation Assay*

547 To visualize phosphorylated RTKs each receptor was subcloned into an expression vector
548 driven by either the *Mesp* (early expression) or *Foxf(TVC)bpFog* (late expression)
549 enhancer and tagged with 3x hemagglutinin (HA). Expression vectors were
550 electroporated into fertilized *Ciona robusta* embryos, which were raised at room
551 temperature to desired stages as indicated. Embryos were fixed by washing in 1ml of cold

552 100% methanol for 10 minutes and stored in 100% methanol at -20 C until processed.
553 Fisherbrand slides were coated with 0.1% w/v Poly-L-Lysine (Sigma P8920) two times
554 and allowed to dry. Embryos were rehydrated in a 0.1% PBT (0.1% Tween-20 in PBS):
555 MeOH series of 3:7, 1:1, 7:3, 100% PBT for 15 minutes and mounted on the Poly-L-Lysine
556 coated slides. Slides were blocked in three drops of Duolink Blocking reagent for 30
557 minutes at 37°C. Rabbit Anti-HA and mouse anti-pTyr were used to visualize
558 phosphorylated RTKs and a rat anti-HA antibody was used to visualize total amount of
559 RTK expressed in the embryo. Antibodies were used at a concentration of 1:500 diluted
560 in Duolink Antibody Diluent. Slides were incubated with primary antibody for 1 hour at
561 37 degrees and washed 2x in home made Duolink Buffer A for 15 minutes each in a
562 Coplin Jar. Secondary Duolink anti-mouse and anti-rabbit antibodies conjugated to a
563 DNA probe were used as directed by the Duolink manual. AlexaFluor-555 anti-rat
564 secondary antibody (Life Technology #A21434) was added at a 1:1000 dilution to the mix
565 of secondary antibodies to detect total amounts of HA. 100µl total of secondary
566 antibodies were used per slide. Slides were incubated for 1 hour at 37°C, washed two
567 times in home-made Duolink Buffer A for 15 minutes each in Coplin jars. Ligation
568 reactions were carried out as recommended in the Duolink manual, but extended to 1
569 hour at 37°C. Slides were washed again two times in Duolink Buffer A for 15 minutes
570 each in Coplin jars. Polymerase reaction was carried out as recommended, and extended
571 to 2 hours at 37°C. Slides were washed two times in home-made Duolink Buffer B for 15
572 minutes each and one time in 0.01% Buffer B for 15 minutes. 15 µl Duolink Mounting
573 Media was added to each slide, 22x22 Fisherbrand coverslips were placed on top and the
574 slides were sealed with clear nail polish.

575

576 *Fluorescent in situ hybridization – FISH-IHC*

577 RNA hybridization probes were generated from late tailbud derived cDNA by subcloning
578 the coding regions of the genes of interest into the pCRII-TOPO dual-promoter cloning
579 vector (Invitrogen, KH461020) and PCR was performed to confirm the orientation of the
580 insert. Coding regions were amplified using M13 Forward and Reverse primers and the
581 PCR product was used as a template to synthesize RNA probes labeled with either
582 digoxigenin or fluorescein using the SP6 polymerase. Primers for probe generation are
583 listed in Supplemental Table 1. Single and double FISH were performed as described⁶⁶,
584 ⁶⁹.

585

586 *CRISPR/Cas9 - guide RNA design*

587 Single guide RNAs (sgRNAs) targeting the *Col9-a1* locus (KH.C8.248) were designed and
588 tested essentially as described^{36, 43}. To test the efficiency of the sgRNAs, we first mutated
589 the *Col9-a1* locus using the ubiquitous *Efta>nls::Cas9::nls* and the *U6>sgRNA*
590 constructs. Cutting efficiency was calculated based on Sanger sequencing of the sgRNA
591 target region as described³⁶. Cutting efficiency of *Col9-a1* sgRNAs peaked at 0.8 and 1.1
592 for sgRNAs targeting the 1st and 29th exon, respectively. We targeted the *Col9-a1* locus by
593 combining two high efficiency *Col9-a1* sgRNAs, expected to generate large deletions. To
594 target the *Col9-a1* locus in endoderm progenitors, we used the vegetal hemisphere
595 enhancer from *FoxD* to drive *nlns::Cas9::nls* expression. 25µg of Cas9-expressing vector
596 and a total of 80µg of sgRNA-expressing constructs were used for each experiment. As a

597 control, sgRNAs targeting the late-expressed *Ebf* gene were used throughout the
598 manuscript.

599

600 *Design and cloning of short hairpin RNAs (shRNAs)*

601 shRNA were designed as described^{22, 69}. We designed the *Ciona* short hairpin microRNA
602 (Ci-shmiR) cassette based on the primiR structure of *Cirobu.mir-2213-a*⁷⁰. The Ci-shmiR
603 cassette (aaa gcgccgc aaa gctagca taa tga acttcgtggccgctgatcgtttaaaggaggtagtga ggtacc
604 tctagt ggatcc
605 [cgcgccgctaggttcgtttaatggtctaaaaatcaGagcgttagt**GTTTG**gagaccgagagaggggtctcactaaaactgc
606 gcttattatcttctacgaacctgtaagtggc] agatct ggccgca ctcgag ttg atgaattccagctgagcg) was cloned
607 downstream of the *Mesp* enhancer. Hairpins were cloned into the Ci-shmiR cassette
608 using *Bsa*I. Cloned hairpins were validated by testing for a knockdown of target genes
609 tagged with a GFP compared to cell markers that are not fused to gene targets. Hairpins
610 that knocked down the reporter were further validated by *in situ* hybridization for the
611 target gene.

612

613 *BMP-Smad biosensor, pSmad staining and quantification*

614 The *Mesp*>HA::Smad1/5/8^{Hs} was built by replacing the C-terminus of *Ciona* Smad1/5/8
615 by that of human SMAD5, using standard cloning procedures. A monoclonal Rat anti-HA
616 antibody was used to evaluate total levels of expressed protein and to normalize pSMAD
617 levels across multiple embryos. Polyclonal Rabbit anti-phospho-SMAD5 was used to
618 label phosphorylated HA::Smad1/5/8^{Hs} proteins, and anti-beta-galactosidase against the
619 protein product of *Mesp*>LacZ was used to visualize the B7.5 lineage. To quantitate
620 pSMAD levels as ratios, we use the anti-beta-galactosidase signal to identified TVC
621 nuclei as spots in Bitplane Imaris. We then took the quantified the average fluorescence
622 of anti-pSmad and anti-HA in the leader and trailer TVC. Ratios of pSmad to HA were
623 calculated to normalize for variable transgene expression. The sensor's response to BMP-
624 Smad signaling was validated using expression of published constitutively active BMP
625 receptor and Noggin⁴⁸, an extracellular inhibitor (Figure S6).

626

627 *Image acquisition*

628 All images were acquired using the Leica SP8 X WLL Confocal microscope using the 63x
629 oil immersion lens. Z-stacks of fixed embryos were acquired at the system optimized Z-
630 step, 512x512 resolution, 600 Hz, and bi-directional scanning. Multiple HyD detectors
631 were used to capture images at various wavelengths.

632

633 *Morphometrics analysis and surface contact calculation*

634 The membrane marker *Mesp*>hCD4::GFP was used to segment the TVCs and derive
635 morphometric measurements such as sphericity, area, and volume in Bitplane Imaris
636 using the Cell function. Z-steps were normalized to achieve equal voxel size in X, Y, and
637 Z planes. TVCs were then segmented and resulting cells were exported to separate
638 surfaces. Another surface was created for the epidermis using the *EfnB*>hCD4::tagRFP
639 or mCherry marker. Distance transformation was performed on each TVC and the
640 epidermal surface was then used to mask the distance from the epidermal surface to the
641 distance-transformed surface of each TVC. A new surface representing the surface of

