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Intracellular enhancement of BMP signaling by LIM-domain protein FHL3 controls spatiotemporal emergence of the neural crest driven by WNT signaling.

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1 **Summary**

2 The spatiotemporal coordination of multiple morphogens is essential for embryonic
3 patterning yet poorly understood. During neural crest (NC) formation dynamic BMP, FGF and
4 WNT signals cooperate by acting on mesoderm and ectoderm. Here we show that Fhl3, a
5 scaffold LIM domain protein, modulates BMP gradient interpretation during NC induction.
6 During gastrulation low BMP signaling neuralizes the neural border (NB) ectoderm while Fhl3
7 enhances Smad1 intracellular response in underlying paraxial mesoderm triggering the high
8 WNT8 signals needed to pattern the NB. During neurulation *fh13* activation in NC ectoderm
9 promotes simultaneous high BMP and BMP-dependent WNT activity required for
10 specification. Mechanistically, Fhl3 interacts with Smad1 and promotes Smad1 binding to
11 *wnt8* promoter in a BMP-dependent manner. Consequently, differential Fhl3 expression in
12 adjacent cells ensure a finely tuned coordination of BMP and WNT signaling at several
13 stages of NC development, starting by positioning the NC-inducing mesoderm center under
14 competent NB ectoderm.

15

16 **Keywords:** four and a half LIM domain protein 3; neural crest gene regulatory network

17 **Introduction**

18 A strict spatiotemporal control of bone morphogenetic proteins (BMP) activity is
19 crucial for both mesoderm and ectoderm patterning (Bier and De Robertis, 2015). Intricate
20 additional morphogen gradients further refine germ layer subdivision. Neural crest (NC)
21 development is an ideal paradigm to model multiple tightly controlled inputs in patterning.
22 The NC is induced from the neural border ectoderm (NB), precisely located lateral to the
23 neural plate (NP) of early gastrulas in response to BMPs, WNTs and fibroblast growth factors
24 (FGFs) diffusing from the ectoderm and/or from the underlying mesoderm (De Croz e et al.,
25 2011; Garnett et al., 2012). Later on during neurulation, additional BMP and WNT planar
26 ectodermal signals control further NC development (Pla and Monsoro-Burq, 2018). While
27 each pathway has been studied separately in NC formation, how these signals are
28 coordinated and how adjacent cells, submitted to similar extracellular inputs, may interpret
29 differentially this complex information remains poorly understood.

30 A fine-tuned dynamic modulation of BMP signaling positions the NC and promotes
31 sequential steps of NC formation (Kuriyama et al., 2006; Piacentino and Bronner, 2018; Wu
32 et al., 2011). Specifically, ventral BMPs diffuse towards dorsal tissues, while dorsal BMP
33 antagonists diffuse ventrally along Brachet's cleft, the space separating mesoderm from
34 ectoderm (Plouhinec et al., 2013). This creates a net gradient of extracellular BMP activity
35 which patterns ectoderm and mesoderm simultaneously. Consequently the paraxial and
36 intermediate mesoderm and the overlying NB ectoderm receive similar moderate BMP
37 signals at gastrula stage (Harland and Gerhart, 1997). In the gastrula ectoderm, low BMP
38 signaling allows NP induction, intermediate levels pattern NB and NC, while high BMP
39 activity promotes non-neural ectoderm fates (De Robertis and Kuroda, 2004; LaBonne and
40 Bronner-Fraser, 1998). Later during neurulation however, enhanced BMP signaling in the
41 neural folds promote further NC development (Litsiou et al., 2005; Steventon et al., 2012; Wu
42 et al., 2011).

43 BMP signaling molecular cascade involves BMP ligand binding to membrane
44 receptors triggering Smad1/5/8 phosphorylation and dimerization. Smad1/5/8 complex with

45 Smad4, translocate into the nucleus and regulate target genes in a BMP dose-dependent
46 manner (Massagué et al., 2005). A first major question during NC induction is whether the
47 moderate levels of BMP signaling are interpreted similarly by the mesoderm and the
48 overlying ectoderm. Moreover, intermediate levels of BMP activity are not sufficient for a
49 robust NB induction: WNT and FGF signals from the mesoderm cooperate with BMPs to
50 activate the NB/NC gene regulatory network (GRN) in the ectoderm: when NB is specified in
51 frog gastrulas, the underlying mesoderm expresses FGF and WNT ligands diffusing towards
52 the overlying ectoderm, all essential for NB-NC activation (Chang and Hemmati Brivanlou,
53 1998; Garnett et al., 2012; Monsoro-Burq et al., 2003; Saint-Jeannet et al., 1997). BMP
54 signaling also triggers *wnt8* expression in the mesoderm; in turn Wnt8 patterns the paraxial
55 mesoderm (Hoppler and Moon, 1998). Importantly BMP and WNT relative signaling levels
56 are dynamically orchestrated during gastrulation and neurulation: low BMP/high WNT signals
57 induce NB, followed by high BMP/high WNT to induce NC (Steventon et al., 2009). Thus, a
58 second major question is how these various signals are modulated and coordinated with the
59 initial BMP gradient, either in the responding ectoderm or mesoderm (spatial coordination),
60 or during gastrulation and neurulation (temporal coordination).

61 We found that the four-and-a-half LIM domain scaffold protein Fhl3 is critical for the
62 intracellular interpretation of BMP signals during frog NB and NC development. FHL proteins
63 broadly regulate cell proliferation, differentiation and apoptosis (Bach, 2000). Fhl3 modulates
64 cell signaling and gene transcription in adult and cancer tissues by protein-protein
65 interactions but its developmental function is unknown (Kadmas and Beckerle, 2004). Fhl3
66 interacts with Smad2/3/4 and modulates TGF β signaling in cancer, while Fhl3 promotes
67 myoblast proliferation *in vitro* by binding MyoD and Sox15 (Ding et al., 2009; Han et al.,
68 2018; Meeson et al., 2007). Here, we find that Fhl3 is dynamically and differentially
69 expressed in mesoderm and ectoderm during gastrulation and neurulation. Fhl3 enhances
70 paraxial mesoderm response to BMPs and promotes WNT signaling towards the prospective
71 NB ectoderm. Later on, Fhl3 activity promotes BMP and WNT signals in premigratory NC
72 itself. We find that Fhl3 binds Smad1 and promotes its activity. Last, we show how by their

73 differential expression of *fh13*, apposed ectoderm and mesoderm exhibit different levels of
74 response to the intermediate BMP signals during gastrulation. Our results identify Fhl3 as a
75 key modulator of cell response to morphogens and show how this impacts the activation of
76 several steps of the NC-GRN.

77

78 **Results**

79 **Fhl3, first expressed in gastrulating mesoderm and later in NC cells, is essential for** 80 **NC development**

81 *Fhl3* developmental expression was compared to the patterns of ectoderm regional
82 markers in *Xenopus laevis* embryos: *pax3*, *sox2* and *snai2* for NB, NP and NC respectively.
83 At early gastrula stage, *fh13* labeled the involuting mesoderm (dorsal marginal zone/DMZ and
84 dorsal-lateral marginal zone/DLMZ). *Fhl3* remained expressed in the axial (notochord) and
85 paraxial/intermediate mesoderm until tailbud stage (Figures 1A, S1A-F). *Fhl3* expression
86 then labeled NB ectoderm at late gastrula stage (Figure S1B). In neurulas *fh13* was seen in
87 all premigratory NC cells, starting with the hyoid stream (Figure 1A, S1D). At tailbud stage
88 *fh13* was found in the migratory NCCs with stronger levels in the hyoid stream (Figure S1D).
89 *Fhl3* was later observed in branchial arches, notochord and somites (Figure S1D-F). In this
90 study, we focused on Fhl3 function in the two key tissues involved in NC induction: the dorsal
91 mesoderm inducing the overlying NB in the ectoderm at gastrula stage, and the NB and
92 premigratory NC in neurulas.

