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1 **Regulation of anterior neurectoderm specification and differentiation by BMP signaling in**
2 **ascidians**

3

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10

11 **Running title**

12 Palps and BMP in ascidians

13

14 **Keywords**

15 palps, ascidian, BMP, anterior neural boundary, placode, peripheral nervous system

16

17 **Summary statement**

18 BMP signaling regulates two steps of ascidian palp formation: presumptive territory
19 specification at the anterior neural plate border during gastrulation, and ventral palp vs
20 inter-palp segregation during neurulation.

21

22

23 **Abstract**

24 The most anterior structure of the ascidian larva is made of three palps with sensory and
25 adhesive functions essential for metamorphosis. They derive from the anterior neural
26 border and their formation is regulated by FGF and Wnt. Since they also share gene
27 expression profiles with vertebrate anterior neural tissue and cranial placodes, their study
28 should shed light on the emergence of the unique vertebrate telencephalon. We show that
29 BMP signaling regulates two phases of palp formation in *Ciona intestinalis*. During
30 gastrulation, the anterior neural border is specified in a domain of inactive BMP signaling,
31 and activating BMP prevented its formation. During neurulation, BMP defines ventral palp
32 identity and indirectly specifies the inter-papilla territory separating the ventral and dorsal
33 palps. Finally, we showed that BMP has similar functions in the ascidian *Phallusia*
34 *mammillata* for which we identified novel palp markers. Collectively, we provide a better
35 molecular description of palp formation in ascidians that will be instrumental for
36 comparative studies.

37

38

39 **Introduction**

40 Ascidians (or sea squirts) belong to a group of marine invertebrates, the tunicates, that is the
41 sister group of vertebrates (Delsuc et al., 2006). This phylogenetic position associated with a
42 stereotyped embryonic development with few cells puts ascidians as interesting models for
43 developmental biology and comparative approaches to address questions regarding
44 chordates evolution and the emergence of vertebrates. Ascidians have a biphasic life cycle:
45 following external development, the embryo gives rise to a swimming tadpole-like larva with
46 typical chordate features (notochord, dorsal neural tube) that is going to attach to a
47 substrate before metamorphosing into a sessile adult ascidian with a radically different body
48 plan, a 'bag' with two siphons. Metamorphosis is controlled by a specific organ, the palps
49 (also referred to as the adhesive organ or the adhesive papillae), that is located at the
50 anterior end of the larva. The palps are a specialized part of the ectoderm that has adhesive
51 and sensory properties (Cloney, 1977; Imai and Meinertzhagen, 2007; Satoh, 1994). They
52 enable the larva to select a suitable substrate for metamorphosis, hence a chemo- and/or
53 mechano-sensory function, and to attach to it through the secretion of adhesive materials
54 (reviewed in (Pennati and Rothbächer, 2015)). It contains at least four cells types whose
55 specification and function have not yet been deciphered in details (Johnson et al., 2020;
56 Zeng et al., 2019). Three cell types - the ciliated sensory neurons, the colocytes (containing
57 vesicles filled with adhesive material), and the axial columnar cells (ACCs) (myoepithelial
58 cells controlling palp retraction following adhesion) - are elongated cells forming a
59 protrusion. Three protrusions or papillae (two dorsal papillae that are bilaterally symmetrical,
60 and a ventral papilla located at the midline; Fig. 1C) make up the palps and are separated by
61 the fourth cell type, the non-elongated inter-papillae cells.

62 Palps belong to the peripheral nervous system and have been instrumental for proposing
63 evolutionary scenarios on the nervous system in chordates (Cao et al., 2019; Horie et al.,
64 2018; Poncelet and Shimeld, 2020; Thawani and Groves, 2020). In the ascidian *Ciona*
65 *intestinalis*, palp cell lineage and topology, together with gene expression data and
66 functional studies, have shown affinities with anterior derivatives of the vertebrate nervous
67 system, the olfactory placodes and the telencephalon (Cao et al., 2019; Horie et al., 2018;
68 Hudson et al., 2003; Liu and Satou, 2019; Poncelet and Shimeld, 2020; Thawani and Groves,
69 2020; Wagner and Levine, 2012; Wagner et al., 2014). Palps originate from precursors that
70 are located at the anterior edge of the neural plate during gastrulation, that we will refer to

71 as the anterior neural border (ANB) (Fig. 1W,X) (Horie et al., 2018; Liu and Satou, 2019).
72 While the ANB is not part of the central nervous system (CNS), it originates from the same
73 lineage specified by FGF-mediated neural induction at the 32-cell stage and expresses neural
74 markers such as *Celf3/4/5/6* (also known as *Etr* and *Celf3.a*) and *Otx* (Horie et al., 2018;
75 Hudson, 2016; Hudson et al., 2003; Nishida, 1987). The separation between these two
76 lineages is regulated by FGF/Erk signaling at gastrula/neurula stages, FGF being active in the
77 CNS precursors (Hudson, 2016; Hudson et al., 2003; Wagner and Levine, 2012). FGF signaling
78 thus regulates positively and negatively two separate phases of palp specification. The ANB
79 also expresses *Dmrt* and *Foxc*, coding for transcription factors that are essential for palp
80 formation (Imai et al., 2006; Wagner and Levine, 2012). From neurulation and through
81 differentiation, palps express genes such as *Dlx.c*, *Foxg*, *Isl* or *Sp6/7/8/9* (also known as
82 *Zf220* and *Btd*) whose orthologs specify anterior neural territories and placodes in
83 vertebrates (Cao et al., 2019; Liu and Satou, 2019; Wagner et al., 2014). In particular, *Foxg*
84 and *Isl* are essential for palp formation (Liu and Satou, 2019; Wagner et al., 2014). The ANB
85 thus shares similarities with vertebrate anterior cranial placodes; and the palps share
86 similarities with derivatives of the vertebrate telencephalon such as the olfactory bulb and
87 of the anterior placodes. It has been proposed that co-option of ANB/palp gene network to
88 the anterior CNS led to the emergence of the vertebrate telencephalon (Cao et al., 2019).
89 While knowledge on transcription factors functions and interactions in palp formation has
90 been elucidated in some detail (Horie et al., 2018; Liu and Satou, 2019; Wagner et al., 2014),
91 the role of cell-cell communication is scarce except for the involvement of FGF/Erk pathway
92 (Hudson et al., 2003; Wagner and Levine, 2012). While we have previously shown that
93 inhibition of canonical Wnt pathway is essential for ANB specification (Feinberg et al., 2019),
94 the description of a later function for Wnt signaling is still lacking. In a distantly related
95 ascidian species *Halocynthia roretzi*, morphological data indicate that activating BMP
96 pathway abolishes palp formation while BMP inhibition results in palps made of a single
97 protrusion instead of three (Darras and Nishida, 2001). But the lack of molecular analysis
98 prevents from precisely determining the function of BMP signaling.
99 We have directly addressed the function of BMP signaling pathway in palp formation during
100 the embryogenesis of the ascidian *C. intestinalis*. We show that BMP is involved in two
101 consecutive phases. Up to neurulation, ANB specification is incompatible with active BMP
102 signaling; and the ANB forms in a region devoid of active BMP (as revealed by phospho-

103 Smad1/5/8 immunostaining). Consequently, early activation of BMP prevents palp formation
104 through the inhibition of ANB precursors formation. Following gastrulation, BMP participates
105 in the differentiation of the palps through the specification of the ventral papilla and the
106 regulation of the papillae vs inter-papillae fate decision. In particular, BMP-inhibited larvae
107 harbor a single large protrusion made of elongated cells, the *Cyrano* phenotype, with an
108 increased number of sensory neurons and ACCs. We propose that the competence to
109 become a papilla is regulated by BMP through the transcription factors coding genes *Foxg*
110 and *Sp6/7/8/9*. Interestingly, we show that modulating BMP pathway in the ascidian
111 *Phallusia mammillata* (275 My of divergence time) produces the same phenotypes as in *C.*
112 *intestinalis*. This allowed us to use previously published RNA-seq data (Chowdhury et al.,
113 2022) to identify a number of novel genes expressed in the ANB and the palps. Altogether,
114 our work points to a role for signaling pathways inhibition in ANB specification, similarly to
115 early anterior neurectoderm formation in vertebrates. Moreover, we provide a significant
116 enrichment of the palp gene network, an essential requisite to probe its conservation with
117 the networks regulating cranial placodes and telencephalon formation in vertebrates.

118

119

120 **Results**

121

122 **BMP activation abolishes palp formation**

123 When we activated the BMP signaling pathway by overexpressing, in the ectoderm, the BMP
124 ligand *Admp* by electroporation using the *pFog* driver (active from the 16-cell stage) (Pasini
125 et al., 2006; Rothbacher et al., 2007), we observed an absence of protrusions that are
126 obvious features of the adhesive palps. The anterior end of the larvae were smooth, and
127 epidermal cells were flat and did not display the typical elongated shape (Fig. 1A-D). This
128 morphological evidence was accompanied by the repression of the expression at mid-tailbud
129 stages of all the genes expressed in the palps that we have examined: *Sp6/7/8/9*, *Isl*, *Foxg*,
130 *Celf3/4/5/6*, *Pou4* and *Emx* (Fig. 1E-P). Palps derive from the median anterior neural border
131 (ANB) at gastrula stages (Fig. 1W,X) and can be tracked by the expression of genes essential
132 for palp formation, *Foxg* at neurula stages (Fig. 1Q,R) and *Foxc* at gastrula stages (Fig. 1U,V)
133 (Liu and Satou, 2019; Wagner and Levine, 2012). Both genes were repressed by BMP
134 activation; and this repression is sufficient to explain the later lack of palp gene expression
135 and differentiation. Interestingly, palps were not converted into general epidermis since the
136 epidermal marker *Sox14/15/21* (also known as *SoxB2*) was normally expressed and did not
137 show ectopic expression in the palp area (Fig. 1S,T).