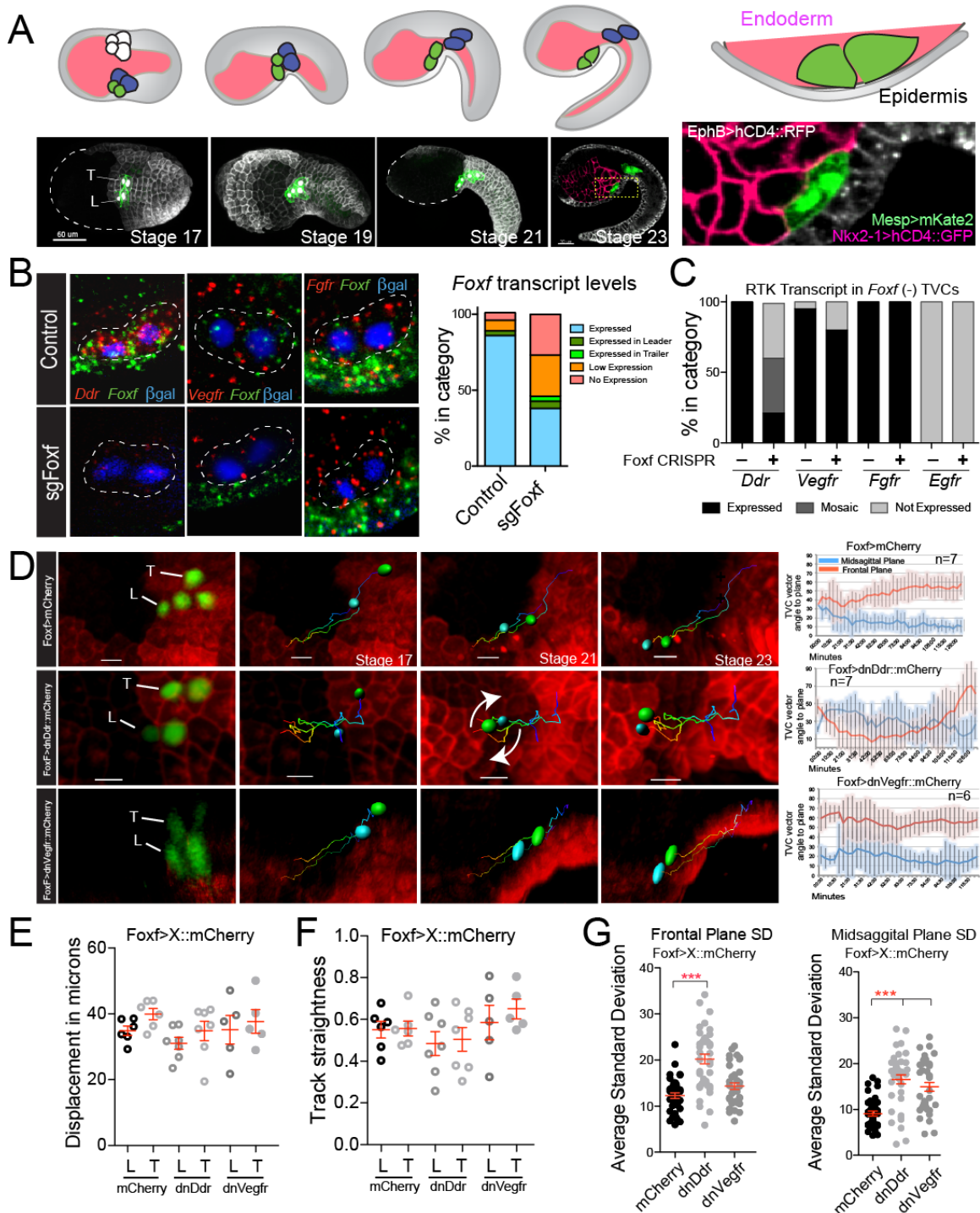
642 contact between the TVC and the epidermis was created using the masked distances. To
643 calculate the percent of the TVC surface in contact with the epidermis the area of the
644 surface contact was divided by two and then divided by the total area of the TVC.

645

646 *Colocalization*

647 To identify spots of colocalization the CoLoc module of Bitplane Imaris was used to
648 create a colocalization channel. The threshold of each image was set by the signal of
649 interest, e.g. to find colocalization between the *Mesp>hCD4::mCherry* and *Nkx2-1>Col9-*
650 *a1::GFP* the threshold was set to include the red channel. A separate channel was then
651 created based on the colocalization and each area of that channel was transformed into a
652 spot. We then calculated the number of spots close to the leader and trailer by identifying
653 spots within 5 microns of the TVC surface.

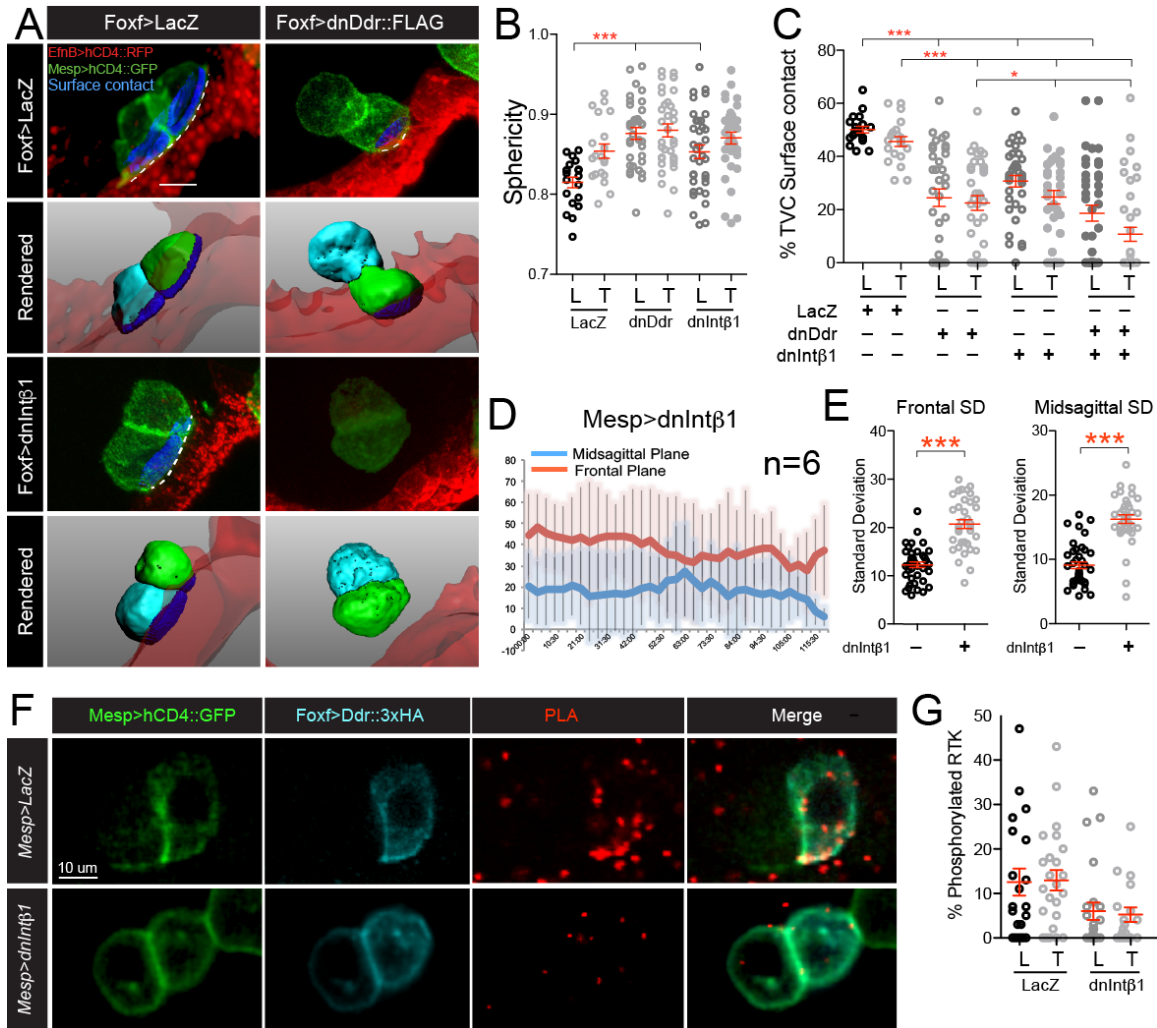
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Figure 1. Ddr and Vegfr regulate trunk ventral cell (TVC) migration. **A.** Schematic of TVC migration between the endoderm and epidermis. Cousin TVCs in green, ATMs are blue, endoderm in pink, and epidermis in grey. Micrographs of TVC migration and tissue contact. Epidermal cells are marked with *EfnB>hCD4::mCherry*, B7.5 lineage marked with *Mesp>hCD4::GFP* and nuclear *Mesp>H2B::mCherry* for stages 17-19, markers used for 3-channel acquisition are given on the magnified image. Close-up shows the positioning of the TVCs between the endoderm and epidermis. **B.** Fluorescent *in situ* hybridization (FISH) carried out in migrating TVCs at stage 23 in