93 To assess its developmental function *in vivo*, we depleted Fhl3 unilaterally using a
94 translation-blocking morpholino (MO), a splice-blocking MO or CRISPR/cas9-mediated *fh13*
95 mutation (Figure 1B-F, S2A-F). The phenotype rescue by human FHL3 mRNA assessed MO
96 specificity and indicated that FHL3 function was evolutionarily conserved (Figure S2C). Upon
97 Fhl3 knockdown (KD, Figure 1B-D, S2D-F), NB markers *tfap2a*, *pax3* and *msx1* were
98 severely downregulated while others (e.g. *zic1*) were unchanged or upregulated, as was the
99 NP marker *sox2*. This showed that *fh13* morphant NB ectoderm formed (*zic1*-positive) but

100 was mispatterned. Expression of *tbxt* (*xbra*, pan-mesoderm) and *myod* (paraxial mesoderm)
101 was unaffected indicating globally normal mesoderm formation. Thus, Fhl3 KD did not affect
102 the initial formation of dorsal mesoderm and ectoderm during gastrulation but was essential
103 for NB early patterning. At neurula stage Fhl3 KD downregulated all premigratory NC
104 markers (*snai2*, *foxd3*, *twist1*, *sox10*, Figure 1C). Instead, the NP was enlarged (*sox2*) at the
105 expense of NC (*snai2* or *twist1*) and of non-neural ectoderm (*epidermal keratin epK*).
106 Transverse sections show the lateral expansion of the neural tissue (*sox2*) and the lateral
107 shift of the reduced *snai2/twist1* territories while the underlying mesoderm was normal
108 (Figure 1D, S2F). At tailbud stage, migration of Fhl3 morphant cranial NC cells was markedly
109 reduced (*twist1*, Figure 1E) while other tissues (e.g. eye) were unaffected. This resulted in
110 severe craniofacial skeleton defects (Figure 1F): the *trabeculae cranii* derived from the
111 mandibular and hyoid arches were missing and branchial arches were atrophied. Together,
112 these results showed that Fhl3 was necessary for NB and NC induction by causing strong
113 early developmental defects.

114 Last, we injected *fhl3* mRNA unilaterally to test Fhl3 gain-of-function (GOF) on NB
115 and NP formation compared to the uninjected contralateral side. Both at late gastrula and
116 neurula stages, increased and ectopic expression of *Fhl3* disrupted NB/NP patterning. NP
117 size was reduced and *sox2* expression levels were decreased. In contrast, the NB was
118 strongly expanded (*pax3*, Figure 1G) towards both the midline and the lateral ectoderm. This
119 suggested that an ectopic NB state was imposed on NP progenitors, while NP was reduced.
120 However, this ectopic NB was incompletely specified, lacking expansion of other NB markers
121 (*tfap2a*, not shown). Those disruptions resulted in decreased *snai2* a usual consequence of
122 perturbing the NC-GRN equilibrium. This NP/NC reduction phenotype phenocopied an
123 increased BMP signaling obtained by injecting constitutively active (ca) BMP receptor mRNA
124 unilaterally (Figure 1G).

125 Altogether expression, KD and GOF results suggested that Fhl3 was essential for
126 NB/NC formation, potentially acting in mesoderm or ectoderm at different developmental
127 stages.

128

129 Fhl3 controls BMP signaling in the paraxial mesoderm

130 The pattern alterations observed above could result from abnormal signaling in the
131 early NB/NC-GRN. Lower BMP signaling after Fhl3 depletion could expand the NP and
132 shift/reduce the NB laterally while higher BMP signaling after Fhl3 GOF would have the
133 converse phenotype. During NB induction, low BMP signaling triggers *zic1* expression, while
134 high WNT signaling activates *tfap2a*, *pax3*, and *msx1* (De Croz e et al., 2011; Mizuseki et al.,
135 1998). Thus, low BMP and low WNT signals could result in the pattern deregulations
136 observed in Fhl3-depleted embryos. We found that *Fhl3* mesodermal expression increased
137 in the DLMZ at late gastrula stages, i.e. when NB ectoderm was induced, compared to early
138 gastrula stage (Figure S3A). We then measured the response to BMP signaling in the
139 mesoderm after co-injecting a BMP reporter (Kusanagi et al., 2000) with *fhl3* MO and
140 dissecting the DLMZ out of early gastrulas. Response was significantly reduced in *fhl3*
141 morphant DLMZ compared to controls (Figure 2A-B). Thirdly we used tissue recombination
142 to test how the paraxial mesoderm induced NB/NC after Fhl3 KD, by apposing a gastrula-
143 stage DLMZ to a piece of late blastula-stage animal cap ectoderm (AC, Bonstein et al., 1998;
144 Figure 2C). Compared to control DLMZ, *fhl3* morphant DLMZ failed to activate NB and NC
145 markers (*pax3*, *snai2*, *sox10*). Last, we tested if the altered BMP signaling observed in the
146 morphant mesoderm caused its lack of inducing activity by a phenotype rescue, i.e. by co-
147 injecting *fhl3* MO with caSmad1 or of caBMPR1. The co-injected DLMZ activated *pax3* and
148 *snai2* expression better than morphant DLMZ but lower than uninjected controls (Figure 2C).
149 These results showed that Fhl3 function in the paraxial mesoderm was critical for NC
150 induction in the ectoderm, at least partly by promoting BMP signaling.

151

152 Fhl3 controls vertical Wnt8 signaling acting between paraxial mesoderm and ectoderm
153 during NC induction

154 WNTs and FGFs from the paraxial mesoderm are crucial for inducing the NC. We
155 quantified the expression of *dusp5* (FGF-responsive gene) and *axin2* (canonical WNT
156 signaling readout) in mesoderm-ectoderm recombinants (Figure 2D). *Axin2* expression was
157 reduced in the *fh13* morphant recombinants but not *dusp5* levels, suggesting altered WNT
158 activity and normal FGF function. To confirm that Fhl3 KD, when restricted to the mesoderm,
159 resulted in altered canonical WNT signaling specifically in the ectoderm, we injected the
160 WNT/ β -catenin signaling reporter into the ectoderm only, and juxtaposed it to either
161 uninjected or *fh13* morphant DLMZ. Compared to controls, the ectoderm combined to
162 morphant mesoderm showed a strongly decreased response to WNT signaling (Figure 2E)
163 showing that the levels of WNT activity in the ectoderm depended on Fhl3 function
164 specifically in the mesoderm. We tested if the decreased WNT signaling observed in
165 ectoderm after Fhl3 KD in the mesoderm explained the lack of NC induction with a
166 phenotype rescue using either an inducible form of caTCF3 or *wnt8* GOF, injected in the
167 ectoderm only (Figure 2F). When uninduced ectoderm was juxtaposed to Fhl3-depleted
168 DLMZ, *pax3* and *snai2* expression levels were as low as in uninjected ectoderm above. In
169 contrast induction of caTCF3 or Wnt8 GOF in the ectoderm fully restored *pax3* and *snai2*
170 levels. Together, these results demonstrated that Fhl3 early function in the paraxial
171 mesoderm was critical for its ability to activate WNT signaling at levels required for NC
172 induction in the ectoderm.