138

139 **Dynamic BMP activity in the palp forming region**

140 The above results suggest that active BMP signaling is incompatible with palp formation.
141 Active BMP signaling can be determined by examining the phosphorylated (active) form of
142 the BMP transducer *Smad1/5/8*. It has been previously shown that BMP is active from late
143 gastrula to early tailbud stages in the ventral epidermis midline of *C. robusta* embryos (Waki
144 et al., 2015). We obtained similar results in *C. intestinalis* using a different antibody (Fig. 2).
145 More specifically, up to gastrulation, we did not detect significant levels for P-*Smad1/5/8*
146 except in a few posterior endomesodermal cells (Fig. 2A). At mid-gastrula, P-*Smad1/5/8* was
147 present in the nuclei of the posterior (b-line) ventral midline epidermis (Fig. 2B).
148 Consequently, at the onset of *Foxc* expression in palp precursors (St. 10), BMP is not active in
149 the palp forming region (Fig. 2L). Shortly later, at early neurula stages, P-*Smad1/5/8*
150 extended into the anterior (a-line) ventral midline epidermis (Fig. 2C). During neurulation,
151 the posterior limit of P-*Smad1/5/8* gradually shifted anteriorly in agreement with the

152 dynamic posterior to anterior expression of candidate target genes (Roure and Darras,
153 2016); by mid-tailbud stages, P-Smad1/5/8 was restricted to the trunk ventral epidermis (Fig.
154 2D,E,J). In addition, active Smad1/5/8 was also detected in the endoderm underlying the
155 ventral epidermis midline with a similar temporal dynamic (Fig. 2E,F,H,J,K), and in a group of
156 cells of the anterior sensory vesicle at mid-tailbud stages (Fig. 2E,J,K). We validated the
157 specificity of these results by modulating BMP pathway: when embryos were treated with
158 BMP2 protein, P-Smad1/5/8 was ectopically detected in the entire epidermis at early
159 neurula stages, while no staining was observed following inhibition using the
160 pharmacological inhibitor DMH1 (Fig. S1).

161 To precisely relate the location of active signaling in the ectoderm and palp precursors, we
162 performed double staining: P-Smad1/5/8 and *in situ* hybridization for the early palp markers
163 *Foxc* and *Foxg* (Fig. 2F-J). At late gastrula stages, P-Smad1/5/8 abutted *Foxc* expression
164 domain, confirming that palp precursors were specified in a BMP-negative domain (Fig.
165 2F,G,L). Later, we observed P-Smad1/5/8 in the median *Foxc* expression domain at mid
166 neurula stages (not shown) and in the median part of the U-shaped *Foxg* expression domain
167 in late neurulae (Fig. 2H,I,L), corresponding presumably to the future ventral palp. This was
168 confirmed by co-expression of P-Smad1/5/8 and *Foxg* in ventral cells of early tailbuds (Fig.
169 2J,K,L). In summary (Fig. 2K-L), BMP signaling is absent from the ANB marked by *Foxc* until
170 the early neurula stages; active BMP signaling is detected from mid neurula stages in the
171 future protrusion (*Foxg*⁺ cells) of the ventral palp.

172

173 **BMP inhibition participates in ANB definition**

174 We next tested whether BMP inhibition was sufficient to induce an ANB fate. When BMP
175 signaling was blocked either by overexpression of the secreted inhibitor Noggin or by
176 treatment with the BMP receptor inhibitor DMH1, *Foxc* expression at late gastrula stages
177 was unchanged (Fig. 3A-C). The fact that *Foxc* was not ectopically expressed following BMP
178 inhibition could be explained by an incomplete BMP blockade. However, DMH1 treatment
179 led to undetectable P-Smad1/5/8 levels (Fig. S1). Alternatively, it could be that the number
180 of cells that are competent to become ANB in response to BMP inhibition could be restricted
181 to the cells already expressing *Foxc*. *Foxc* expression and palp fate are regulated by FGF
182 signaling following neural induction and cell fate segregation (Wagner and Levine, 2012). We
183 thus aimed at increasing the number of cells competent to form ANB by early activation of

184 FGF signaling using treatment with recombinant bFGF protein, and testing the effects of
185 BMP pathway modulations in this context. As expected, bFGF treatment from the 8-cell
186 stage neuralized the entire ectoderm as revealed by the ectopic expression of the neural
187 markers *Otx* and *Celf3/4/5/6* and the downregulation of the epidermal marker *Tfap2-r.b*
188 (also known as *Ap2-like2*) at late gastrula stages (Fig. 3iv). *Foxc* behaved somewhat
189 unexpectedly: it was either ectopically expressed in a fraction of the embryos (53%, n=69;
190 Fig. 3Giv) or repressed in the others (38%, n=69; not shown). The repression of *Foxc* might
191 be explained by the fact that FGF/Erk is downregulated in the palp lineage during
192 gastrulation (Wagner and Levine, 2012), hence our continuous treatment might inhibit *Foxc*
193 expression. Nevertheless, when BMP pathway was inhibited on top of FGF activation, *Foxc*
194 was strongly expressed, in all embryos, as a cup covering the anterior end including the
195 ventral epidermis (Fig. 3Gvi). Our observations demonstrate that *Foxc* expression and ANB
196 formation can only occur in a domain devoid of active BMP signaling.
197 Importantly, the loss of *Foxc* following BMP activation using recombinant BMP2 protein
198 treatment was specific to this gene and did not result from neural tissue inhibition as in
199 vertebrates since the neural markers *Otx* and *Celf3/4/5/6* were still expressed in the CNS but
200 downregulated in the ANB (Fig. 3ii). Reciprocally, BMP inhibition was not sufficient to lead to
201 ectopic neural tissue formation (Fig. 3iii). This is in agreement with similar data produced in
202 the distantly related ascidian *Halocynthia roretzi* (Darras and Nishida, 2001). Interestingly,
203 while *Foxc* and *Tfap2-r.b* were co-expressed in the ANB, only *Foxc* was repressed by BMP (Fig.
204 S2). However, since the ANB does not convert into epidermis (Fig. 1), it is likely that, while
205 inhibiting palp fate, BMP signaling does not abolish all the effects of the induction by FGF.

206

207 **The BMP signaling pathway regulates palp formation after ANB is specified**

208 We examined whether modulating BMP had any impact on palp formation besides ANB
209 specification. We thus performed whole embryo treatments starting at progressively later
210 stages of embryonic development and examined the early marker *Foxc* and the late marker
211 *Isl* (Fig. 4).

212 Activating BMP at early gastrula stages (St. 10) partially repressed *Foxc* while mid-gastrula
213 (St. 12) treatment had no effect. The time-dependent effects of BMP activation coincide
214 with the dynamics of *Foxc* expression: before it was expressed (8-cell stage), the repression
215 was complete (Fig. 3Gii); at the onset of expression (St. 10), the repression was milder; and

216 once *Foxc* was robustly expressed (St. 12), there was no repression. This is further supported
217 by the fact that BMP2 treatment led to fast P-Smad1/5/8 nuclear accumulation (the shortest
218 treatment we have tested is 30 min; Fig. S1). The ventral spot of *Isl* expression was lost for
219 treatments starting at St. 10 and St. 12, while later treatments did not change *Isl* expression.
220 Inhibiting BMP had no effect on *Foxc* expression similarly to the earliest treatment (Fig. 3Giii).
221 *Isl*, that is normally expressed as 3 spots, had a U-shaped expression. The same effect was
222 observed by overexpressing Noggin using electroporation (Fig. S3). This phenotype was
223 much less frequent when the DMH1 treatment started at late neurula stages (St. 16).
224 The changes in *Isl* expression prompted us to determine how palps differentiate when BMP
225 is modulated from gastrula stages.

226

227 **A single protrusion with additional neurons following BMP inhibition**

228 In DMH1-treated embryos, while *Isl* was expressed following a large U at mid-tailbud stages
229 (St. 23) (Fig. 4), it was concentrated in a protruding structure at the anterior tip at late
230 tailbud stages (St. 25) (Fig. 5E). In larvae, this single large protrusion was made of elongated
231 cells as visualized by phalloidin staining (Fig. 5F-H). We coined this phenotype *Cyrano* (in
232 memory of the famous character depicted by Edmond Rostand). In *Ciona*, it is thought that
233 palps contain a fixed number of the different cell types (Zeng et al., 2019). We performed
234 fluorescent *in situ* hybridization at late-tailbud stages (St. 23) using four genes and made 3D
235 reconstruction of the z-stacks acquired by confocal microscopy (see Material and Methods)
236 in order to determine the differentiation of the palps in the *Cyrano* embryos. In agreement
237 with previous reports, we found that the ACC marker *Isl* was expressed in 12 cells in control
238 embryos (4 cells par papilla) (Fig. 5I-J,Q). By contrast, we found that the sensory neuron
239 marker *Pou4* was expressed in 10 cells instead of 12 (Fig. 5I-J,Q). Interestingly, both dorsal
240 palps had four cells that surrounded the *Isl*-positive cells while the ventral palp contained
241 two *Pou4*-positive cells located dorsally to the *Isl*-positive cells. In DMH1-treated embryos,
242 the number of neurons increased to 18 on average and the number of ACCs to 18 (Fig. 5Q).
243 The increase of *Celf3/4/5/6* cells was not statistically significant. Interestingly, the number of
244 cells expressing *Sp6/7/8/9*, that has been described as an inter-papillae marker (Wagner et
245 al., 2014), was decreased in DMH1-treated embryos (Fig. 5Q). These data are in agreement
246 with the interpretation that the number of cells with papilla fate has increased, however
247 their physical proximity likely leads to the formation of a single protrusion.