664 control and CRISPR targeting the *Foxf* locus for mutagenesis. All micrographs are
665 oriented with the leader TVC to the left. TVC pair is outlined with dotted line and nuclei
666 are marked with *Mesp>LacZ* and stained for β -galactose. Quantification of *Foxf*
667 knockout efficiency is shown to the right. **C.** RTK expression in the proportion of
668 embryos that lose *Foxf* expression. Guide RNAs targeting the *Ebf* locus for mutagenesis
669 are used as controls as *Ebf* is not required for TVC migration and does not affect
670 expression of TVC-specific genes. **D.** TVC positional tracking. TVC (and ATM) nuclei are
671 marked with *Mesp>H2B::GFP* and epidermis with *EfnB>hCD4::mCherry*. Tracks of
672 migrating TVCs are shown in each panel, cyan sphere = leader, green sphere = trailer.
673 Arrows show the tumbling of the TVCs as leader and trailer switch positions. L = leader,
674 T= Trailer. Average position angle to sagittal and frontal planes are shown with standard
675 deviation. **E.** Average displacement of leader and trailer TVCs during migration. **F.** Track
676 straightness of leader and trailer TVCs. **G.** Average standard deviation at the sagittal and
677 frontal planes. *** = $p < 0.0005$.
678



679
 680 **Figure 2. Integrin-β1 and Ddr promote TVC adhesion to the ventral**
 681 **epidermis.** **A.** Micrographs of surface contacts (blue) made by migrating TVCs with the
 682 underlying epidermis. On the upper panels, the length of the surface contact is indicated
 683 with a dotted line. TVCs are marked and segmented based on the B7.5 lineage-specific
 684 expression of *Mesp>hCD4::GFP* and epidermal surface is visualized with
 685 *EfnB>hCD4::tagRFP*. Lower panels show rendered surfaces, leader is shown in cyan,
 686 trailer in green, epidermal surface in transparent red. For segmentation and derivation
 687 of surface contacts see Materials and Methods. **B.** Morphology of TVC under
 688 perturbation conditions measured as sphericity of Leader/Trailer cells during migration.
 689 **C.** Quantitation of percent TVC surface in contact with the epidermis. All transgenes are
 690 expressed from the Foxf TVC-specific enhancer. Results shown are pooled from 3
 691 biological replicate experiments. **D.** Quantitation of migration angles of *Mesp>dnIntβ1*-
 692 expressing TVCs, averages at each time point and standard deviation shown. **E.** Average
 693 standard deviation of the angle between the LT axis and the frontal and sagittal planes in
 694 *Mesp>dnIntβ1*-expressing TVCs. **F.** Proximity ligation assay (PLA) of phosphorylated
 695 full-length Ddr under control and dnIntβ1 conditions. **G.** Quantitation of phosphorylated
 696 Ddr compared to the total amount of Ddr. All images are oriented with leader TVC to the
 697 left. L=leader, T=Trailer. * = p<0.05, ** = p<0.005, *** = p<0.0005.
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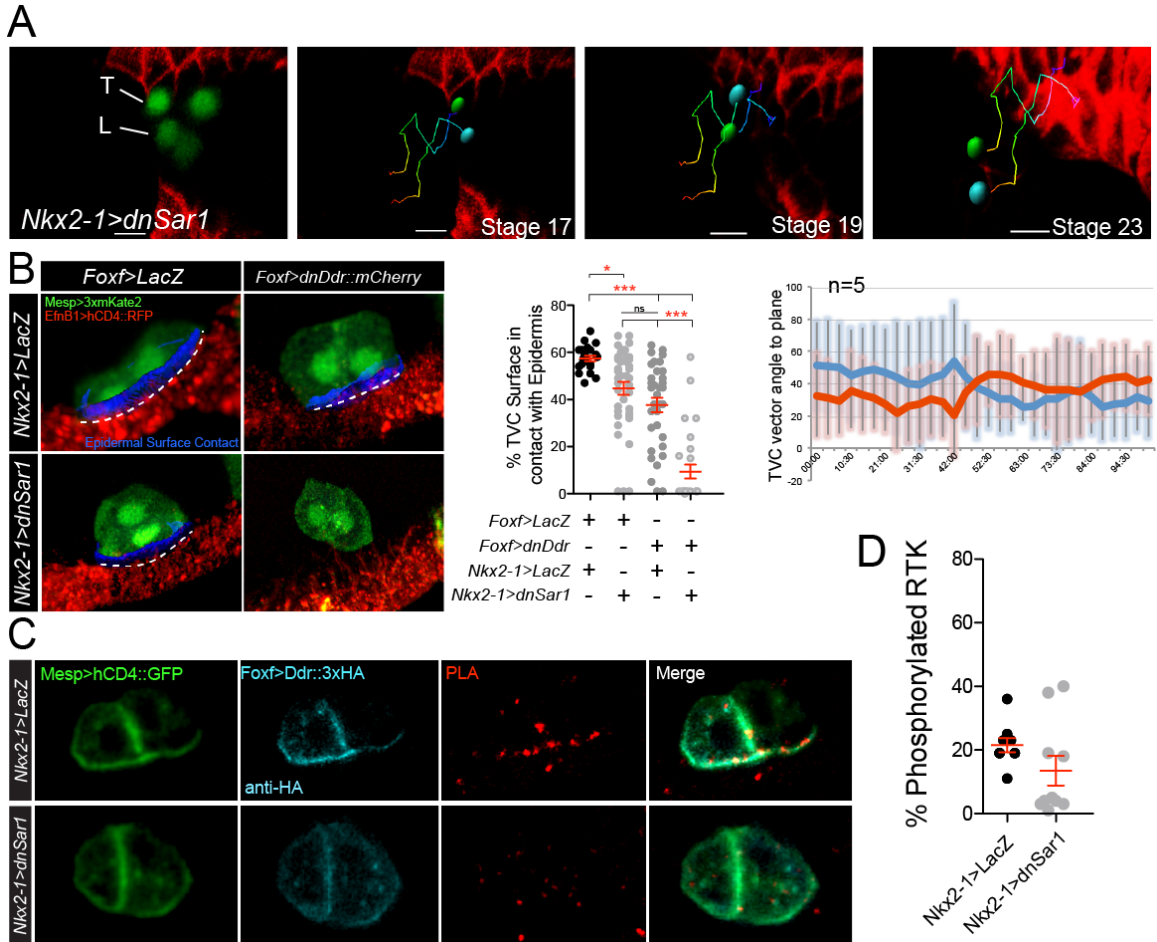
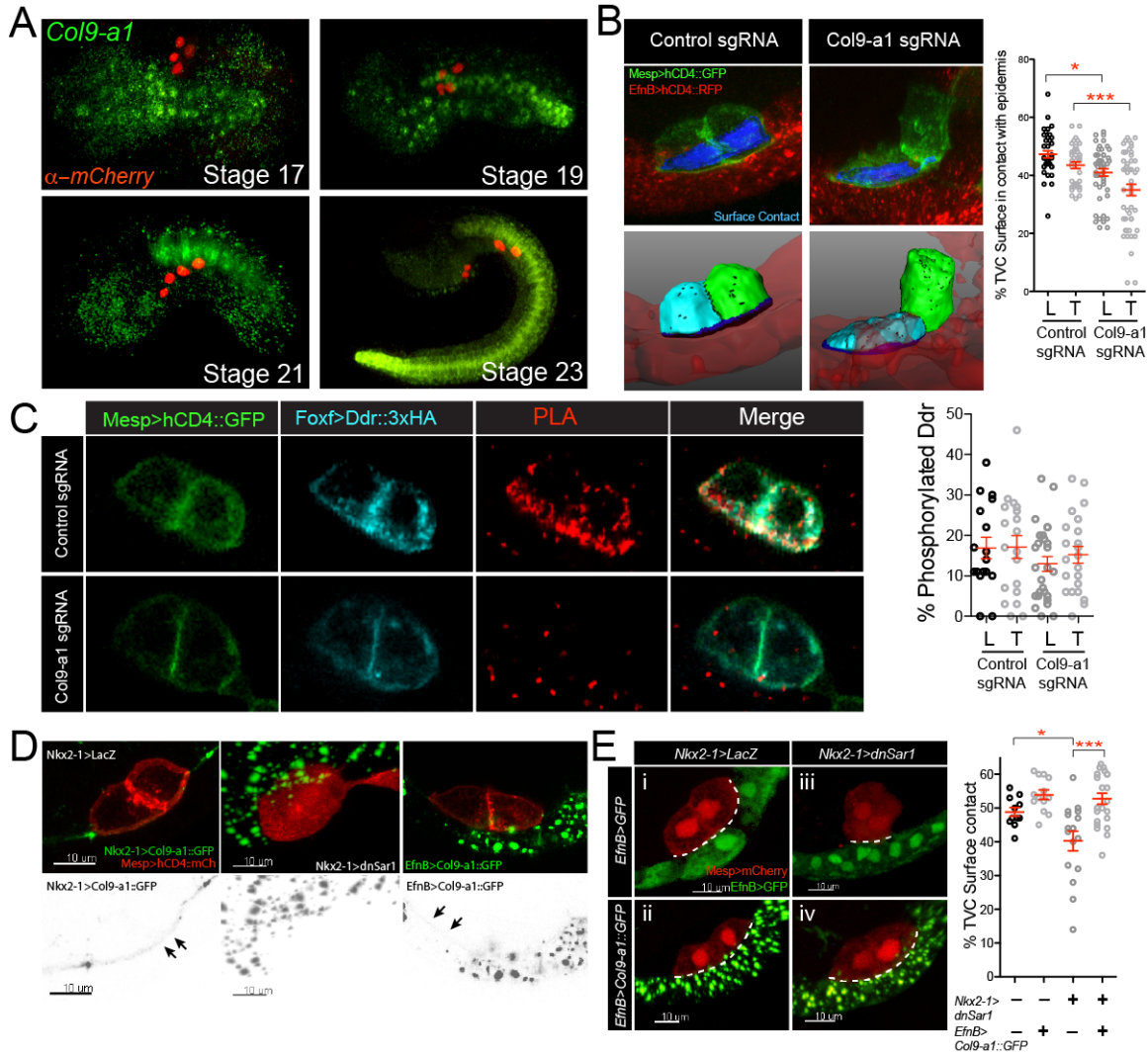