173

174 **During neurulation, Fhl3 is necessary for NC induction by NB specifiers and controls**
175 **both BMP and WNT signaling levels in the ectoderm**

176 Interestingly, neurula-stage *fh13* ectodermal expression partly overlapped with *pax3*
177 pattern consistent with the identification of *fh13* as a putative target of Pax3 (Bae et al., 2014).
178 Indeed, unilateral Pax3 GOF or KD *in vivo* modestly expanded or fully eliminated *fh13*
179 expression respectively (Figure S4A). This replaced *fh13* as a new member of the NC-GRN
180 activated in the ectoderm, downstream of NB specification.

181 We then directly measured *fh13* expression in ectoderm (Figure S4B-C). In AC grown
182 *in vitro* forming non-neural ectoderm, the levels of *fh13* increased at neurula stage. In
183 ectoderm-specific transcriptomes (Plouhinec et al., 2017) we found high *fh13* expression in
184 the ventral non-neural ectoderm, intermediate levels in NB ectoderm and low levels in the
185 NP. Last, Fhl3 depletion promoted neural induction (*sox2*) in AC ectoderm (Figure S4D)
186 suggesting that Fhl3 globally modulated tissue response to BMP signaling in the ectoderm
187 too.

188 We have then used a direct NC induction assay (iNC, Figure 3A) in order to separate
189 the roles of *fh13* within the ectoderm from its mesodermal functions. Calibrated GOF of Pax3
190 and Zic1 triggers the NC-GRN in AC ectoderm (Milet et al., 2013). Compared to control iNC,
191 co-injecting *fh13* MO prevented NC activation (*snai2*, *sox10*) suggesting that Fhl3 was also
192 required for premigratory NC specification downstream of NB regulators (Figure 3B).
193 Moreover, *pax3* expression was also decreased suggesting that Pax3 positive autoregulatory
194 loop was also defective (Plouhinec et al., 2014).

195 Next, we tested if Fhl3 depletion in iNC affected BMP and WNT signaling as
196 observed in the mesoderm. After *fh13* KD, BMP and WNT signaling reporters activity was
197 severely reduced compared control iNC while co-injection of either caSmad1 or *wnt8* in *fh13*
198 morphant iNC rescued *snai2* and *pax3* expression (Figure 3C-D). As in the mesoderm,
199 caSmad1 rescued *snai2* with lower efficiency than Wnt8. Thus, at neurula-stage steps the
200 NC-GRN, Fhl3 is essential for NC specification by promoting both BMP and WNT signaling in
201 the prospective NC.

202 The striking parallel observed between BMP activity and WNT signaling in all the
203 results above, either in the mesoderm or in the ectoderm, led us to hypothesize that Fhl3
204 participated to the known transcriptional cross-talk between the two signaling pathways
205 (Hoppler et al., 1998). We tested this potential interaction directly by Fhl3 KD either in the
206 mesoderm or in the iNC combined to BMP activation. Either caSmad1 GOF or caBMPR GOF
207 restored *wnt8* and *axin2* expression in morphant DLMZ recombined to control ectoderm and
208 in morphant iNC (Figure 3E). Here, the rescue was efficiently achieved with both caSmad1

209 and caBMPR suggesting that the mechanisms controlling *wnt8* expression after Fhl3 KD
210 were more direct than those controlling *snai2* (Figure 2C, 3D). This also suggested that in
211 both assays Fhl3 might enhance Smad1 activity resulting in up-regulated *wnt8* expression.
212 We thus hypothesized that Fhl3 potentiated BMP signaling in a non-essential manner, since
213 decreased Fhl3 activity was compensated by increased BMP signaling, indicating that the
214 BMP molecular cascade remained functional in the absence of Fhl3.

215

216 **Fhl3 interacts with Smad1 and promotes its binding to *wnt8* promoter**

217 We thus examined *wnt8* expression *in vivo* after Fhl3 KD: Fhl3 morphant mesoderm
218 displayed diminished expression of *wnt8* and WNT-responsive gene *myf5* compared to the
219 uninjected control side (Figures 4A, S2D). Although Smad1/5/8 phosphorylation was strongly
220 decreased in DLMZ, we did not find defective BMPR activity or phospho-Smad stability
221 (Figure S3). We thus hypothesized that Fhl3 may potentiate Smad1 nuclear shuttling, binding
222 or activity on target sequences by protein-protein interactions. As BMP signals regulate
223 mesodermal *wnt8* transcription (Hoopler et al., 1998), we searched for and found two
224 putative Smad-binding/BMP-responsive (BRE) elements upstream of *wnt8* transcriptional
225 start (BS1 at position -1571; BS2 at position -674). To validate BS1 we used EMSA with a
226 wild-type or a mutated BS1 primer (mut-BS1). Cell nuclear extracts with GFP shifted BS1
227 primer indicating endogenous Smad1 binding. Both Smad1 and Fhl3 transfections enhanced
228 BS1 shifting, which was lost with mut-BS1 (Figure 4A). This validated the importance of the
229 TAGAC motif in BS1 for Smad1 binding. Using ChIP-qPCR at late gastrula stage (Figure 4A-
230 B), we further tested if Smad1 and Fhl3 were found in protein complexes bound to BS1 and
231 BS2 *in vivo* and if this was regulated by BMP signaling. *Ventx1.1* gene BRE was a positive
232 control (Lee et al., 2011). BS1, BS2 and *ventx1.1* BRE were all found strongly enriched in the
233 Fhl3-bound chromatin compared to background. This enrichment was no longer detected
234 after co-injection of *noggin* showing that Fhl3 specifically bound *wnt8* promoter when BMP
235 signaling was active. We next validated Smad1 occupancy at BS1, BS2 and *ventx1.1* BRE
236 sites. Furthermore, the efficiency of Smad1 binding on BS1, BS2 and *ventx1.1* BRE *in vivo*

237 was reduced after Fhl3 KD and increased after Fhl3 GOF (Figure 4B). Altogether, these
238 experiments identify an Fhl3-dependent binding of Smad1 on *wnt8* promoter. Fhl3 binding to
239 the same chromatin regions suggests that Smad1 and Fhl3 are likely engaged together in a
240 transcriptional complex regulating *wnt8* expression. The response of *ventx1.1* BRE showed a
241 similar dependency to Fhl3 levels, suggesting a broad role of Fhl3 in the regulation of BMP-
242 responsive genes in embryos.

243 We then tested if Fhl3 could affect Smad1 nuclear shuttling. *First, at high BMP levels,*
244 *we found Fhl3 enriched in the nucleus of ectoderm cells (AC) and of HEK293 cells while Fhl3*
245 *was mainly in the cytoplasm of AC injected with *noggin* (Figure 4C, S5A). In HEK293 cells,*
246 *Fhl3 transfection increased Smad1 nuclear localization suggesting enhanced shuttling of*
247 *Smad1 in presence of Fhl3 (Figure S5A). We further tested if FHL3 and Smads interacted*
248 *directly by co-immunoprecipitation (IP) using HEK293 cells or whole embryo extracts, but*
249 *detected only a modest enrichment of FHL3 after IP by FLAG-Smad1 or of Smad1 after IP*
250 *with HA-tagged FHL3 (Figures 4D, S5B). To overcome IP limitations to detect transient*
251 *interactions or interactions between proteins in a larger complex, we then used a two-hybrid*
252 *assay tuned for mammalian cells, which robustly detects the transcriptional outcome a given*
253 *protein-protein interactions occurring *in cellulo* prior to cell lysis. We found that Fhl3 robustly*
254 *interacted with Smad1 and Smad4, Smad2 being a positive control (Figure 4E, Han et al.,*
255 *2018) indicating that Fhl3 and Smad1/4 belonged to the same protein complex *in vivo*.*

256 Altogether, our results supported *a model* where, upon BMP signaling, Fhl3
257 interacted with the Smad1/4 complex, shuttled into the nucleus and enhanced Smad1
258 binding to its target genes including *wnt8*.