248 In DMH1 embryos, *Pou4* was expressed all around the *Isl* cells like in the dorsal palps (Fig.
249 5M,N). This suggested that the *Cyrano* protrusion may have a dorsal identity. In support of
250 this interpretation, we found that the expression of the homeobox transcription factor *Msx*,
251 that we found transiently expressed in the future ventral palp at the onset of *Isl* expression
252 (Fig. 5K,L), was lost following BMP inhibition (Fig. 5O,P). While we have performed a detailed
253 analysis of the *Cyrano* phenotype only on embryos generated by DMH1 treatment starting at
254 gastrula stages, a similar phenotype was observed upon Noggin overexpression (a single
255 protrusion visualized by phalloidin staining in Fig. 5F; U-shape/ectopic expression of *Isl* and
256 *Celf3/4/5/6* in Fig. S3). In *Cyrano* embryos, the ventral palp is missing, the number of inter-
257 palp cells is reduced, and there is an excess of dorsal protruding cells. This suggests that BMP
258 is required to specify the ventral palp and inter-palp cells. When this pathway was inhibited,
259 cells that have lost these fates would adopt a 'default' dorsal palp fate.

260

261 **The ventral palp is missing following BMP activation**

262 As expected from the *Isl* profile (Fig. 4), only 2 protruding papillae made of elongated cells
263 developed dorsally in BMP2 treated embryos (Fig. 6G,H). This morphological absence of
264 ventral palp was only partially confirmed at the molecular level. Similarly to *Isl*, *Msx*
265 expression was abolished in treated embryos, but the expression of *Pou4* revealed that one
266 or two neurons were still present (Fig. 6I,J). *Sp6/7/8/9* was repressed in its most ventral
267 expression domain (Fig. 6K). In conclusion, while the ventral protrusion is absent, palp
268 identity is not completely suppressed. Accordingly, we did not detect ectopic expression of
269 the epidermal gene *Sox14/15/21* (Fig. 6L).

270 While BMP is required to define ventral palp fate (Fig. 5), BMP activation does not lead to
271 ectopic ventral palp formation nor ventralizes the dorsal palps (we did not detect obvious
272 defects in dorsal palp differentiation, and identified 4 *Pou4*⁺ cells surrounding *Isl*⁺ cells as in
273 controls, Fig. 6I). Since BMP suppresses ventral palp, it is likely that excessive or precocious
274 BMP signaling levels are responsible for this phenotype.

275

276 **BMP controls ventral palp and inter-palp fates through *Sp6/7/8/9* regulation**

277 The U-shape pattern of *Isl* in DMH1 embryos reminded us of endogenous *Foxg* expression
278 (Liu and Satou, 2019): at neurula stages, *Foxg* was expressed in the future palps following a
279 U-shape that gradually converted into a 3-spots pattern at tailbud stages that prefigures the

280 three papillae protrusions (Fig. 7A-D). It thus seems that inhibiting BMP prevented the
281 refinement of *Foxg* expression. Accordingly, *Foxg* expression was U-shaped following DMH1
282 treatment (Fig. 7E). Interestingly, knockdown of the zinc finger transcription factor coding
283 gene *Sp6/7/8/9* leads to a U-shaped *Foxg* expression (Liu and Satou, 2019). Since *Sp6/7/8/9*
284 and *Foxg* are initially partially co-expressed before showing exclusive patterns (Fig. 2L), it has
285 been proposed that *Foxg* restriction to the future protrusions is the result of repression by
286 *Sp6/7/8/9*. We thus determined *Sp6/7/8/9* and *Foxg* expression following BMP modulation
287 from early gastrula stages (Fig. 7). While we have confirmed initial co-expression using
288 double fluorescent *in situ* hybridization, we have failed to get robust simultaneous
289 expression allowing analysis the effects of the treatments (not shown). At St. 15/16, this is
290 the onset of *Sp6/7/8/9* expression and it was barely detectable (Fig. 7F), and at St. 18/19
291 when *Sp6/7/8/9* expression was strong, *Foxg* showed a transient downregulation (Fig. 7B).
292 We thus analyzed each gene at different stages (*Foxg* at late neurula stages (St. 16) and
293 *Sp6/7/8/9* at initial tailbud stages (St. 18)). When embryos were treated with BMP2 protein,
294 the ventral expression of *Foxg* in the U-shape was missing (Fig 7J) and *Sp6/7/8/9* was
295 ectopically expressed at this location (Fig. 7M). Reciprocally, following DMH1 treatment,
296 *Foxg* was unchanged (Fig. 7K) and ventral *Sp6/7/8/9* expression was shifted to a median
297 position (Fig. 7N). Hence, *Sp6/7/8/9* was no more expressed in the ventral part of the U-
298 shaped palp forming row of cells (Fig 7T). We summarized our understanding of these results
299 on schematic embryos (Fig. 7O-T). Interestingly, DMH1 treatment had limited effects on *Isl*
300 expression at St. 16 (Fig. 4), a timing that coincides with the onset of *Sp6/7/8/9* expression
301 (Figs 2N, 7F).
302 Our results indicate that *Sp6/7/8/9* is positively regulated by BMP signaling. However, since
303 it is not expressed in P-Smad1/5/8⁺ cells (Fig. 2L), we propose that an intermediate, yet
304 unidentified, factor activates *Sp6/7/8/9* downstream of BMP in the neighboring cells (Fig.
305 7U,V). This hypothetical model of gene interaction is sufficient to explain *Sp6/7/8/9* and
306 *Foxg* expression patterns and final phenotypes (Fig. 7U-X). Importantly, our data show that
307 dorsal-most cells of the *Foxg* U-shape and dorsal papils develop independently of BMP
308 signaling. In conclusion, we propose that the ventral papilla and the papilla vs inter-papilla
309 fate choice is controlled by BMP signaling through the indirect regulation of *Sp6/7/8/9*
310 expression.
311

312 **Palp formation is similarly regulated by BMP in *P. mammillata***

313 We aimed at determining the conservation of the role of BMP in palp formation by
314 examining embryos of the ascidian *P. mammillata* that belongs to the same family as *Ciona*,
315 the Phlebobranchia, but with a significant divergence time (275 My) (Fig. 8A) (Delsuc et al.,
316 2018). First, we determined that BMP signaling was active in the ventral part of the embryo
317 with a similar dynamic to *Ciona* as revealed by P-Smad1/5/8 immunostaining (Fig. S4). Next,
318 we identified single orthologs for *Celf3/4/5/6*, *Pou4* and *Isl* genes, that were all expressed in
319 the palps (Fig. 8) (Chowdhury et al., 2022; Coulcher et al., 2020; Dardaillon et al., 2020).

320 Treatment with recombinant BMP2 protein from the 8-cell stage abolished expression of all
321 three markers in the palps, like in *Ciona* (Fig. 8). Following DMH1 treatment from the 8-cell
322 stage, both *Celf3/4/5/6* and *Isl* were expressed in the palp territory following a U-shape
323 pattern like in *Ciona* but not in all cases. For a large fraction of embryos, the pattern
324 appeared as two bars of intense staining resembling the U-shape but without the ventral
325 part. This phenotype that we did not observe in *Ciona* might reveal some differences in the
326 role of BMP in the two species.

327 Given the overall similar effects on palp formation after alterations of BMP signaling, we
328 sought to identify novel palp molecular markers by using a dataset previously generated in *P.*
329 *mammillata* (Chowdhury et al., 2022). We had generated, at several developmental stages,
330 RNA-seq data for whole embryos treated with recombinant BMP4 protein and/or DAPT, a
331 pharmacological Notch inhibitor. We identified 1098 genes repressed by BMP signaling at
332 least at one developmental stage (Table S1). In this list, we found the orthologs for 11 well
333 defined *Ciona* palp markers; and 4 of them (*Otx*, *Isl*, *Atoh1/7* and *Celf3/4/5/6*) were
334 described as expressed in the palp lineage in *Phallusia* (Coulcher et al., 2020; Dardaillon et
335 al., 2020). Using Gene Ontology analysis, we selected a list of 53 genes encoding
336 developmental regulators (transcription factors and signaling molecules) or involved in
337 neural tissue formation, and examined their expression patterns (Table S2). Within this list,
338 the expression patterns of 26 genes were previously determined (from the Aniseed database
339 (Dardaillon et al., 2020) and from our previous data (Chowdhury et al., 2022; Coulcher et al.,
340 2020)); and 12 of them were expressed in the palps. We performed *in situ* hybridization for
341 the remaining 27 genes, and discovered 7 novel palp markers whose expression is shown in
342 Fig. 9.

343 Surprisingly, by examining the expression data generated previously, we found that some
344 genes with palp expression were up-regulated by BMP in our dataset, such as *Chrdl* and *Nos*
345 (Table S2 and Fig. 9A,K). To have a broader view of the potential effect of BMP signaling on
346 gene regulation in the palps, we gathered, from previous publications (Chen et al., 2011;
347 Chowdhury et al., 2022; Coulcher et al., 2020; Joyce Tang et al., 2013; Kusakabe et al., 2012;
348 Liu and Satou, 2019; Pasini et al., 2006; Roure and Darras, 2016; Shimeld et al., 2005;
349 Wagner and Levine, 2012; Wagner et al., 2014), from the Aniseed database (Dardaillon et al.,
350 2020) and from the present study, a list of 68 genes with expression in the palp lineage in
351 *Ciona* and/or *Phallusia* (Table S3). We plotted the results of our *Phallusia* RNA-seq data, and
352 found that 70% of the genes were regulated by BMP signaling. Most of them were repressed
353 by BMP, but 20 genes were activated by BMP, and a smaller fraction was repressed or
354 activated depending on the stage. Consequently, the precise function of BMP that is likely to
355 be dynamic in the course of palp differentiation needs to be further investigated in details.
356 Interestingly, Notch is likely to play a role in the specification of the different cell types that
357 compose the palps. For instance, it has been shown that activating Notch represses palp
358 neuronal markers in *H. roretzi* (Akanuma et al., 2002). We found 30 genes regulated by
359 Notch in our dataset.
360
361

362 **Discussion**

363 We have shown that BMP signaling regulates two distinct steps of palp formation in *C.*
364 *intestinalis*: ANB specification, and ventral papilla vs inter-papilla specification. Moreover,
365 we have shown conservation of gene expression and regulation by BMP in *P. mammillata*.