Figure 3. An endodermal cue potentiates TVC adhesion and Ddr activation.

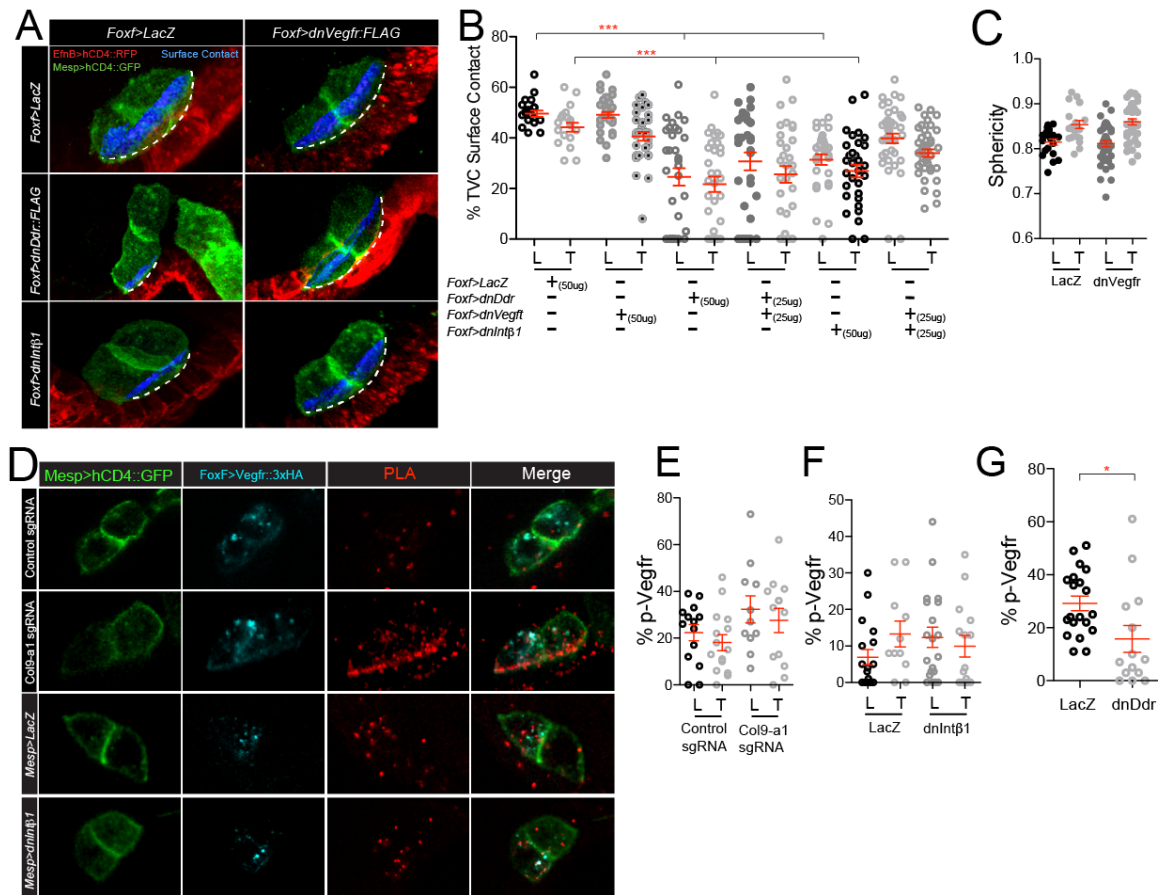
A. Positional tracking of TVC migration in embryos expressing *dnSar1* (*Nkx2-1>dnSar1*) to block secretion in the endoderm. Tracks are time coded from early (blue) to late (red). Leader = cyan, trailer = green. Quantitation of migration angles to frontal and sagittal planes is shown as averages at each time point and standard deviation from average. B. Percent of TVC pair surface in contact with epidermis. The B7.5 lineage is marked with *Mesp>3xmKate2*, epidermis is marked with *EfnB>hCD4::tagRFP*. Surface of contact is shown in blue and marked with a dashed line. Scatter plot shows average surface contact under each condition. C. PLA of *Foxf>Ddr::3xHA* in control (*Nkx2-1>LacZ*) and *Nkx2-1>dnSar1* conditions. D. Quantitation of percent phosphorylated Ddr. All images are oriented with leader TVC to the left. L=leader, T=Trailer. * = $p < 0.05$, ** = $p < 0.005$, *** = $p < 0.0005$.