259

260 **At intermediate BMP levels, tissue-specific Fhl3 expression selectively coordinates**
261 **NB ectoderm neuralization with mesoderm higher response to BMPs**

262 To understand if Fhl3 modulated generic BMP signaling in a homogeneous tissue
263 grown in isolation, we tested Fhl3 effects on BMP response in AC injected with the standard
264 BMP-reporter (Figure 5A). For Fhl3 GOF assay, we lowered the high endogenous BMP

265 levels found in AC with a low dose of *noggin*. Co-injection of increasing doses of *fh13* mRNA
266 resulted in a graded increase in BMP response. Conversely, increasing doses of Fhl3 MO
267 caused a dose-dependent decrease of BMP response in AC. As AC were grown *in vitro*
268 without growth factors, this ensured the lack of interfering regulation. These results showed
269 that, at a given level of BMP signaling, Fhl3 levels influenced the response of a generic BMP-
270 reporter in a dose-dependent manner.

271 Last, we sought to understand the significance of Fhl3 action *in vivo*, in the context of
272 the tissue response to the ventral-to-dorsal BMP gradient, and between the mesoderm and
273 ectoderm germ layers during gastrulation. The results above collectively suggest that, when
274 subjected to the same levels of extracellular BMP signals, cells expressing Fhl3 would
275 respond to BMPs higher than cells without Fhl3. We thus directly measured tissue response
276 to BMP in wt and Fhl3 morphant gastrula-stage mesoderm positioned dorsally (submitted to
277 very low BMP levels), dorsal-laterally (intermediate BMP levels) and ventrally (high BMP
278 levels) (Figure 5B) by dissecting DMZ, DLMZ and VMZ injected with BMP-reporter alone or
279 together with Fhl3 MO. All explants were homogeneous fragments of embryonic mesoderm
280 without any contact with another tissue that might modulate their response to BMP. Thus,
281 this experiment directly measured BMP response in the mesoderm along the endogenous
282 BMP gradient. We find that wt ventral and dorsal-lateral mesoderm respond similarly to
283 BMPs while dorsal mesoderm had a low response (set to basal level). Upon Fhl3 depletion,
284 response of DMZ and VMZ were unchanged, while DLMZ response was abolished. This
285 experiment uncovered three points. First, as expected upon the action of BMP antagonists,
286 the DMZ responded weakly to BMP signaling despite its expression of Fhl3, confirming that
287 Fhl3 action was dependent on BMP signals. Secondly, in the ventral mesoderm, producing
288 high BMP levels, a strong BMP response is achieved with or without Fhl3. In contrast, Fhl3
289 action had a strong impact on the response of intermediate mesoderm: the DLMZ response
290 to intermediate BMP levels was as strong as one observed ventrally and not an "intermediate
291 level" response as previously proposed, and this resulted from Fhl3 activity.

292 Lastly, we hypothesized that at paraxial levels of gastrula-stage embryos, i.e. at the
293 same position along the dorsal-ventral (DV) BMP gradient, the Fhl3-positive DLMZ would
294 respond stronger to the moderate BMP levels than the Fhl3-negative ectoderm. We
295 dissected out separately the gastrulating mesoderm or the adjacent ectoderm, both coming
296 from the same DV position, either injected with BMP-reporter alone or together with Fhl3 MO
297 (Figure 5C). As predicted, the mesoderm (DLMZ) response was higher and Fhl3-dependent
298 while the response by the prospective NB ectoderm (DL-EC) was low (equivalent to the
299 response by the axial DMZ) and unchanged upon Fhl3 depletion, consistent with lack of
300 expression in the ectoderm at gastrula stage. These data thus showed that at paraxial
301 position, Fhl3 controlled the response to BMPs in a tissue-specific manner, such that the
302 mesoderm responded as strongly as ventral mesoderm, while in the ectoderm, the low
303 response was compatible with neuralization of the NB. This differential tissue response
304 during gastrulation, dependent upon Fhl3, thus controlled the relative positioning of the NC-
305 inducing paraxial mesoderm (secreting WNTs) underneath the responsive NB ectoderm,
306 thereby triggering NC formation (Figure 5D).

307

308 **Discussion.**

309 ***Fhl3 is a novel enhancer of BMP signaling which controls several steps in the NC-GRN***
310 ***in vivo.***

311 Early patterning and later organogenesis are the result of a remarkably orchestrated
312 suite of signaling and inductive interactions. However, how each germ layer or subsets of
313 cells in each tissue respond to the broad morphogen gradients set up during early
314 embryogenesis remains incompletely understood. Moreover, the parameters controlling the
315 relative dynamics of multiple signals are still largely unknown. For example, the relative
316 levels of BMP and Wnt signaling needed for the different steps of the NC-GRN are highly
317 dynamic (Steventon et al., 2009) but how these signaling modulations are obtained locally,
318 and how they impact the mesoderm-ectoderm interactions and further regulations in NC
319 remains unexplored. Here, we used tissue-specific gene manipulations, either in isolation or

320 combined to other embryonic tissue recombination at several stages of development to
321 disentangle the sequence of events involving the scaffold protein Fhl3 in early NB/NC
322 induction.

323 Fhl3 is activated in the involuting dorsal mesoderm during gastrulation and in the
324 prospective NC ectoderm during neurulation. Fhl3 depletion or GOF alters the NC-GRN but
325 not initial mesoderm patterning. Fhl3 controls the NC-inducing properties of the paraxial
326 mesoderm at gastrula stages and participates to ectoderm patterning in neurulas by
327 enhancing the cellular response to BMP signaling in both mesoderm and NC at those two
328 different stages. Enhanced mesodermal BMP signaling activates WNT signals, diffusing
329 towards the ectoderm and essential for NB induction. The BMP signaling cascade remains
330 functional in absence of Fhl3 since Fhl3 depletion was compensated by increasing response
331 to BMPs by alternative other means (caBMPR, caSmad1): Fhl3 is an accessory enhancer of
332 BMP signaling. Similar dispensable enhancing roles have been described for Fhl2 on WNT
333 signaling (Labalette et al., 2004). In neurula-stage prospective NC or non-neural ectoderm,
334 Fhl3 regulated both BMP and WNT signaling simultaneously, suggesting that Fhl3 mode of
335 action may be broadly used in embryos. In addition, the incomplete rescue of NB/NC
336 induction by increasing BMP signaling suggests that Fhl3 may also interfere with other
337 pathways in the NB/NC-GRN. In contrast, both caBMPR and caSmad1 efficiently rescued
338 *wnt8* gene expression suggesting a strong link between Fhl3 levels and *wnt8* transcriptional
339 response to BMPs.

340 By direct measure of Fhl3 impact on the intracellular response to the extracellular
341 gradient of BMP signaling in the mesoderm and in the prospective NB ectoderm in gastrulas
342 (Figure 5), we found that Fhl3 was essential to ensure a high level response in the paraxial
343 mesoderm, at similar levels to those observed in the ventral mesoderm, which is subjected to
344 higher BMP signals. This high mesodermal response then controlled the high WNT signal
345 activation essential for NB induction (LaBonne et al., 1998; Steventon et al., 2009).
346 Simultaneously, the prospective NB ectoderm with low Fhl3, although adjacent to the
347 paraxial mesoderm and subjected to the same intermediate levels of extracellular BMP,

348 responded at basal levels allowing early NB neuralization (Piacentino and Bronner, 2018).
349 Thus, Fhl3 tissue-specific expression elicits a differential response to the common external
350 intermediate BMP levels. Our results demonstrate how mesodermal Fhl3 activity controls the
351 low BMP/high WNT signaling levels inducing the NB state in the ectoderm during
352 gastrulation. During neurulation in contrast, elevated BMP and WNT signals cooperate with
353 NB transcription factors such as Pax3, Tfap2a and Zic1, to activate the premigratory NC
354 program (Monsoro-Burq et al., 2005; Sato et al., 2005; Simões-Costa et al., 2015; Steventon
355 et al., 2009; Wu et al., 2011). Fhl3 expression in premigratory NC cells promoted WNT
356 signaling in a Smad-dependent manner thus coordinating the high BMP/high WNT signaling
357 required for premigratory NC specification.