366

367 **Signaling pathway inhibition and ANB specification**

368 ANB specification is regulated by inputs from several signaling pathways: FGF, Wnt and BMP.
369 While FGF is positively required early on, at the time of neural induction (32-cell stage), all 3
370 pathways are inactive at the time of ANB fate acquisition as revealed by the expression of
371 *Foxc* (mid-gastrula). This situation is reminiscent of data from vertebrates where anterior
372 neural fate is determined by the triple inhibition of BMP, Nodal and Wnt pathways
373 (Andoniadou and Martinez-Barbera, 2013; Niehrs et al., 2003; Wilson and Houart, 2004). It
374 would thus be interesting to test the function of Nodal inhibition in ANB specification since
375 we have already shown that it is involved in posterior neural fate determination in *Ciona*
376 (Roure et al., 2014). While it appears that active FGF, Wnt or BMP signaling is incompatible
377 with ANB determination, the specific function of each pathway seems different. FGF appears
378 to regulate anterior CNS vs ANB fate decision along the antero-posterior axis (Wagner and
379 Levine, 2012). Wnt seems to regulate *Foxc*+ ANB fate vs *Foxc*- ANB fate along the medio-
380 lateral/dorso-ventral axis (Feinberg et al., 2019). Finally, BMP might participate in the
381 segregation between ANB and immediately anterior/ventral epidermal fates. Finer details on
382 the function of these pathways in ANB fate determination and on their likely cross-talk
383 should be an exciting line of research in this simple and geometric model system.

384

385 **From ANB to palp differentiation**

386 Our results of late inhibition of BMP signaling (from gastrula stages) indicate that *Foxg*,
387 expressed in a single row of cells with a U-shape, delineates cells competent to become
388 papilla. A network of gene interactions has previously been identified that regulates the
389 transition of *Foxg* from a U-shape to 3-spots eventually forming protruding papillae (Liu and
390 Satou, 2019). BMP is an input to this network, presumably through the indirect regulation of
391 ventral *Sp6/7/8/9* expression. We hypothesized the involvement of a signaling molecule that
392 would be a direct target of BMP (Fig. 7). Interestingly, MAPK inhibition during neurulation
393 results in a U-shaped expression of *Isl* (Wagner et al., 2014) similar to what we observed by

394 inhibiting BMP. It is tempting to propose an FGF ligand to be the factor downstream of BMP,
395 however none has been described with a discrete pattern in the palps (Imai et al., 2004).
396 Again, studying epistatic relationships and cross-talks between these signaling pathways is a
397 future line of research.

398 Importantly, we have shown that BMP signaling is active and required in the median palp
399 forming region, most likely corresponding to the future ventral palp, before the onset of
400 *Foxg* and *Sp6/7/8/9* expression. However, activating BMP at this stage, does not result in
401 ectopic palp formation but to an absence of the ventral palp. This discrepancy might be
402 better understood by more finely controlling levels of BMP signaling but also its timing and
403 cells that receive it, through optogenetics for example. Nevertheless, our observations point
404 to differences between the two symmetrically bilateral dorsal palps and the single median
405 ventral palp. While we are not aware of ventral palp-specific marker, we have shown that
406 *Msx* is transiently expressed only in the ventral palp (Fig. 5); this may also be the case for
407 *Hes.a* (Chowdhury et al., 2022). In addition, the *Pou4+* sensory neurons are located dorsally
408 in the ventral palp, while they are located around *Isl+* cells in the dorsal palps. Specific dorsal
409 and ventral genetic sub-network would thus be interesting to uncover.

410

411 **Anterior adhesive organs formation in chordates.**

412 The specification of the 3 cell types (ACCs, neurons and colocytes) that make the papillae
413 and their relationships (lineage, alternative cell fate...) are still poorly understood, but will
414 most likely be the subject of future research (Zeng et al., 2019). For example, we are not
415 aware of colocytes specific gene marker, however these cells can be distinguished from
416 other palp cells with the peanut agglutinin (Cao et al., 2019; Sato and Morisawa, 1999; Zeng
417 et al., 2019). They are involved in the secretion of adhesive materials for the larva to attach
418 to a substrate before metamorphosis. The palps thus constitute an adhesive organ whose
419 homology with adhesive organs that exist in the larvae of some vertebrates (*e.g.* the cement
420 gland of *Xenopus*) has been previously proposed (Yoshida et al., 2012). Our present work
421 adds to the similarities observed between frog cement gland and ascidian palps: they are
422 ectodermal derivatives specialized in adhesion, they are located at the anterior-most part of
423 the larva, they share the expression of the transcription factors coding genes *Otx* and *Pitx*,
424 and their formation is regulated by BMP signaling (Gammill and Sive, 2000; Jin and
425 Weinstein, 2018; Yoshida et al., 2012). Further detailed comparison of the shared but also

426 divergent parts of the developmental networks regulating adhesive organ/sensory organ
427 formation in ascidians and vertebrates should be of great interest.

428

429 **Conservation of PNS formation in chordates?**

430 The ascidian larval PNS, palps included, originates from the neural plate border with the
431 exception of the ventral tail PNS that originates from a region at the opposite end of the
432 embryo, the ventral epidermis. Signaling pathways are pleiotropic and are consequently
433 poor indicators of possible evolutionary conservation. Nevertheless, it is striking that FGF,
434 Wnt and BMP are deployed in ascidians to regulate neural plate border specification and
435 differentiation of its derivatives, most likely with changing dynamic requirements at diverse
436 developmental stages. This is reminiscent of the mechanisms regulating neural plate border
437 and its derivatives, the cranial placodes and the neural crest (Martik and Bronner, 2021; Pla
438 and Monsoro-Burq, 2018; Stundl et al., 2021). The similarities extend beyond signaling
439 pathways since a suite of genes have conserved expression between ascidians and
440 vertebrates, and have led to several evolutionary scenarios (Cao et al., 2019; Horie et al.,
441 2018; Pasini et al., 2006; Poncelet and Shimeld, 2020). Our present study add material to
442 gene network level comparisons.

443 The ascidian PNS is made of epidermal sensory neurons that have different morphologies,
444 connectivity and sensory capacities depending on their location (Abitua et al., 2015; Imai and
445 Meinertzhagen, 2007; Ryan et al., 2018). However, they share a number of genes marking
446 the presumptive domains or differentiating neurons. Yet, what regulates their specific
447 identities is still incompletely understood (Chacha et al., 2022). For example, a number of
448 genes expressed in the tail PNS are also expressed in the palps, and these expression
449 domains are conserved in species that have diverged almost 400 My ago (Table S3)
450 (Akanuma et al., 2002; Coulcher et al., 2020; Joyce Tang et al., 2013; Pasini et al., 2006;
451 Roure and Darras, 2016). Comparative approaches of PNS formation between divergent
452 ascidian species and across chordates (vertebrates and cephalochordates) promise to yield
453 insights into PNS evolution and the flexibility of developmental mechanisms.

454

455

456 **Materials and methods**

457

458 **Embryo obtention and manipulation**

459 Adults from *Ciona intestinalis* (formerly referred to *Ciona intestinalis* type B (Brunetti et al.,
460 2015)) were provided by the Centre de Ressources Biologiques Marines in Roscoff (EMBRC-
461 France). Adults of *Phallusia mammillata* were provided by the Centre de Ressources
462 Biologiques Marines in Banyuls-sur-mer (EMBRC-France) following diving or by professional
463 fishermen following trawling in the Banyuls-sur-mer (France) area. Gametes collection, *in*
464 *vitro* fertilization, dechoriation and electroporation were performed as previously
465 described (Coulcher et al., 2020; Darras, 2021); and staging of embryos was performed
466 according to the developmental table of *Ciona robusta* (Hotta et al., 2007).

467 Electroporation constructs used in this study have been previously described (Pasini et al.,
468 2006). Embryos were treated with 150 ng/ml of recombinant mouse BMP2 protein (355-BEC,
469 R&D Systems Inc, 100 µg/mL stock solution in HCl 4 mM + BSA 0.1 %), 100 ng/ml of
470 recombinant human bFGF (F0291, Sigma-Aldrich, 50 µg/mL stock solution in 20 mM Tris
471 pH=7.5 + BSA 0.1 %) complemented with 0.1% BSA, or 2.5 µM of the BMP receptor inhibitor
472 DMH1 (S7146, Euromedex, 10 mM stock solution in DMSO) at the stages indicated in the
473 text and figures. These concentrations were determined following pilot experiments. Control
474 embryos were incubated with sea water containing 0.1 % BSA and/or 0.025% DMSO.

475

476 ***In situ* hybridization and immunostaining**

477 For all labeling experiments, embryos were fixed in 0.5 M NaCl, 100 mM MOPS pH=7.5 and
478 3.7% formaldehyde. Whole mount chromogenic *in situ* hybridization were performed using
479 plasmid cDNA or synthetic DNA (eBlocks Gene Fragment, IDT) as templates for probe
480 synthesis (Tables S2 and S4) as described previously (Chowdhury et al., 2022). Gene models
481 and identifiers correspond to the following genome assemblies, KH2012 for *Ciona robusta*
482 (Satou et al., 2008) and MTP2014 for *Phallusia mammillata*, that were retrieved from the
483 Aniseed database (Dardaillon et al., 2020). Images were acquired using an AxioCam ERc5s
484 digital camera mounted on a stereomicroscope (Discovery V20, Zeiss). The number of
485 experiments and embryos for phenotypic effects by gene expression analysis are shown in
486 the figures and their legends.