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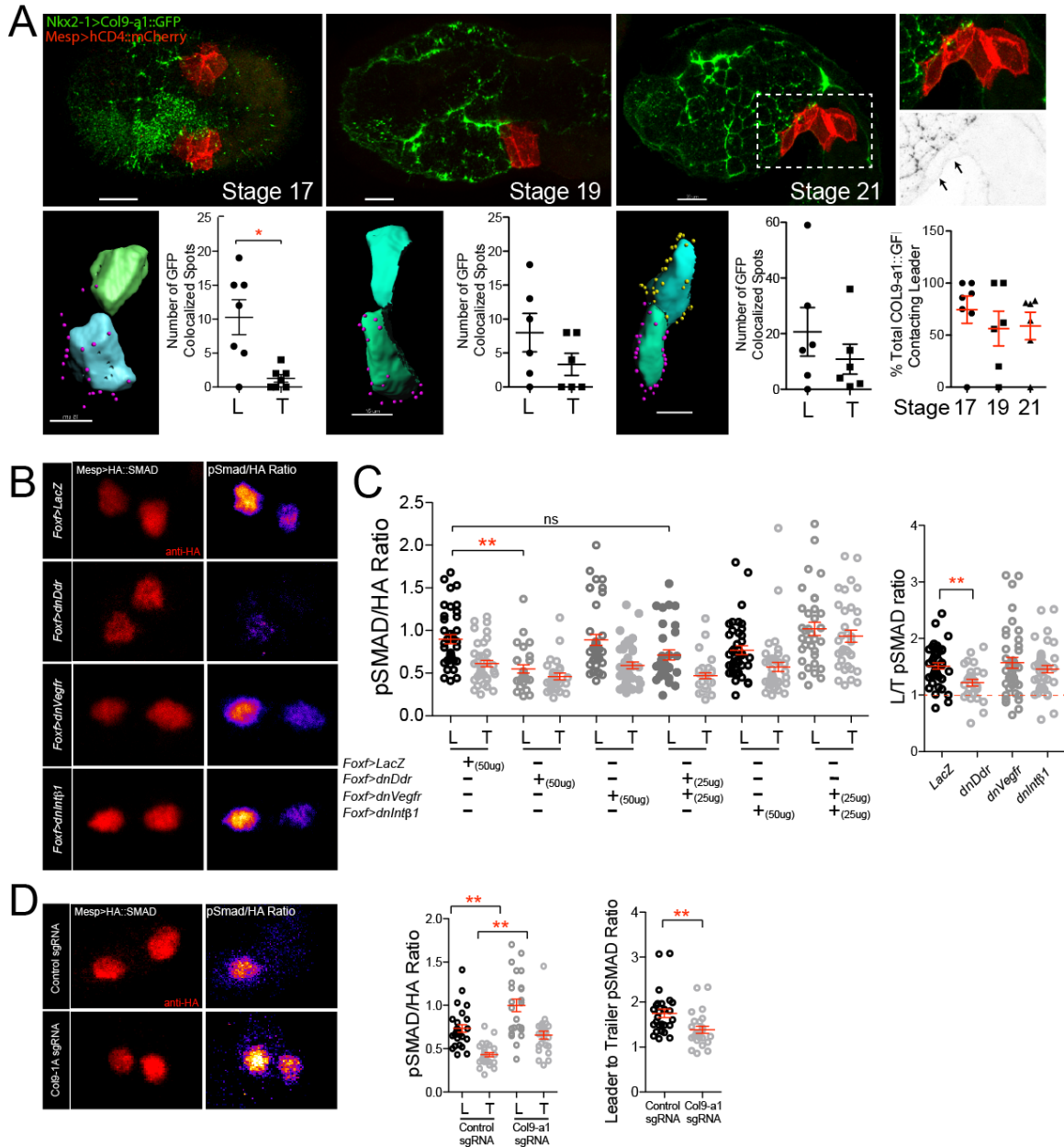
Figure 4. *Col9-a1* is the endodermal cue that promotes TVC adhesion and Ddr activation. **A.** Endogenous expression of *Col9-a1* in the developing embryo. Micrographs are oriented with anterior to the left, an *in situ* RNA probe is used to visualize *Col9-a1* transcripts. B7.5 lineage is visualized using *Mesp>H2B::mCherry*. **B.** CRISPR mutagenesis of the *Col9-a1* locus in the endoderm using a *FoxD*-driven Cas9. Blue surfaces are sites of contact. Quantitation of TVC surface area in contact with epidermis adhesion is shown on the right. **C.** PLA to detect phosphorylated full-length Ddr in control (Ebf CRISPR) and CRISPR conditions targeting the *Col9-a1* locus for mutagenesis. **D.** Secretion and localization of a *Col9A::GFP* fusion expressed in the endoderm (*Nkx2-1*) or in the epidermis (*EfnB*). TVC membrane is visualized using *Mesp>hCD4::mCherry*. Middle panel shows secretion inhibition in the endoderm using *dnSar1*. Gray scale panels show the corresponding inverted GFP channel. Arrows point to the accumulation of *Col9-a1* in the extracellular matrix. **E.** Rescue of *Nkx2-1>dnSar1* TVC adhesion defects by expressing *Col9-a1::GFP* from the epidermis. Control epidermis is marked with *EfnB>GFP*. Note that in the lower panels *EfnB1>GFP* is expressed but is less bright in comparison to the *Col9-a1::GFP* trafficking vesicles. Dotted line outlines the surface of contact. Quantitation of TVC adhesion is shown to the right of the micrographs. All images are oriented with leader TVC to the left. L=leader, T=Trailer. * = p < 0.05, ** = p < 0.005, *** = p < 0.0005.



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Figure 5. Vegfr modulates TVC adhesion by antagonizing Col9-a1/Ddr/Intβ1.

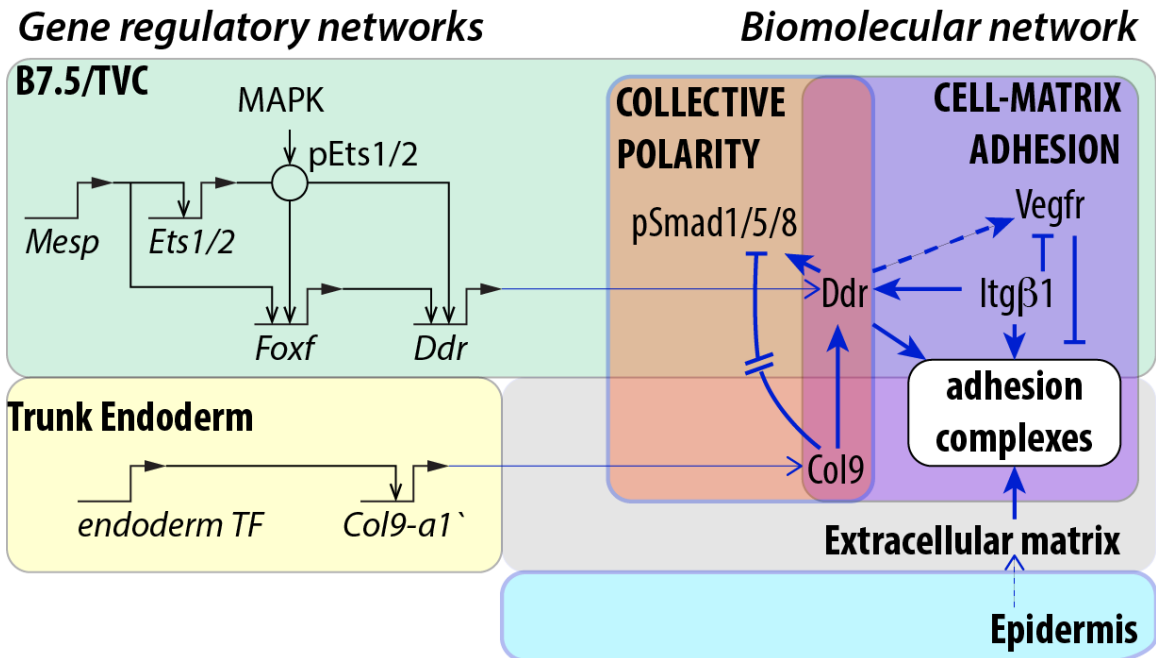
A. TVC adhesion under double dn conditions. TVCs are visualized using *Mesp>hCD4::GFP*, epidermis is visualized using *EfnB>hCD4::tagRFP*. Extent of epidermal surface content is shown with dotted line. Blue surfaces represent portion of TVC in contact with epidermis. **B**. Quantitation of TVC L/T surface in contact with epidermis under conditions that combine dnVegfr and dnDdr or dnIntβ1. Note that this data set originally included the mCherry, dnDdr, and dnIntβ1 conditions that are shown in Figure 2. **C**. Sphericity of leader/trailer TVCs under control and dnVegfr conditions. **D**. PLA assay to detect levels of phosphorylated Vegfr in Col9-a1 CRISPR, dnIntβ1, and dnDdr conditions. **E-F**. Percent phosphorylated Vegfr under perturbation conditions. All images are oriented with leader TVC to the left. L=leader, T=Trailer. * = $p < 0.05$, ** = $p < 0.005$, *** = $p < 0.0005$.



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Figure 6. Asymmetric exposure to Col9-a1 polarizes TVCs through an adhesion-independent function of Ddr. **A.** Distribution of Col9-a1::GFP fusion expressed in the endoderm at stages 17-21, prior to and at the onset of TVC migration, respectively. Boxed and expanded region shows localization of Col9-a1::GFP fusion ventral to the migrating TVCs. Calculation of total exposure of leader or trailer to endodermal collagen at each embryonic stage. Presumptive leader is shown in blue, presumptive trailer in green. Purple spheres indicate collagen spots proximal to the leader TVC, yellow spheres are proximal to the trailer TVC. Graphs show total number of collagen contact sites for leader or trailer at each given time point. Total number of Col9-a1::GFP spots contacting leader TVC over time. **B.** Quantitation of pSmad activity under conditions perturbing TVC adhesion. Ratios between the pSmad and HA channels are calculated by dividing the pSmad channel by the HA channel. Ratio is color-coded using the Fire Look Up Table (LUT) with lighter colors indicating a higher ratio. **C.** Normalized levels of pSmad in L/T under perturbation conditions and ratios of total leader:trailer

761 pSmad levels. **D.** pSMAD staining of control migrating TVCs and TVCs under
762 endodermal Col9-a1 CRISPR mutagenesis conditions. L:T ratios and normalized L/T
763 pSmad levels are shown to the right. **E.** All images are oriented with leader TVC to the
764 left. L=leader, T=Trailer. * = $p < 0.05$, ** = $p < 0.005$, *** = $p < 0.0005$.