358

359 **Limitations of the study.**

360 ***Mechanisms of BMP signaling enhancement by Fhl3 and challenges in detecting Fhl3*** 361 ***interactions with Smad proteins in vitro.***

362 Intense research has explored the BMP signaling cascade highlighting many actors
363 and modulators (Massague 2005; Piacentino and Bronner, 2018; Wu et al., 2011). We have
364 tried to narrow-down the mechanisms allowing Fhl3 to enhance BMP response. *In vivo* and
365 *in cellulo* assays support a model where Fhl3 binds to Smad1 or Smad4 and is present on
366 target genes BRE (*wnt8*, *ventx1.1*; Figures 4, S5). In HEK293 cells, two-hybrid assay shows
367 protein-protein interactions between Fhl3 and Smad1/4. In embryos, Fhl3 is bound to BREs
368 in a BMP-dependent manner by chromatin immunoprecipitation. Moreover, efficient binding
369 of Smad1 to its target sequences (*wnt8*, *ventx1.1*) depends on the levels of Fhl3. However,
370 we only detected a weak interaction between Fhl3 and Smad1 by direct immunoprecipitation.
371 This suggests that the two proteins could be part of a larger complex, which is found *in vivo*
372 and remains to be defined biochemically. Secondly, the subcellular localization of the Fhl3-
373 Smad first interaction remains unknown: our data suggest that Fhl3 may facilitate Smad
374 nuclear shuttling. Indeed, Fhl3 is enriched in the nucleus of AC cells with high BMP activity
375 (Figure 4). Moreover, transfection of Fhl3 in HEK293 cells promotes Smad1 nuclear

376 enrichment (Figure S5). However, an analysis of Smad nuclear shuttling dynamics by
377 fluorescence recovery after photobleaching (FRAP), of Fhl3-Smad interactions by Proximity
378 Ligation Assay, or of the enhancement of Fhl3-Smad interaction by BMP in the mammalian
379 two-hybrid assay, all coupled to Fhl3 protein domains' mutagenesis, would allow the full
380 demonstration of this model.

381

382 **Conclusion**

383 Altogether, our experiments suggest model where the scaffold LIM-domain protein
384 Fhl3 would interact with Smad1/4 and promote the transcriptional activation of *wnt8* and
385 other BMP targets such as *ventx1.1*, in a broad register of cell types including the NC, the
386 paraxial mesoderm and the ectoderm lineages. The enhancement/facilitation of Smad
387 activity by Fhl3 is reminiscent of the synergistic co-activator function of Fhl2 on CBP-p300
388 and β -catenin in cell lines (Labalette et al., 2004).

389 Fhl3 is a novel agonist of BMP signaling during development, cell-autonomously
390 promoting Smad transcriptional activity during mesoderm and NC patterning. The
391 developmental dynamics and tissue-specific expression of Fhl3 at different stages of the NC-
392 GRN regulate the specific response of either mesoderm or ectoderm cells to the intermediate
393 BMP levels controlling NC development, and coordinates BMP and WNT signaling. In the
394 global view of early development, our findings highlight how complex patterns are refined
395 locally in vertebrate embryos, following the initial and broad dorsal-ventral patterning. The
396 cooperation between dorsal (Fhl3) and ventral (BMP) cues positions a secondary WNT-
397 secreting signaling center in the paraxial mesoderm, sending the WNT signals towards an
398 ectoderm area neuralized by low BMP signaling. The strictly controlled and coordinated
399 signals ultimately induce the neural crest.

400

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 407 not quoting more important references.

408

409 **Authors contribution**

410 M.A. PhD supervision: P.P., A.H.M-B; Analyses, Visualization: M.A., A.H.M-B; Investigation:
 411 M.A; Original Draft: M.A., P.P., A.H.M-B; Editing and Funding: A.H.M-B.

412

413 **Declaration of interest**

414 The authors declare no competing interests.

415

416 **Figure Legends**

417

418 **Figure 1. Fhl3 is required for NB and NC induction *in vivo***

419 **A:** *Fhl3* expression is compared to neural plate/tube (np/nt), NB (nb) and NC (n) marker
 420 patterns during frog development. n, notochord; bl, blastocoel; m, paraxial mesoderm; e,
 421 endoderm.

422 **B-F:** *Fhl3* depletion prevents NB and NC but not neural (*sox2*) and mesoderm (*tbxt*) gene
 423 activation. It blocks NC migration and cranial cartilage formation.

424 **G:** *Fhl3* GOF reduces NP size (*sox2*), expands NB (*pax3*) and affects NC (*snai2*). Due to NP
 425 reduction, NC is closer to the midline (dotted line) as seen after caBMPR1 injection.
 426 Blue/green lines measure width on the uninjected or injected (*) sides respectively.

427 [Biological triplicates or more. The most frequent phenotype is shown; phenotype](#)
 428 [quantification in Table 1. Scale bars: 500 \$\mu\$ m. See Figures S1-S2-S3A.](#)

429

430 **Figure 2. Fhl3 is necessary for BMP signaling and WNT-dependent NC induction by** 431 **the mesoderm**

432 **A-B:** *Fhl3* is essential for BMP activity in late gastrula paraxial mesoderm.

433 **C:** In mesoderm (DLMZ)/ectoderm (AC) NC-inducing assay, depleting *Fhl3* selectively in the
 434 mesoderm affects NB/NC induction in the ectoderm, a phenotype partially rescued by *ca-*
 435 *smad1* or *caBMPR* co-injection in the mesoderm.

436 **D:** In DLMZ/AC assay, WNT target expression (*axin2*) and TOPFLASH WNT reporter activity
 437 are decreased in wild-type AC juxtaposed to *fhl3* morphant DLMZ. FGF target expression
 438 (*dusp5*) is unchanged.

439 **E:** WNT reporter-injected ectoderm shows decreased response when juxtaposed to a *fhl3*
 440 morphant DLMZ, compared to a control DLMZ.

441 **F:** Injection of either *TCF3* or *wnt8* mRNAs in the ectoderm rescues NC induction in AC
 442 juxtaposed to *fh13* morphant DLMZ.

443 Biological triplicates (or more, A-B, D-F) or duplicates (C). Data are represented as mean +/-
 444 S.E.M. See Figure S3A.

445

446 **Figure 3. Fhl3 is necessary for NC specification in the ectoderm, downstream of NB**
 447 **formation**

448 **A-B:** In a direct NC induction assay, key NC specifiers are poorly induced in *fh13* morphant
 449 iNC compared to control.

450 **C:** BMP and WNT activities are reduced in *fh13* morphant iNC.

451 **D:** Injection of *ca-smad1* or *wnt8* rescues NC induction in *fh13* morphant iNC.

452 **E:** *Wnt8* and *axin2* expression are rescued by *ca-smad1* or *caBMPR* either in morphant iNC
 453 or in *fh13* morphant recombinants.