487 Fluorescent *in situ* hybridization were adapted from (Racioppi et al., 2014). Briefly,
488 digoxigenin-labeled probes were recognized using an anti-DIG antibody coupled to
489 peroxidase (11207733910, Roche), and fluorescein-labeled probes were recognized using an
490 anti-FLUO antibody coupled to peroxidase (11426346910, Roche). Fluorescence signal was
491 produced using the TSA plus kit (NEL753001KT, Perkin-Elmer) following manufacturer's
492 recommendations with cyanin3 and fluorescein for DIG- and FLUO-probes respectively.
493 Active BMP signaling was visualized by immunostaining using a rabbit monoclonal antibody
494 against mammal Smad1, Smad5 and Smad8 phosphorylated at two serine residues at the C-
495 terminal end (clone 41D10, #9516, Cell Signaling Technology) diluted at 1:200. The epitope is
496 present in the single ortholog Smad1/5/8 of both *Ciona intestinalis* and *Phallusia*
497 *mammillata*. Anti-rabbit coupled to Alexa Fluor 568 (A11011, Invitrogen) was used at 1:400
498 for visualization. Similar data were obtained using another antibody (clone D5B10, #13820,
499 Cell Signaling Technology) (data not shown). Membranes were stained using Alexa Fluor 594
500 phalloidin (A12381, Invitrogen) used at 1:1000. Nuclei were stained using DAPI. Image
501 acquisition was performed using confocal microscopy (Leica SP8-X, BioPiC platform, Banyuls-
502 sur-mer). Confocal z-stacks were visualized and analyzed in 3D using the Imaris 8.3 software
503 (Bitplane). In particular, this software was used to count the number of cells expressing a
504 gene of interest. In brief, fluorescent signals were converted as 3D objects: *in situ*
505 hybridization signals as surface objects, and DAPI-labeled nuclei as spots. The number of
506 spots within a given surface was used as a proxy for the number of cells expressing a gene.
507 Snapshots of such analyses and 3D renderings are shown in Figs 1,2,5,6,S1,S2. Maximum
508 intensity projections of Fig. S4 were performed using ImageJ.
509 Image panels and figures were constructed with Affinity Photo and Affinity Designer.
510
511

512 **Conflict of interest.**

513 The authors declare that they have no conflict of interest.

514

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527

528 **Authors' contributions**

529 AR and SD designed the project. AR performed most of the experiments and analyses with
530 the help of RC and SD. SD supervised the project, wrote the manuscript and obtained
531 funding. All authors edited the manuscript, read and approved the final version.

532

533 **Data availability**

534 RNA-seq data are available under the BioProject ID PRJNA779382. All other data generated
535 or analyzed during this study are included in the manuscript and supporting files.

536

537

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688

689

690 **Figure legends**

691

692 **Figure 1. Early BMP activation prevents palp formation.** BMP pathway was activated by
693 overexpressing the BMP ligand *Admp* using the *Fog* ectodermal promoter. Experimental
694 embryos were compared to control (overexpressing the fluorescent protein *Venus*). **(A-D)**
695 Papilla protrusions and elongated cells were absent following BMP activation as revealed by
696 confocal stacks for phalloidin (white) and DAPI (cyan) staining at larval stages (A,B: confocal
697 sections; C,D: surface rendering). Scale bar: 20 μ m. **(E-V)** BMP activation repressed genes
698 expressed in the palps as determined by *in situ* hybridization for *Sp6/7/8/9* (E,F), *Isl* (G,H),
699 *Foxg* (I,J), *Celf3/4/5/6* (K,L), *Pou4* (M,N) and *Emx* (O,P) at mid-tailbud stages (St. 23); and
700 *Foxg* (Q,R) and *Foxc* (U,V) at neurula stages. The expression of the epidermis marker
701 *Sox14/15/21* which is excluded from the palps at neurula stages was unchanged (S,T). For
702 each panel, n indicates the number of embryos examined. The percentages correspond to
703 normal expression for pFog>*Venus*, and to gene repression (except for *Sox14/15/21*) in the
704 palp territory for pFog>*Admp*. Experiments have been performed at least twice, except for
705 *Celf3/4/5/6*, *Pou4*, *Emx* and *Sox14/15/21* where results come from a single experiment. In
706 tailbud embryos, a bulging mass of cells was often visible in the dorsal posterior trunk. It
707 most likely corresponds to the CNS as revealed by *Celf3/4/5/6* expression that was outside
708 of the embryo due to abnormal neural tube closure. Anterior to the left in lateral views
709 except Q-T (frontal views) and U-V (neural plate views). Scale bar: 50 μ m. **(W-X)** Schematic
710 representation of the progeny of the neural plate: CNS in orange, palp region in light purple
711 and aATENS (sensory neurons of the trunk PNS) precursors in gray (adapted from (Horie et
712 al., 2018; Liu and Satou, 2019)).

713

714 **Figure 2. Dynamic BMP activity in the palp forming region. (A-E)** P-Smad1/5/8
715 immunostaining (magenta) at various developmental stages in control embryos: (A) early
716 gastrula (St. 11, vegetal view), (B) late gastrula (St. 13, ventral view), (C) neurula (St. 14/15,
717 ventral view), (D) initial tailbud (St. 18, lateral view), and (E) late tailbud (St. 23, lateral view)
718 stages. **(F-J)** P-Smad1/5/8 immunostaining (magenta) and *in situ* hybridization (green) for
719 *Foxc* at early neurula stages (St. 14) (F,G), and *Foxg* at late neurula (St. 16) (H,I) and mid
720 tailbud (St. 21) (J) stages of control embryos. Dorsal is to the top with lateral views and
721 anterior to the left (F,H,J), or frontal views (G,I). F and G are different views of the same

722 embryo. H and I are different views of another embryo. All data have been obtained from at
723 least two independent experiments. Scale bar: 20 μ m. The embryos have been outlined with
724 white dotted lines. **(K)** Schematic representation of the dynamics of P-Smad1/5/8 (magenta
725 circles) with respect to the palp forming region (light purple). The schemes depict sagittal
726 sections with anterior to the left and dorsal to the top at early gastrula (St. 10/11), late
727 gastrula (St.13), early neurula (St. 14), late neurula (St. 16) and mid tailbud (St. 21) stages.
728 Main sites of expression are depicted: a few endomesodermal cells (St. 10/11), posterior
729 ventral epidermis and endoderm (St. 13), ventral epidermis and endoderm throughout the
730 antero-posterior axis (St. 14 and 16), ventral part of the palp forming region (St 16 and 21),
731 trunk ventral epidermis, endoderm and sensory vesicle (St 21). **(L)** Active BMP signaling (P-
732 Smad1/5/8 in magenta) and palp gene expression (green) for *Foxc*, *Foxg* and *Sp6/7/8/9* were
733 mapped to schematic embryos according to the above data and previous reports (Horie et
734 al., 2018; Liu and Satou, 2019). Schemes and lineages representing the frontal view of
735 embryos during gastrulation and neurulation were drawn following *Phallusia mammillata* 4D
736 reconstructions available at <https://morphonet.org/> (Guignard et al., 2020; Leggio et al.,
737 2019).

738

739 **Figure 3. Inactive BMP signaling is required for ANB specification. (A-C)** *Foxc* expression by
740 *in situ* hybridization at early neurula stages (St. 14) was unchanged following BMP pathway
741 inhibition by Noggin overexpression (B) or DMH1 treatment from the 8-cell stage (C). **(D-G)**
742 Embryos were treated from the 8-cell stage to the fixation at early neurula stages (St. 14)
743 with BMP2 protein or DMH1 alone, or in combination with bFGF protein. Gene expression
744 was assessed by *in situ* hybridization for *Celf3/4/5/6* (D), *Otx* (E), *Tfap2-r.b* (F) and *Foxc* (G).
745 For each panel, n indicates the number of embryos examined. The percentages indicate the
746 frequency of the phenotype depicted in the picture. The results come from two independent
747 experiments. Embryos are shown with anterior to the left in neural plate views except insets
748 that are lateral views with dorsal to the top. The arrows in Dii and Eii mark the
749 downregulation of *Celf3/4/5/6* and *Otx* in the palp precursors. Scale bar: 50 μ m. **(H)**
750 Schematic interpretations of the consequences of the various treatments (i: control, ii: BMP2,
751 iii: DMH1, iv: bFGF, v: BMP2+bFGF and vi: DMH1+bFGF) on some ectodermal derivatives:
752 palp precursors (light purple), a-line neural tissue (orange), a-line epidermis (light gray) and
753 b-line epidermis (dark grey). The embryo schemes show a neural plate view (top) and a

754 lateral view (bottom) with anterior to the left. The schemes were drawn using *Phallusia*
755 *mammillata* 4D reconstructions available at <https://morphonet.org/> (Guignard et al., 2020;
756 Leggio et al., 2019).

757

758 **Figure 4. Late effects of BMP pathway modulations on palp formation.** Embryos were
759 treated with BMP2 protein (left panels) or DMH1 (right panels) from the stage indicated on
760 the figure up to fixation and *in situ* hybridization for *Foxc* at early neurula stages (St. 14) and
761 *Isl* at late tailbud stages (St. 23). For each panel, n indicates the number of embryos
762 examined. The percentages indicate the frequency of the phenotype depicted in the picture.
763 The results come from two or more independent experiments. Embryos are shown in neural
764 plate views with anterior to the left for *Foxc* and frontal view with dorsal to the top for *Isl*.
765 White arrowheads highlight the absence of the ventral spot of *Isl*. Scale bar: 50 μ m.