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Figure 7. Integration of transcriptional and biomolecular networks to produce directional migration. Intersection of fate-specification through the cardiac GRN in the B7.5/TVC lineage (green) and downstream cellular effectors regulating ECM adhesion and polarity (purple and orange) produces directional migration. Black arrows show transcriptional regulation, blue arrows show activity regulation. Gene regulatory connections are after^{14-16, 18, 21}. Exposure to extracellular Col9-a1 activates Ddr and Intβ1 while blocking Vegfr activity. Solid blue lines show interactions confirmed in this report. Dashed lines show indirect relationships.

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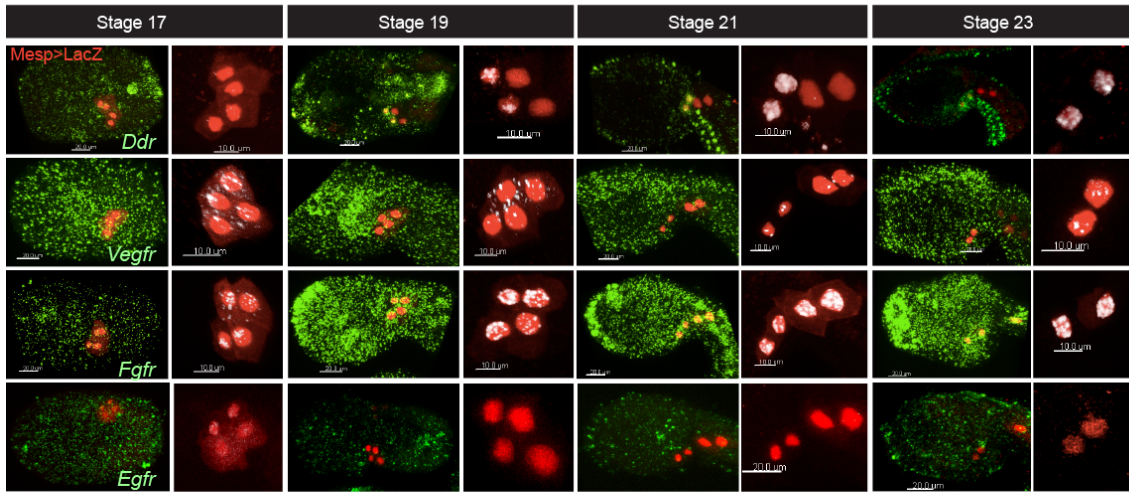
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Supplemental Figure 1

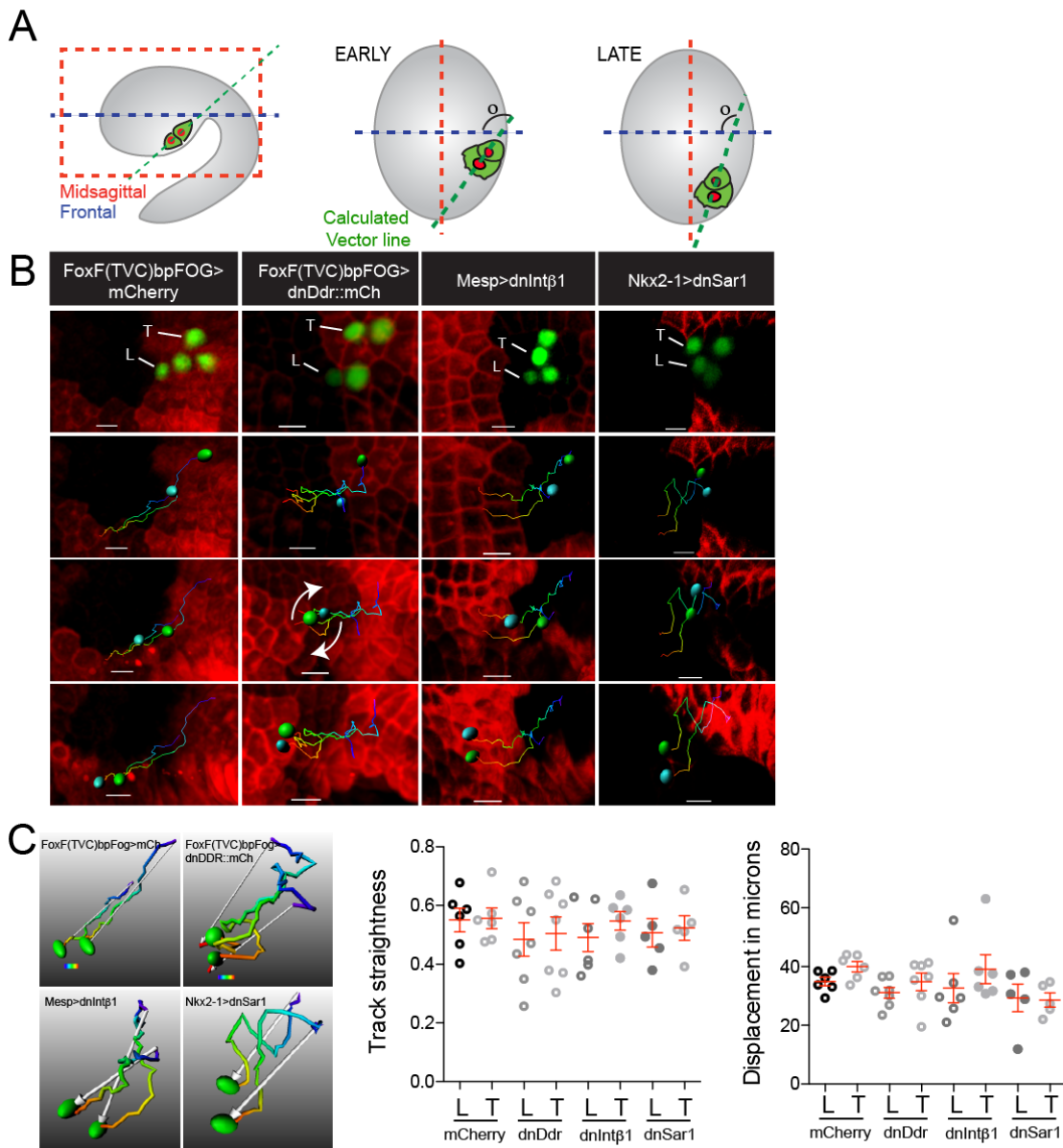


Supplemental Figure 1. RTK expression during TVC migration. Fluorescent *in situ* hybridization for *Ddr*, *Vegfr*, *Fgfr*, and *Egfr* at indicated developmental stages. B7.5 lineage is marked with *Mesp>LacZ* and stained for beta-galactosidase (red in the micrographs). Close ups show colocalization of transcripts with B7.5 lineage nuclei.