454 Biological triplicates (or more, A-D) or duplicates (E). Data are represented as mean +/-
 455 S.E.M. See Figure S4.

456

457 **Figure 4. Fhl3 interacts with Smad1/5/8 and enhances Smad-binding to *wnt8* promoter.**

458 **A:** *Wnt8* expression decreases in *fh13* morphant mesoderm *in vivo* after unilateral injection (*,
 459 decrease in 96% of cases, arrow) while *wnt8* is symmetrical in 93% of control siblings. Fhl3
 460 and Smad1 bind to BS1 but not mutated BS1 (EMSA, shifted wt-BS1 primer pointed by
 461 arrow). At late gastrula stage *in vivo*, Fhl3 binds to BS1, BS2 and to *ventx1.1* BRE but not to
 462 EF1a sequences. In presence of Noggin, Fhl3 binding on those sequences is strongly
 463 reduced. Scale bar 500 μ m.

464 **B:** Smad1 binding to BS1, BS2 and *ventx1.1-BRE* was analyzed either alone or after Fhl3
 465 KD or GOF. Smad1-bound chromatin enrichment depended upon Fhl3 levels on all three
 466 promoter elements.

467 **C:** Fhl3 is found in the nucleus of BMP-positive animal cap cells. FHL3 nucleus/cytoplasm
 468 ratio is strongly decreased in Noggin-treated cells. See Figure S5A.

469 **D:** Modest Fhl3 enrichment by co-immunoprecipitation (Smad1-FLAG IP).

470 **E:** Strong direct protein-protein interactions between Fhl3 and Smad 1,2,4, by a mammalian
 471 two-hybrid reporter assay.

472 Biological triplicates or duplicates (Fhl3-FLAG/noggin CHIP, Smad1-FLAG/Fhl3 MO/mRNA
 473 CHIP and EMSA). Data are represented as mean +/- S.E.M. See Figure S5B.

474

475 **Figure 5. Direct measurement of BMP signaling output in isolated ectoderm or**
 476 **mesoderm explants evidences specific Fhl3 requirement in the paraxial mesoderm for**
 477 **high BMP response.**

478 **A:** Direct impact of Fhl3 levels was measured in AC. Increasing doses of Fhl3 mRNA
479 promoted a dose-dependent BMP response while increasing Fhl3 MO gradually decreased
480 the reporter response.

481 **B:** Fhl3 role in the BMP-responsive mesoderm was directly assessed by dissection and
482 separate analysis of each type of tissue. High BMP response is seen in VMZ and DLMZ,
483 while DMZ shows lower response (set to basal level). In contrast to the dorsal and ventral
484 mesoderm, Fhl3 depletion specifically affects BMP activity in the dorsal-lateral mesoderm,
485 changing the high response to background level response.

486 **C:** Prospective dorsal-lateral NB ectoderm (DL-EC) displays a basal-level response to BMPs
487 (same scale as in B). Fhl3 KD affects BMP activity in the dorsal-lateral mesoderm (DLMZ)
488 but not the adjacent DL-EC.

489 **D:** Model of Fhl3 role during of NB induction, resulting in low BMP/high WNT signaling in the
490 prospective NB ectoderm. During gastrulation, combined activity of the ventral-dorsal BMP
491 gradient and of dorsally-expressed Fhl3 positions the NC-inducing signaling center, secreting
492 high levels of Wnt8 in the paraxial mesoderm located underneath the NB ectoderm.

493 [Biological triplicate or more. Data are represented as mean +/- S.E.M.](#)

494

495 **STAR Methods Text**

496

497 **RESOURCE AVAILABILITY**

498 **Lead Contact**

499 Further information and requests for resources and reagents should be directed to and will
500 be fulfilled by the lead contact, Anne H. Monsoro-Burq; anne-helene.monsoro-burq@curie.fr.

501

502 **Materials availability**

503 Plasmids and materials generated in this study will be available upon request to the lead
504 contact. Newly generated reagents are listed in [Key Resources Table](#).

505

506 **Data and code availability**

507 This study did not generate any unique datasets or code.

508

509 **EXPERIMENTAL MODEL DETAILS**

510 **Xenopus laevis embryos**

511 Ovation, *in vitro* fertilization and embryo culture were performed using standard methods
512 (www.xenbase.org). Embryos were staged according to Nieuwkoop and Faber table (NF
513 stage). All procedures were performed according to the European animal use and care law
514 (animal care and housing approval license #C91-471-108, Direction Départementale de

515 Protection de la Population, Courcouronnes, France). Stages were described as follows:
516 early gastrula = NF stage 10.5; late gastrula = stage 12; mid neurula = NF stage 14-15; NT
517 closure late neurula = NF stage 18), tailbud =(NF stage 25), tadpole (NF stage > 30).

518

519 **HEK293 cells**

520 HEK293 cells were maintained under standard conditions, 5%CO₂, 37°C, in DMEM with 10%
521 heat-inactivated fetal bovine serum, penicillin (100 U/ml) and streptomycin (100 µg/ml).

522

523 **METHOD DETAILS**

524 **Sub-cloning of *Xenopus laevis* *fh13* cDNA**

525 An *fh13* expression vector was obtained by amplifying the full-length *fh13* cDNA by PCR from
526 late neurula stage whole embryo cDNA and inserting it into pGEM-T Easy by TA cloning
527 (Promega). The insert was then subcloned into pCS107 (*NotI* site). *Fhl3* PCR primers are
528 listed in Key Resources Table.

529

530 ***In vivo* gene depletion, gain of function (GOF) and rescue experiments**

531 For knockdown experiments, two antisense morpholino oligonucleotides (MO) were used to
532 block *fh13* translation or splicing (GeneTools). 15 ng of control MO or *fh13* MO (either ATG
533 MO or splice MO) were injected into one or two blastomere(s) of two-cell stage embryos,
534 according to the assay. For Pax3 depletion experiments, 20 ng of previously validated MO
535 (Monsoro-Burq et al., 2005) was injected into one blastomere of a two-cell stage embryo.

536 Capped mRNAs were synthesized using mMessage mMachine SP6 /T7 kits (Invitrogen). For
537 Pax3 and Fhl3 GOF experiments, *pax3* mRNA (75 pg) or *fh13* mRNA (400-1000 pg) was
538 injected into one blastomere at two/four-cell stage. For rescue experiments, h*FHL3* mRNA
539 (300 pg) was co-injected with the *fh13* MO into one blastomere of two-cell stage embryos;
540 caSmad1 (3SD) mRNA (200 pg) or caBMPR1 mRNA (200 pg) or TCF3 mRNA (80 pg) or
541 Wnt8 mRNA (100 pg) were co-injected with the *fh13* MO into both blastomeres of two-cell
542 stage embryos.

543 In each case, the injected blastomeres were traced by β-gal mRNA (250pg) or GFP mRNA
544 (200pg) co-injection to mark the injected side (marked by an asterisk).

545

546 For CRIPSR/cas9 experiments, *fh13* gRNA was synthesized using MEGAShortscript T7
547 transcription kit. Cas9 protein (2.5 ng) and *fh13* sgRNA (300 pg) were co-injected into one
548 blastomere of two-cell stage embryos.

549

550 **Neural crest (NC) induction in mesoderm/ectoderm recombination assay**

551 To recombine mesoderm at NC-inducing stage to a responsive "naive" ectoderm, a
552 heterochronic recombination was used (Bonstein et al., 1998). Animal caps were dissected
553 out from blastula stage embryos (stage 9), dorsal-lateral marginal zone (DLMZ) explants
554 were dissected out from early gastrula stage embryos (stage 10.25). The two tissues were
555 juxtaposed to form ectoderm-mesoderm recombinants and cultured in $\frac{3}{4}$ NAM (Slack and
556 Forman, 1980) until sibling embryos reached the late neurula stage (stage 18).