766

767 **Figure 5. BMP inhibition leads to the formation of a single large palp of dorsal character.**

768 **(A-P)** Embryos where BMP signaling was inhibited using treatment with DMH1 from St. 10
769 (E,G,H,M-P)) or Noggin overexpression (F) were compared to control embryos (A-D,I-L) for
770 morphology and gene expression by *in situ* hybridization. *Isl*, normally expressed in each of
771 the 3 protruding palps (A; 86%, n=7), was expressed as a large spot in a single protrusion at
772 late tailbud stages (St. 25) in treated embryos (E; 100%, n=21). The single large protrusion is
773 made of elongated cells (F-H; DAPI in cyan and phalloidin in white). Double fluorescent *in*
774 *situ* hybridization for *Pou4* (magenta) and *Isl* (green) in control (I) and treated embryo (M) at
775 late tailbud stages (St. 23). 3D representation of nuclei for cells expressing each gene (J,N).
776 Double fluorescent *in situ* hybridization for *Msx* (magenta) and *Isl* (green) in control (K) and
777 treated embryo (O) at early tailbud stages (St. 19). 3D representation of nuclei for cells
778 expressing each gene (L,P). Co-expression of *Isl* and *Msx* appears white. Embryos are shown
779 with dorsal to the top in lateral views (A-H) or frontal views (I-P). Scale bars: 50 μ m, except
780 for D and H: 20 μ m. **(Q)** Count of the number of cells expressing each gene at late tailbud
781 stages (St. 23) using 3D reconstructions as in J and N. The graph represents the average
782 values from two or more independent experiments, with error bars denoting the standard
783 deviation. Differences in cell number were evaluated using the Mann-Whitney U test, and p-
784 values are indicated (n.s.: non statistically significant). The numbers of embryos examined

785 are as follows: control embryos (*Isl*: 14, *Pou4*: 5, *Celf3/4/5/6*: 5, and *Sp6/7/8/9*: 6) and
786 DMH1-treated embryos (*Isl*: 9, *Pou4*: 6, *Celf3/4/5/6*: 5, and *Sp6/7/8/9*: 5).

787

788 **Figure 6. Late BMP activation prevents ventral palp formation.** Embryos for which BMP
789 signaling was activated using BMP2 treatment from St. 10 (G-L) were compared to control
790 embryos (A-F) for morphology and gene expression by *in situ* hybridization. While 3
791 protruding papillae made of elongated cells were clearly seen in control embryos (A-B), only
792 2 dorsal protruding papillae were present in treated larvae (G-H) (B,H: phalloidin (white) and
793 DAPI (cyan) in confocal sections; A,G: resulting surface rendering). **(C,I)** Double fluorescent *in*
794 *situ* hybridization for *Pou4* (magenta) and *Isl* (green) in control (C) and treated embryo (I) at
795 late tailbud stages (St. 23). While 3 spots of *Isl* expression were seen in control embryos
796 (n=3), 2 dorsal spots were detected in all treated embryos (n=4). In the ventral region, *Pou4*
797 was expressed in 2 cells in controls (as described in Fig. 5). In treated embryos, we found 2
798 embryos with 2 *Pou4*⁺ cells and 2 embryos with 1 *Pou4*⁺ cell. White arrows in I point to two
799 *Pou4*⁺ cells in the ventral area of a treated embryo. **(D,J)** Fluorescent *in situ* hybridization for
800 *Msx* (magenta) in control (D) and treated embryo (J) at early tailbud stages (St. 19). Control
801 embryos: 100% with *Msx* expression in the ventral palp region (n=4). BMP2-treated
802 embryos: 100% without *Msx* expression (n=12). **(E,F,K,L)** Colorimetric *in situ* hybridization for
803 *Sp6/7/8/9* (E,K) and *Sox14/15/21* (F,L). In these panels, n indicates the number of embryos
804 examined. The percentages indicate the frequency of the phenotype depicted in the picture
805 (the results come from two independent experiments). White stars in I-K highlight the
806 absence of staining in the ventral region. Embryos are shown with dorsal to the top in frontal
807 views except lateral views in B and H. Scale bars: 25 μm (displayed for each type of imaging
808 data).

809

810 **Figure 7. Regulation of *Foxg* and *Sp6/7/8/9* by BMP signaling.** **(A-D)** Expression of *Foxg* at
811 different developmental stages in the palp forming area (the specific stage is indicated at the
812 top of each picture). Note that, at early stages (A), *Foxg* was expressed in two anterior
813 ectodermal territories, the U-shaped palp forming region and a more dorsal row of cells
814 likely contributing to the oral siphon primordium (Liu and Satou, 2019). At initial tailbud
815 stages (B) *Foxg* expression was dramatically downregulated in the U-shaped region.
816 Concomitantly, a transient strong expression in the ventral trunk epidermis was detected.

817 **(E)** At mid tailbud stages, *Foxg* was expressed following a U-shape when BMP pathway was
818 inhibited from early gastrula (St. 10) with DMH1. **(F-H)** *Sp6/7/8/9* expression at different
819 developmental stages in the palp forming area. **(I-T)** Expression of *Foxg* (I-K) at late neurula
820 stages (St. 16) and *Sp6/7/8/9* (L-N) at initial tailbud stages (St. 18) in control embryos (I,L),
821 BMP2-treated embryos (J,M), and DMH1-treated embryos (K,N). n indicates the number of
822 embryos examined. The percentages indicate the frequency of the phenotype depicted in
823 the picture. The results come from two independent experiments. Embryos are shown in
824 frontal view with dorsal to the top. Scale bar: 50 μ m. A schematic representation of our
825 interpretation of the expression patterns is shown for *Foxg* (O-Q) and *Sp6/7/8/9* (R-T) with
826 the same color code as in Fig. 2 (light purple: palp precursors; orange: a-line CNS; gray:
827 aATEN precursors; and green: gene expression). **(U-X)** Model for the action of BMP signaling
828 on protruding papilla vs inter-palp fate specification. The model focuses on the 8 *Foxg*⁺ cells
829 making a U-shape at neurula stages that have the potential to become protruding papillae.
830 Importantly, dorsal palp formation is independent of BMP signaling, and expression data (I-
831 N) show that only the 4 median cells are affected by BMP signaling. Hence, the model
832 focuses only on these 4 cells where we postulate some genetic interactions (U). During
833 normal development (V), active BMP signaling (magenta) in the two median cells induces
834 ventral fate and the expression of an unidentified factor (green) that activates the
835 expression of *Sp6/7/8/9* (yellow) in the neighboring cells (*Sp6/7/8/9* is also activated
836 independently of BMP in the most dorsal cells). Next, *Sp6/7/8/9* represses the expression of
837 *Foxg* (light purple) leading to alternate and excluded patterns of expression of these two
838 genes and subsequent specification of protruding and non-protruding cells. Following BMP
839 activation (W), the unidentified factor activates *Sp6/7/8/9* in the 4 median cells, abolishing
840 *Foxg* expression and the formation of the ventral protrusion. In absence of BMP signaling (X),
841 median cells do not acquire a ventral identity and do not express the unidentified factor.
842 Hence *Sp6/7/8/9* expression is not activated and *Foxg* not repressed.

843

844 **Figure 8. The BMP signaling pathway regulates palp formation in *Phallusia mammillata*.**

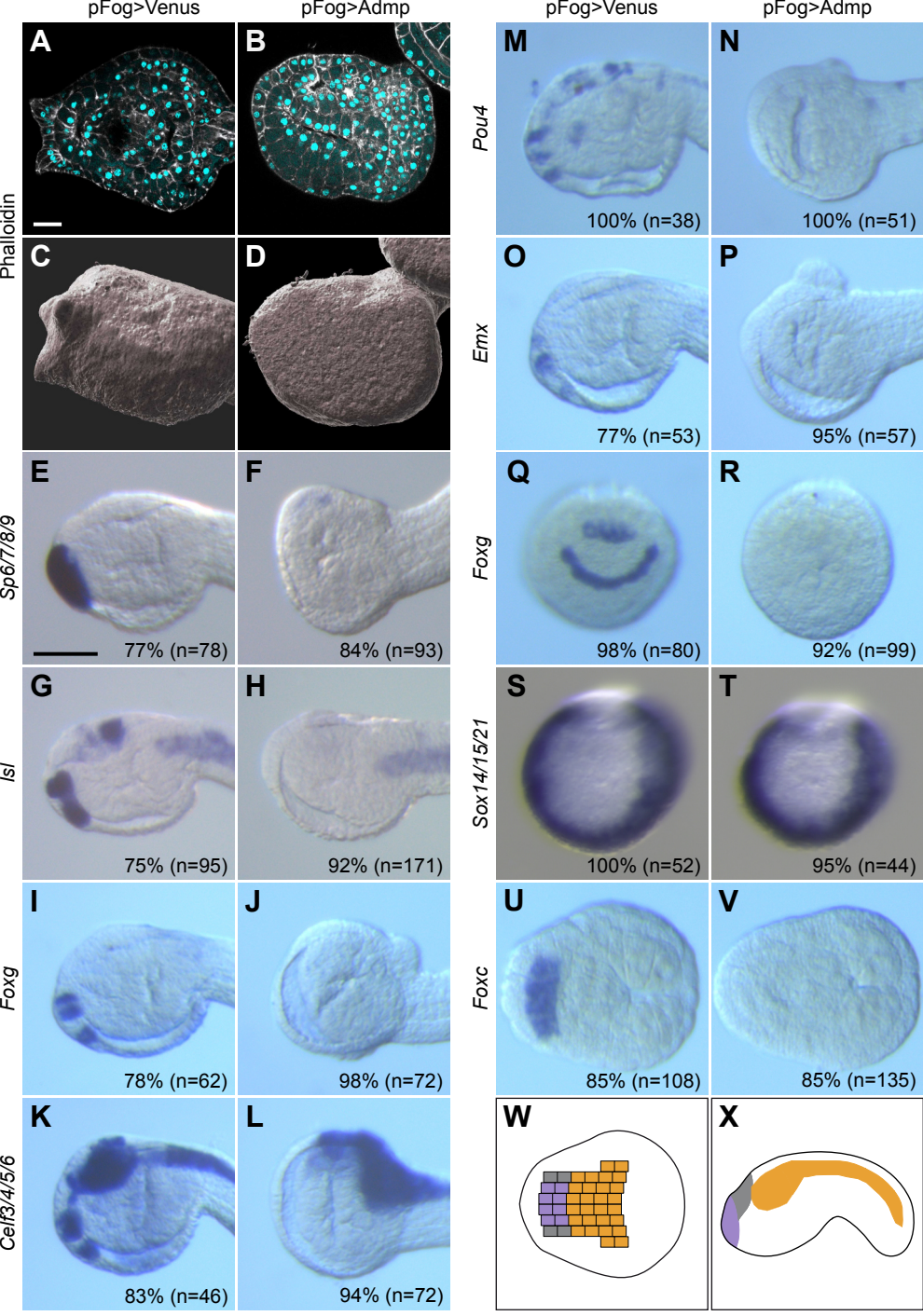
845 **(A)** Schematic representation of the appearance of adults *C. intestinalis* and *P. mammillata*,
846 and their phylogenetic distance. **(B-N)** *P. mammillata* embryos were treated from the 8-cell
847 stage with 150 ng/ml recombinant BMP2 protein (C,F,J,N) or 2.5 μ M DMH1 (D,G,H,K,L). They
848 were fixed at neurula stages (B-D) and mid/late tailbud stages (E-N). Expression patterns for