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Supplemental Figure 2

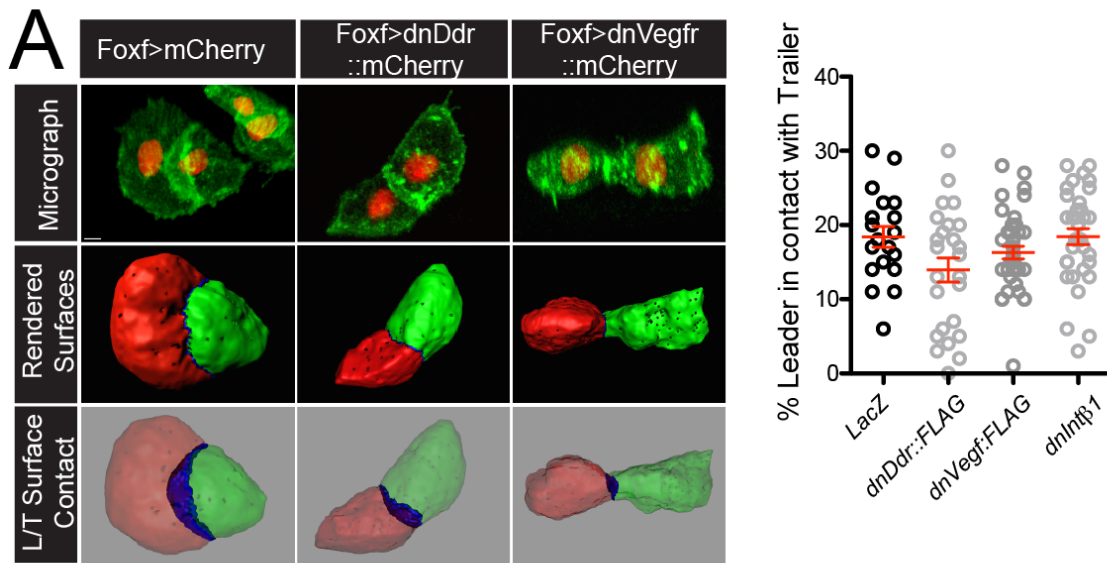


Supplemental Figure 2. Quantitative analysis of TVC migration. **A.** Schematic of calculating TVC position relative to frontal (blue) or sagittal (red) planes. The vector line (green) is calculated using the axes of the TVC nuclei. **B.** Positional tracking of TVC migration under perturbations of ECM adhesion. Graphs show average positions of TVCs relative to orthogonal planes and standard deviation at each time point. **C.** Rendered images of total displacement of the TVC under adhesion perturbation. White arrows point to the final position of TVCs prior to the first asymmetric division. TVC tracks are time code blue for early time points and red for late time points. **D.** Average track straightness and total displacement of TVCs.

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Supplemental Figure 3



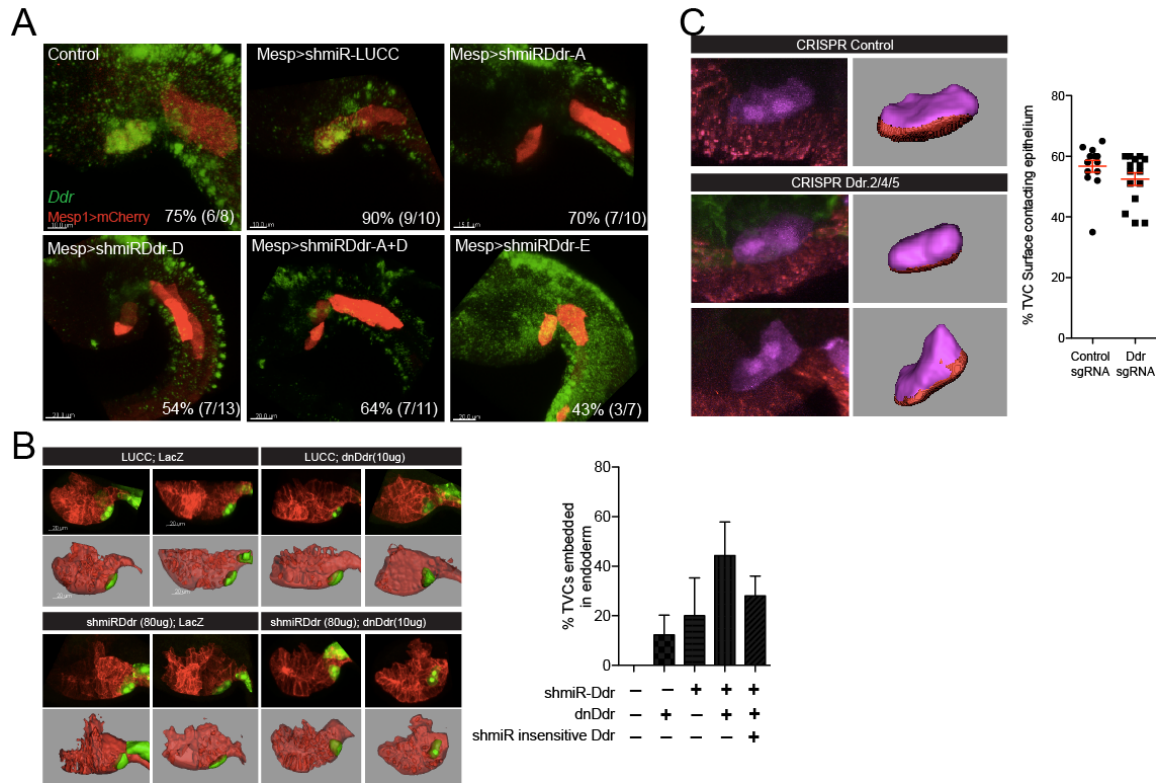
Supplemental Figure 3. Ddr is required for leader/trailer cell-cell junction. Micrographs of TVCs undergoing migration (top row). Nuclei are marked with *Mesp>H2B::mCherry*, cells are visualized using *Mesp>hCD4::GFP*. TVC pairs are segmented using Imaris Bitplane software, middle row. Leaders are in red, trailers in green. Surface of contact between leader and trailer is calculated (bottom row). Average size of the cell-cell junction is reported as percent surface of leader in contact with the trailer.

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Supplemental Figure 4

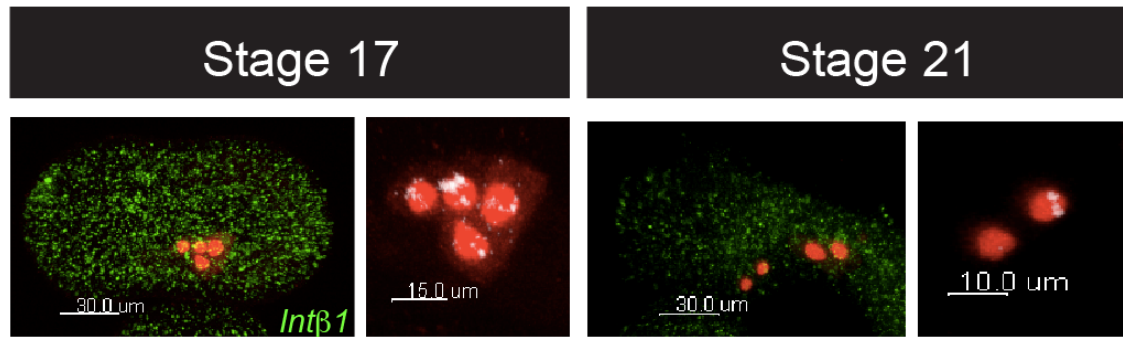


Supplemental Figure 4. Ddr loss-of-function phenotypes. **A.** short hairpin microRNA (shmiR) knockdown of Ddr transcripts. B7.5 lineage is marked with *Mesp>mCherry*. **B.** Sensitized shmiR test. Two representative embryos are shown for each condition. Top rows show raw data, bottom rows show rendered images. Graph shows % TVC that become embedded in the endoderm as a result of detachment from the epidermis. **C.** Mutagenesis of the *Ddr* locus using CRISPR. *EBF* CRISPR is used as control. Graph shows average percent of the TVC pair surface in contact with the epidermis.

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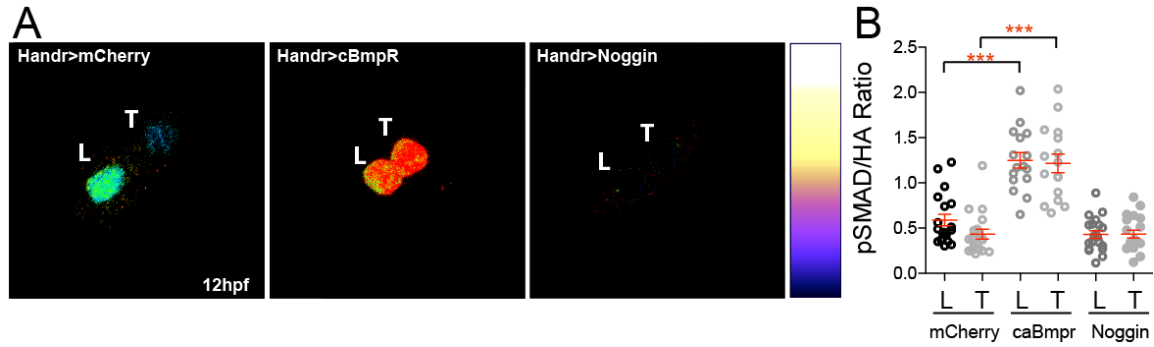
Supplemental Figure 5



Supplemental Figure 5. IntgB1 expression in the B7.5 lineage. *In situ* hybridization of RNA probe against Intβ1 transcripts. B7.5 lineage is marked with *Mesp>LacZ*.