557

558 **Direct neural crest induction (iNC)**

559 Animal cap ectoderm are pluripotent cells that respond to Pax3/Zic1 by activating a *bona fide*
560 NC development (Milet et al., 2013). For this induced Neural Crest (iNC) assay, *pax3-GR*
561 mRNA (35 pg) and *zic1-GR* mRNA (72 pg) synthesized with mMessage mMachine kits
562 (Invitrogen), were co-injected into both blastomeres of two-cell stage embryos, with or
563 without 15 ng *fh13* MO. For rescue experiments, *caSmad1* (200 pg) or *wnt8* mRNA (100 pg)
564 were co-injected with *pax3-GR* mRNA, *zic1-GR* mRNA and *fh13* MO. Animal cap explants
565 were dissected out at stage 9 and dexamethasone (or its solvent ethanol as a control) was
566 added at stage 10.5. Explants were lysed at stage 18.

567

568 **Whole mount *in situ* hybridization (WISH) and sectioning**

569 Embryos were stained using a fast whole-mount *in situ* hybridization protocol (Monsoro-Burq,
570 2007). *Pax3*, *tfap2a*, *msx1* and *zic1* are expressed in the neural border and *snai2*, *foxd3*,
571 *twist1* and *sox10* are expressed in the neural crest. *Sox2* is a general neural plate and neural
572 tube marker and *epK* is a non-neural ectoderm marker. *Tbxt* (*xbra*) is a general mesoderm
573 marker. *Myod* and *myf5* are expressed in the paraxial-fated mesoderm.

574 Late gastrula (stage 12.5), mid-neurula (stage 15) and late neurula (stage 18) embryos are
575 shown in dorsal views with anterior to the top, except for the late gastrula stage embryo
576 stained with *tbxt* shown in vegetal view, dorsal to the top (Figure 1A-C). The blastopore of
577 late gastrulas is marked by a white dashed circlet. The dotted lines in Figure 1A, C, G
578 indicate the midline. Tailbud stage embryos are seen in side views, anterior to the left. A
579 sagittal view of a dissected late gastrula embryo is shown in the top right panel in Figure 1A.
580 Mid- and late neurula embryos were embedded in paraffin and 12- μ m-thick microtome
581 transverse sections were cut (Figures 1, S1, S2).

582

583 **Cartilage staining**

584 Stage 45 tadpoles were fixed, dehydrated and stained in Alcian Blue for 24 hours. After
585 several washes in ethanol until no blue stain is released, embryos were rehydrated, cleared
586 with 4% KOH and transferred into graded glycerol solution in 2% KOH. Finally, ventral head
587 cartilage was dissected. Whole tadpoles are shown from the dorsal side, anterior to the top

588 and the matching dissected cartilages are viewed from the ventral side, anterior to the top
589 (Figure 1F).

590

591 **Luciferase assay**

592 Each assay was repeated for biological replicates (each point on the graphs), each replicate
593 using the number of explants per condition indicated below.

594 For BMP reporter assay after Fhl3 depletion in Figures 2B, 5A-C, Renilla luciferase DNA
595 (pRL; Promega, 25 pg) and BMP reporter DNA (pGL2-15xGCCCG-lux; 50 pg) were co-
596 injected into both blastomeres at two-cell stage with or without *fhl3* MO (7.5 ng/10nl or 15
597 ng/10nl). Twenty animal caps were dissected at stage 9 per sample; or 20 mesoderm
598 explants either DMZ, or DLMZ, or VMZ or DL-EC were dissected at stage 10.5 per sample.
599 All explants were grown individually in $\frac{3}{4}$ NAM, pooled (at stage 18 for animal caps and at
600 stage 12.5 for mesoderm explants) and lysed in 60 μ l of Passive lysis buffer (Promega). For
601 the BMP reporter dose-response experiment after Fhl3 GOF (Figure 5A), each blastomere at
602 two-cell stage was co-injected with Renilla luciferase DNA (25pg), BMP reporter DNA (50pg)
603 and *noggin* mRNA (0.2pg) with or without *fhl3* mRNA (400 pg or 1ng per cell), and 20 animal
604 caps per sample were prepared as described above.

605 For WNT reporter assay in ectoderm/mesoderm recombinants (Figure 2E), Renilla luciferase
606 DNA (25 pg) and WNT reporter DNA (TOPFLASH, 50 pg) were co-injected into both
607 blastomeres at two-cell stage. Animal cap (ectoderm) explants were dissected at stage 9 and
608 juxtaposed to control or Fhl3-depleted DLMZ. Fifteen (15) recombinants
609 (ectoderm+mesoderm) were grown individually then lysed in 60 μ l Passive lysis buffer at
610 stage 18.

611 For BMP and WNT reporter assays in iNC (Figure 3C), Renilla luciferase DNA (25 pg), *pax3-GR*
612 mRNA (35 pg) and *zic1-GR* mRNA (72 pg) were co-injected with either BMP reporter
613 DNA (50 pg) or WNT reporter DNA (50 pg) into both blastomeres at two-cell stage with or
614 without 15 ng of *fhl3* MO. Twenty animal cap (ectoderm) explants were dissected out from
615 stage 9 embryos and lysed in 60 μ l of Passive lysis buffer at stage 18.

616 In all assays, Firefly and Renilla luciferase activities were measured using the Dual-Glo
617 Luciferase Assay System (Promega).

618

619 **qRT-PCR**

620 Total RNA was extracted using Phenol/Chloroform/Isoamyl Alcohol from *Xenopus laevis*
621 embryos or tissue explants at late neurula stage (stage 18). We used M-MLV reverse
622 transcriptase for reverse transcription and SYBR Green mix (BIO-RAD) for quantitative RT-
623 PCR. Results were normalized against the reference genes *odc* and *ef1a*. For primers
624 sequences see Key Resources Table.

625

626 **Chromatin immunoprecipitation (ChIP)**

627 Embryos were injected in both blastomeres at two-cell stage with mRNA encoding FLAG-
 628 Smad1 (200 pg) and FLAG-Fhl3 cDNA separately (200 pg) or FLAG-Fhl3 cDNA with noggin
 629 mRNA (25 pg). Injected embryos were collected at late gastrula stage (stage 12, 80-100
 630 embryos per sample) and processed according to Wills et al., 2014. Sonication was
 631 performed using a 3.2 mm tapered microtip for 13 mm horn (Branson 450 sonicator). Anti-
 632 FLAG antibodies coupled to magnetic beads (42 μ g) were added to the chromatin.
 633 Quantitative PCR was performed on the immunoprecipitated and fragmented chromatin
 634 using primers amplifying *wnt8*, *ef1a* (background control) or *ventx1.1* promoter regions
 635 (positive control). *Wnt8* promoter primers amplified the two following elements where putative
 636 Smad binding sites are underlined:

637 BS1:AGCTCACCAAGTTGATCATGTGGTCCAGGTGCACCAGTCCTAGACCCCTAACCAC
 638 GGGTCGTGGAAACTGAA;

639 BS2:CCTTCCAGTAGAAAACTCATGTCAATACACATATATAGGCTACAAGAGATAACCAT
 640 CGGCAGACAAAGGCTCACCTTGGTTTCACCA. Data were analyzed by the $\Delta\Delta C(t)$ method.
 641 Once a threshold cycle (C(t)) number is calculated for each sample, $\Delta C(t)$ is calculated by
 642 normalizing the IP values to the input values for each condition by subtracting input values
 643 from each corresponding ChIP value [$\Delta C(t) = \text{ChIP } C(t) - \text{Input } C(t)$]. $\Delta\Delta C(t)$ was next
 644 calculated by subtracting the $\Delta C(t)$ for uninjected samples from the $\Delta C(t)$ for experimental
 645 samples [$\Delta\Delta C(t) = \Delta C(t)_{\text{experimental}} - \Delta C(t)_{\text{uninjected}}$]. Once $\Delta\Delta C(t)$ was determined, the
 646 fold change between samples was determined by using the formula [Fold Change (FC) = $2^{-[\Delta\Delta C(t)]}$]
 647 (Taneyhill and Adams, 2008). Primers and FLAG antibody are listed in Key
 648 Resources Table.