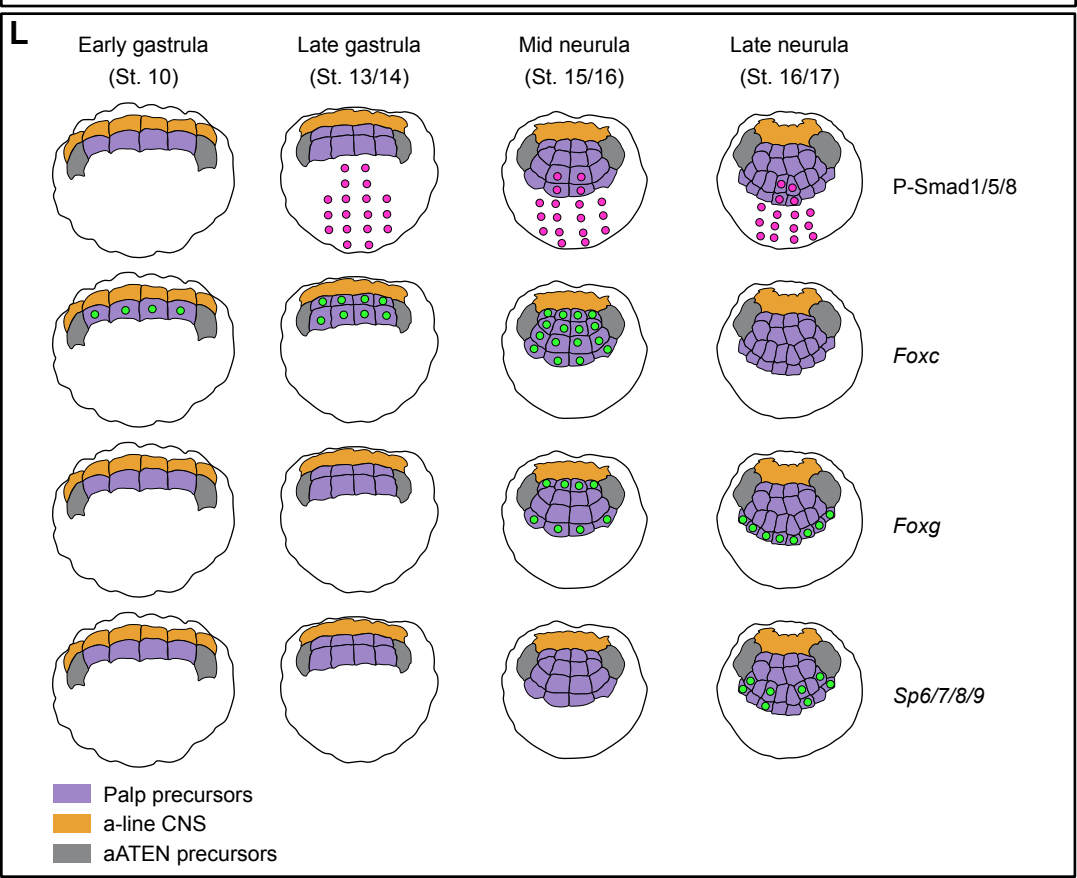
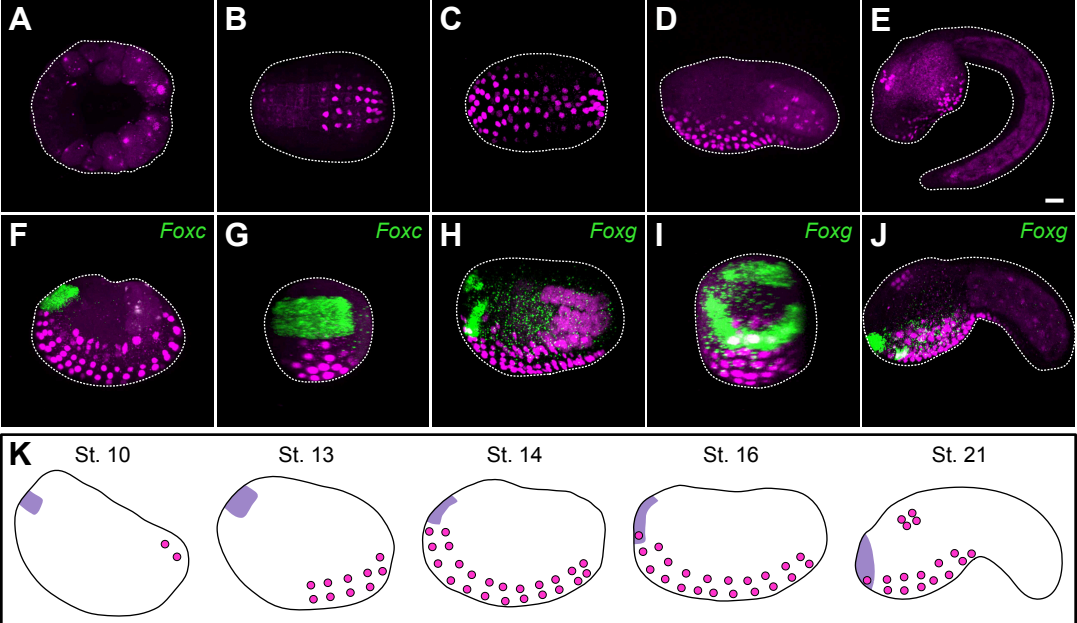
849 *Celf3/4/5/6* (B-H), *Isl* (I-L) and *Pou4* (M,N) was determined by *in situ* hybridization. The arrow
850 in C marks the repression of *Celf3/4/5/6* in the ANB. For each panel, n indicates the number
851 of embryos examined. The percentages indicate the frequency of the phenotype depicted in
852 the picture. The results come from two or more independent experiments. Embryos are
853 shown with anterior to the left in neural plate view (B-D), and in frontal view with dorsal to
854 the top (E-N). Scale bar: 50 μ m.