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Supplemental Figure 6

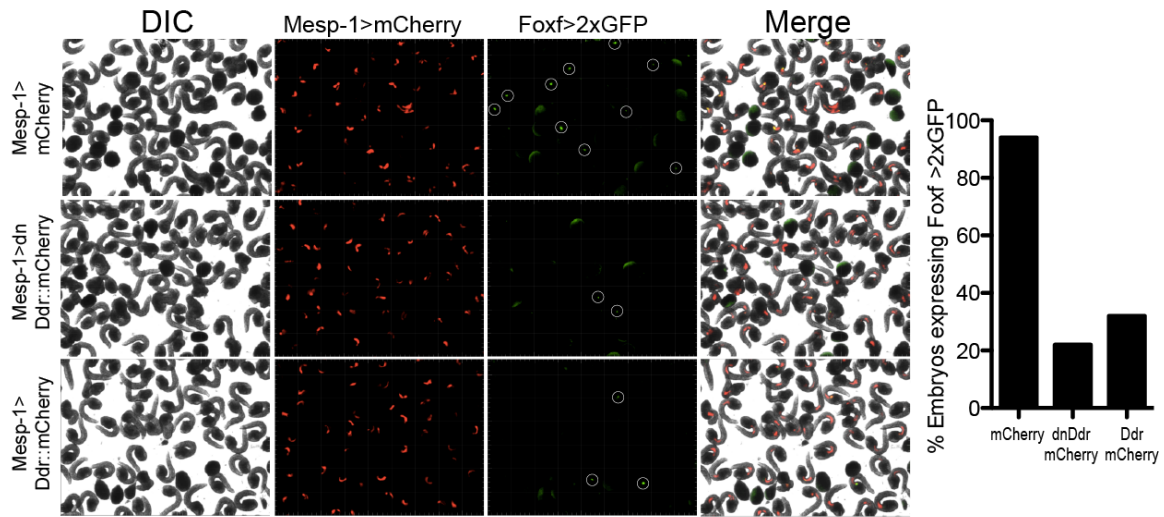


Supplemental Figure 6. Asymmetric induction of BMP-pSmad activity in migrating TVCs. A. Ratios of detected pSmad staining to the total HA levels detected. Levels can be manipulated through addition of a constitutively active BMP receptor (Handr>caBmpR) or by expression of the BMP antagonist Nogging (Handr>Noggin). **B.** Calculated ratios of pSmad/HA under conditions altering BMP-pSmad activity levels.

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Supplemental Figure 7



Supplemental Figure 7. Early expression of Ddr blocks TVC fate induction. Embryos electroporated with Mesp-driven mCherry, a dominant negative Ddr (dnDdr) or full-length Ddr are assayed for expression of Foxf-driven GFP. DIC channel shows embryonic developmental stage. Foxf>2xGFP expressing TVCs are circled in the green channel.

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983 Supplemental Table 1. Primers used for cloning.

Primers for full-length cloning		
Gene	Forward Primer	Reverse Primer
Vegfr-F	acttgattGCGGCCGCAACCATGAACTAACTGTCCAAGTCTGATC	TCACCATGGTCTAGCATTGAGATCCTGAATCCTGGAAGC
Egfr-F	acttgattGCGGCCGCAACCATGAGGCATTGCTATCCAATAGTTG	TCACCATGGTCTAGCCGTTTGGGGTGGCCAC
Fgfr-F	acttgattGCGGCCGCAACCATGATACAACTACAAAATACGTTTATTTTGTGCG	TGCTCACCATACTAGTAAAAACAATACCAAGAATAATGAAAAACCCACC
Ddr-F	acttgattGCGGCCGCAACCATGAAGTCGAAGTCATTACCACCGAGT	TGCTCACCATACTAGTCGCTCTGGGGTGGGAATTGG
Col9-a1-F	ATGCGGCCGCAACCATGTTCTATGAACAAGAGGCGCA	ATACTAGTAACTGAATATCATTATCTC
Ci-shmiR-insensitive Ddr	CGAGGTAGAGTTTGACAATAACGTATTACCACCGAGCGAT	TATTGTCAAACCTACCTCGCTAACCAAGAATATTCGTCCCC

Primers for generating dominant negatives	
Gene	Primer
dnDDR-R	TGCTCACCATACTAGTCTTCTCACGAGGAAACTCGGG
dnVEGFR-R	TGCTCACCATACTAGTTCGATCTCGAGGAACTCCCATTTCC
dnFGFR-R	CCACTACAACAAGCCCTTGATCTTAAAGG
dnEGFR-R	TGCTCACCATACTAGTTTCGCTTCTTAAACAATTCTAAGTTGAGC
dnIntp1-F	AACACACAAGCGGCCCAACCATGTGTGATATACATAAAGATTGCATTCATGCAA
dnIntp1-R	CGCTCAGCTGGAAATCCATATAAGCAGAATAGCAAGCCCA

In Situ Primer list		
Gene	Forward Primer	Reverse Primer
Col9-a1	ATGCGGCCGCAACCATGTTTCATGAACAAGAGGCGCA	ATACTAGTAACTGAATATCATTATCTC
Vegfr	ATGAACTAACTGTCCAAGTCTGATC	ATTGAGATCCTGAATCCTGGAAGC
Fgfr	ATGATACAACATAAAAATACGTTTATTTTGTGCG	TAGTGTAAATACCAGCTCTCTTCAATGTAATCAC
Egfr	ATGAGGCATTGCTATCCAATAGTTG	AGTTATTGTGGATGGGGTTCATTTG
Intp1	acttgattGCGGCCGCAACCATGAAATGAATTATATTACAGTGTATATTGGTGTG	ACTGGTAGTTACAGCAAAAGATTGGTTG
Vegf	GCGGCCGCAaccATGCCAGGATTTAAGAGTCAAATCTC	GAATTCAGTAGTTTTCCCTGGCGAAATGTCC

Ci_shmiR Primers		
Gene	Forward Primer	Reverse Primer
LUCC-C	agatGGGATTTCctTtGATGTAttTGTAAATTaGtAAACATGTACATCGACTGAAATCttttt	aattaaaaaGATTCAGTCGATGTACATGTTaCtAAATTACaaaTACATCaAagGAAATCCC
Ddr-miR-A	gtttGGATAGCAccGtTGGtAAcTGTAATTaGtAAcATTGTCCAGCAATGCTATCC	ttagGGATAGCATTGTGGACAAATGTTaCtAATTACAgTTaaCCaCggTGCTATCc
Ddr-miR-B	gtttGAAATGCGctTtTATTGTAcTGAATTaGtAAcATACAATGAACCTCGCATTCT	ttagAGAATGCGAGTTCATTGTaTGTaCtAATTACAgTACAATaAAagCGCATTTC
Ddr-miR-C	gtttGGTTGGTAgatATGAtATcTGAATTaGtAAcATATGTACATACGATACCAATC	ttagGATTGGTATCGTATGACATATGTTaCtAATTACAgATaTCATAcTcTACCAACC
Ddr-miR-D	gtttGCAAGTCGtTTCGATAAttTGAATTaGtAAcATGTTATCGAAATCGACTTCG	ttagCGAAGTCGAATTCGATAACATGTTaCtAATTACaaaTTATCGAAcaCGACTTCG
Ddr-miR-E	gtttGGTGGTCCtATtGAAATtTGAATTaGtAAcATGATTTCGATCTGGACCAAC	ttagGTTGGTCCAGATCGAAATCATGTTaCtAATTACaaaATTTCaATgaGGACCACC
Ddr-miR-F	gtttGGCGGTACgtTATCAATAcTGAATTaGtAAcATTAGTTGATATGGTACCGTC	ttagGACGGTACCATATCAACTAATGTTaCtAATTACAgTaaTTGATaAcGTACCGCC

Col9-a1 sgRNAs		
Target	Forward Oligo	Reverse Oligo
Col9-a1-Ex1	GAGCAGAAGACAAGGAGAGCGTTTAAAGAGCTATGCTGGAACAG	GCTCTCTTGTCTCTGCTCatctataccatcgatgccttc
Col9-a1-Ex29	GGAGGACCAACAGAACCTGGTTTAAAGAGCTATGCTGGAACAG	CAGGGTTCTGTTGGTCTCCatctataccatcgatgccttc

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