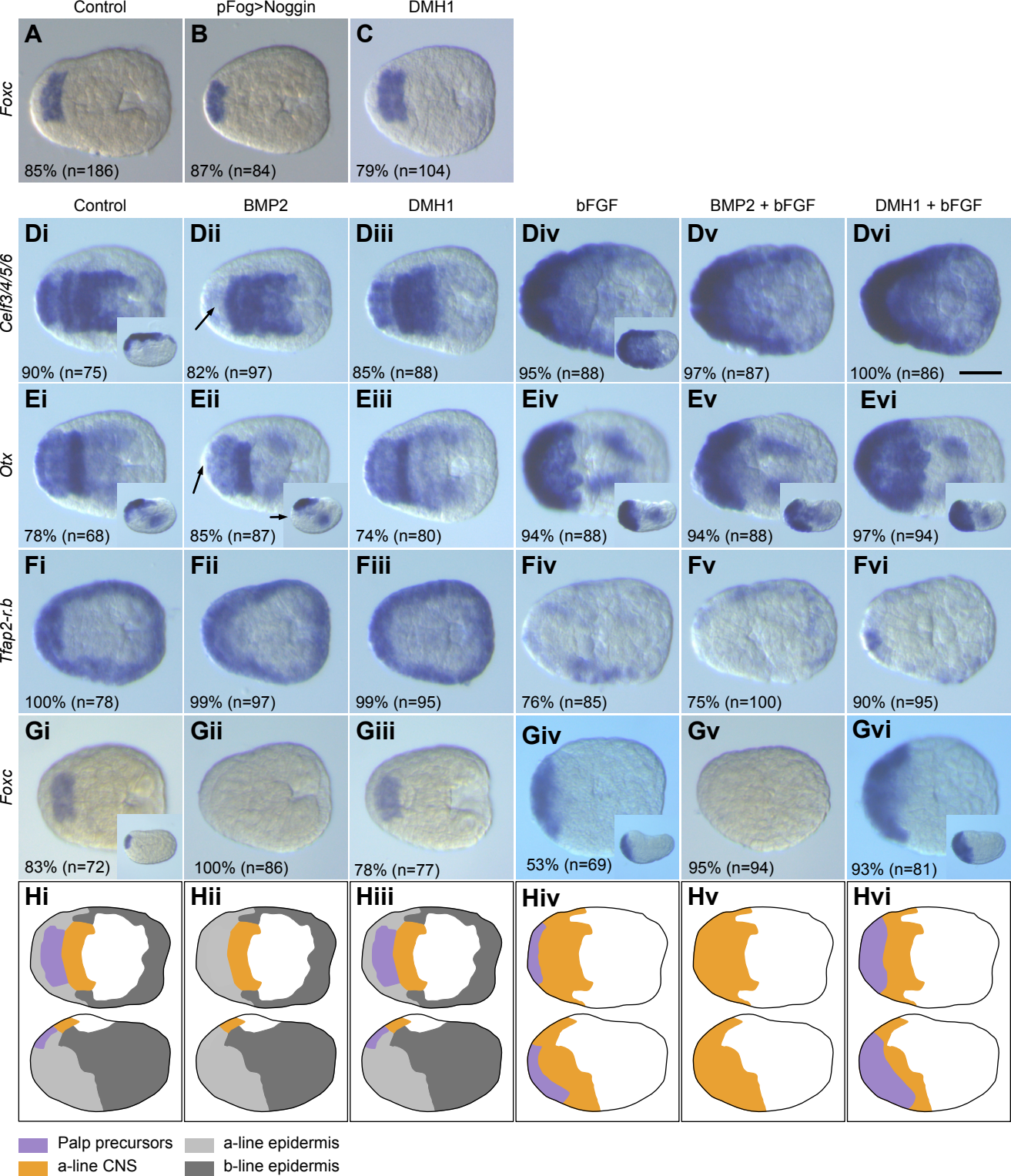
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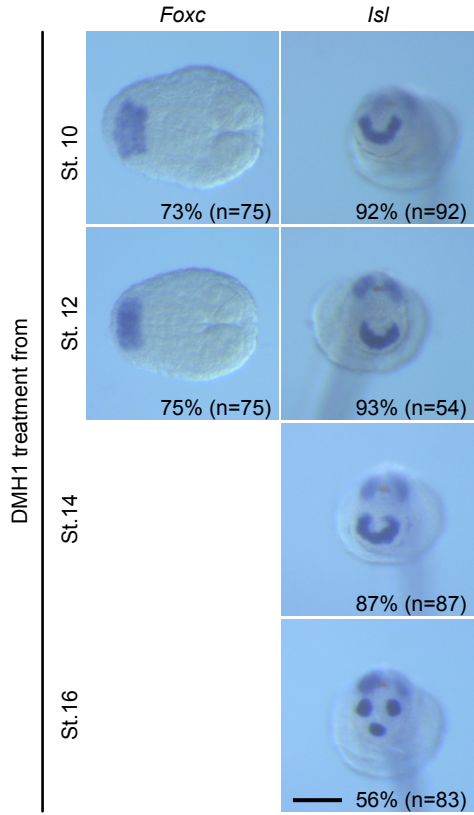
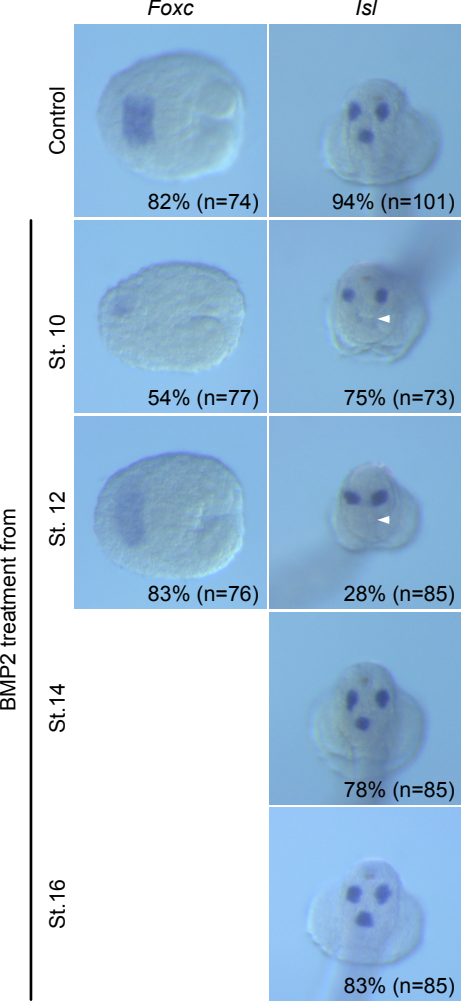
856 **Figure 9. Identification of genes expressed in the palps in *Phallusia mammillata*.** *In situ*
857 hybridization at selected stages for *Chrdl* (A), *Tp53inp* (B,C), *Fzd9/10* (D,E), *Plg* (F,G), *Mucin*
858 (H), *Hes.b* (I), *Barhl* (J), *Nos* (K), *Fbn* (L) and *Wscd* (M). Embryos are shown with anterior to
859 the left in neural plate view (A,B,D), in lateral view with dorsal to the top (B inset,C,D inset,
860 E-M), and in frontal view with dorsal to the top (insets in C,E,F,H,L,M). Scale bar: 50 μ m.

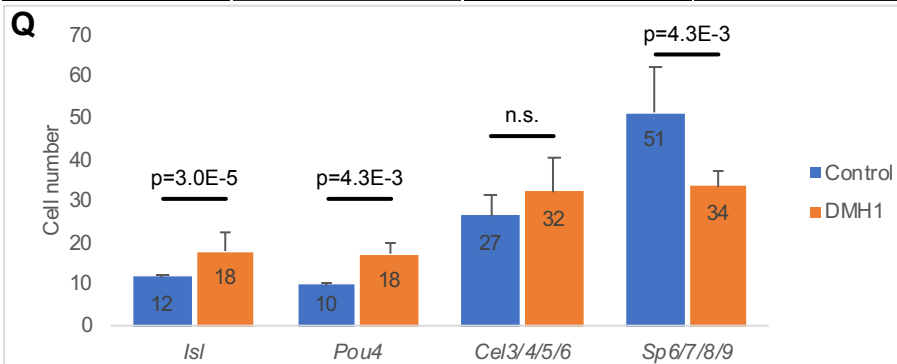
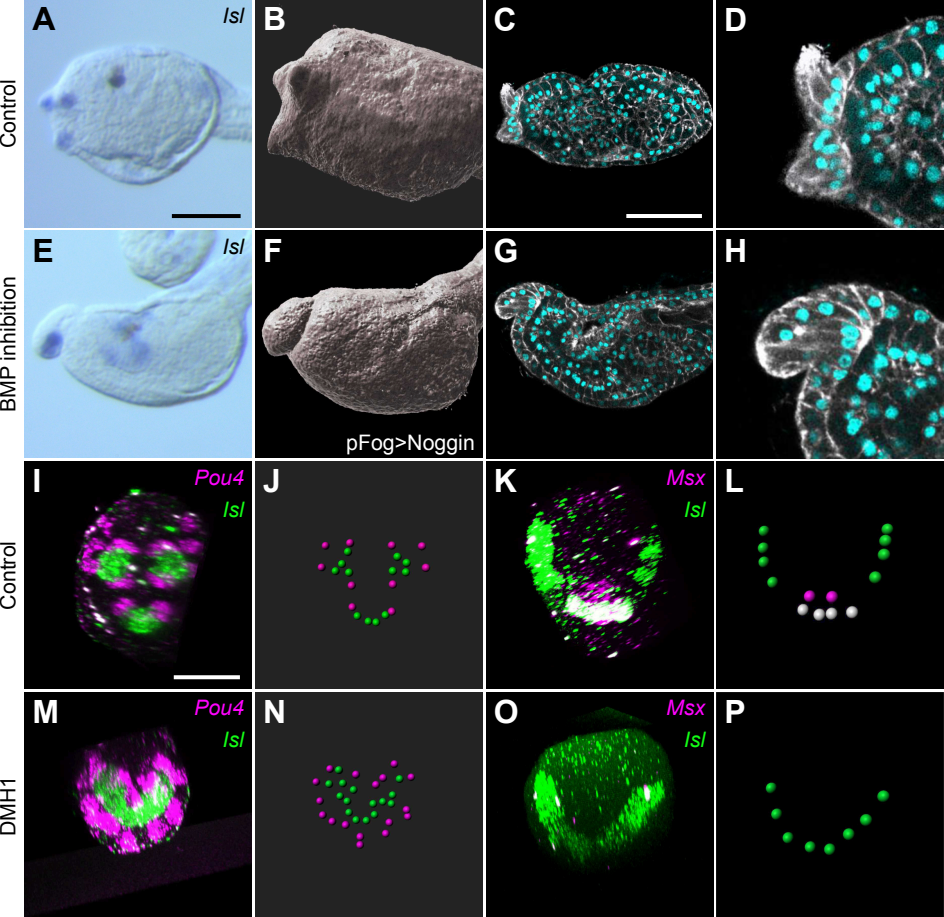
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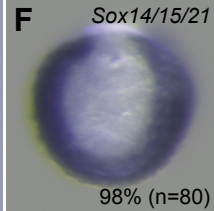
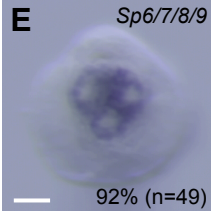
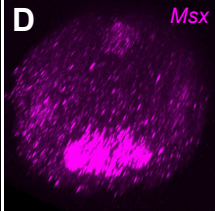
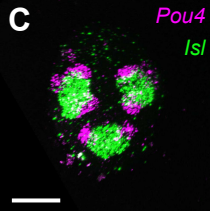
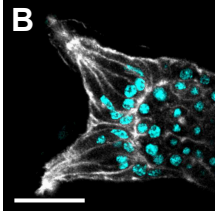
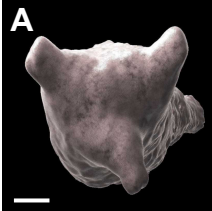








Control



BMP activation