649

650 **Electrophoresis mobility shift assay (EMSA)**

651 HEK293 cells, which do not express endogenous Fhl3, were used to test nuclear protein
 652 binding to the BS1 oligonucleotide. To this end, cells were transfected with plasmids
 653 encoding *gfp*, *smad1* or *fhl3*. Nuclear extracts contained an EDTA-free protease inhibitor
 654 cocktail to avoid Zn^{2+} chelation, Zn^{2+} being necessary for the maintenance of FHL3 structure.
 655 Oligonucleotides containing a wild-type or a mutated form of a BMP-responsive element (see
 656 sequence in Key Resources Table) were labeled with biotin (Biotin 3' End DNA labeling kit,
 657 Thermo Scientific), annealed and incubated with nuclear protein extracts from HEK293T cells
 658 in 50 ng/ μ L Poly [dl-dC], 2.5% glycerol, 5 mM MgCl_2 and 0.05% NP-40 diluted in EMSA
 659 binding buffer (LightShift Chemiluminescent EMSA Kit, Thermo Scientific) during 20 min at
 660 RT. Complexes were separated on a 5% polyacrylamide gel and transferred to a nylon

661 membrane (Hybond-N+, Amersham). Biotin-labeled DNA was detected by
662 chemoluminescence according to manufacturer's instructions.

663

664 **Mammalian two-hybrid assay**

665 For a two-hybrid system adapted to eukaryotes, we used the Check-Mate Mammalian Two-
666 Hybrid System (Promega). Using Gibson cloning method, human Fhl3 was sub-cloned into
667 the pBind vector, which contains the yeast GAL4 DNA-binding domain and human Smad1, 2
668 and 4 were cloned into the pACT vector, which contains the herpes simplex virus VP16
669 activation domain and a nuclear localization sequence. A luciferase reporter driven by five
670 GAL4 binding-sites (pG5-luc) was co-transfected into HEK293T cells. Luciferase activity was
671 measured using the Dual-Glo Luciferase Assay system (Promega). Fhl3-Smad2 interactions
672 serve as a positive control (Ding et al., 2009).

673

674 **Western blot**

675 Detection of phospho-Smads. 10 to 15 DLMZ explants were dissected at stage 10.5, cultured
676 individually and lysed when sibling embryos reached stage 12.5 in presence of phosphatase
677 inhibitor (PhosphoSTOP, Roche) and protease inhibitors (Sigma) and analyzed by western
678 blotting. ECL signal was quantified using ImageJ. Antibodies are listed in Key Resources
679 Table.

680 Assessment of phospho-SMAD dynamics. HEK-293 cells, known to respond to BMP4, were
681 transfected either with GFP or with human FHL3. After 5 hours of starving in serum-free
682 medium, they were incubated with 10 ng/ml BMP4 during 15 min to longer. Proteins
683 extracted at various times were analyzed by western blotting.

684 Detection of nuclear FHL3 (Figure S5A). HEK-293 were transfected with plasmids encoding
685 GFP, SMAD1, caSMAD1 or FHL3. Two days after transfection, cells were starved for 6 hours.
686 One condition with FHL3 transfection was then incubated with 10 ng/ml BMP4 during 30 min.
687 Nuclear proteins were extracted for western blotting with a standard protocol. Western blot
688 signals were measured using the relative signal intensity of nuclear Fhl3 or phospho-
689 Smad1/58 compared to Histone H3 baseline and quantified using ImageJ.

690

691 **Immunoprecipitation**

692 Total proteins were extracted using RIPA buffer (100mM NaCl, 0,5% NP40, 20mM Tris-HCl
693 pH 7,5, 5mM MgCl₂, protease inhibitors and phosphatases inhibitors) from HEK293 cells
694 transfected with plasmids encoding Smad1-FLAG, Fhl3-HA or both (4 µg) or from *X. laevis*
695 embryos injected with Smad1-FLAG mRNA (200 pg), Fhl3-HA plasmids (50 pg) or both. All
696 samples were immunoprecipitated with either 40 µl of FLAG-M2 magnetic beads (Sigma
697 M8823) or 5 µg HA antibody mixed with 40 µl Dynabeads Protein A (Invitrogen) previously

698 washed in RIPA. All samples were incubated with agitation overnight at 4°C. Beads were
699 then washed five times in RIPA buffer. Inputs and immunoprecipitated fractions were
700 analyzed by immunoblotting (see Key Resources Table for antibodies and reagents).

701

702 **Imaging and image processing**

703 WISH images were taken using a Lumar V12 Binocular microscope equipped with bright field
704 or fluorescent filters and color camera (Zeiss), and were processed using Photoshop or
705 Affinity Photo. Live imaging was done on animal caps or HEK293 cells using Leica SP8
706 confocal microscope with a 63x immersion objective. Confocal stacks were analyzed using
707 ImageJ software.

708 For image fluorescence quantification, the cells expressing Fhl3-GFP were stained by the
709 nuclear marker DAPI and the actin filaments marker Phalloidin. Using an ImageJ macro
710 developed by the Curie CoreTech microscopy platform, we delineated cell and nucleus
711 boundaries (using phalloidin staining / cytoplasmic GFP fluorescence and DAPI staining
712 respectively). This allowed measuring fluorescence intensities and automatically calculated
713 nucleus/cytoplasm GFP ratios. All ImageJ plugings are available upon request. In Figure 4C:
714 The normalized fluorescence intensity ratio between nucleus and cytoplasm was measured
715 in 20 injected ectoderm cells for each condition.

716

717 **QUANTIFICATION AND STATISTICAL ANALYSIS**

718 All experiments were performed at least in biological triplicates, except the rescue
719 experiment in Figure 2C and the *axin2* and *wnt8* rescue in the recombinant experiment in
720 Figure 3E, and the ChIP experiments (Fhl3-FLAG + noggin) and (Smad1-FLAG + Fhl3
721 MO/mRNA) (Figure 4A-B), which were performed in biological duplicates. Each dot on the
722 graphs represents one independent experiment (biological replicate). Each sample for
723 reporter assays comprises 10-15 individual explants dissected from sibling embryos. Each
724 sample for ChIP comprises 60-80 sibling embryos. For in situ hybridization, examples of
725 embryos with the most frequent phenotypes are shown, full quantification of the phenotypes
726 is provided in Supplementary materials. We used Prism for statistical analysis, error bars
727 represent S.E.M., [Student's *t*-test was used to determine statistical significance between two groups](#)
728 [and One-way ANOVA test was used for comparison among three groups or more](#)
729 [\(Figures 2E, 2F, 3B, 3D, 4E and 5A\). \(* \$p < 0.05\$; ** \$p < 0.01\$; *** \$p < 0.001\$, ns: not significant\).](#)

730 Figure 2 and 3: Each point on the graphs represents an independent biological replicate,
731 each with 10-15 tissue explants dissected from sibling embryos and pooled together.

732 Figure 4 A,B: Each point on the graphs represents an independent biological replicate, each
733 with ~80 sibling embryos per sample pooled together. C: each point represents one cell.

734 Figure 5: A-C: Each point on the graphs represents an independent biological replicate, each
735 with 10-12 tissue explants dissected from sibling embryos and pooled together.

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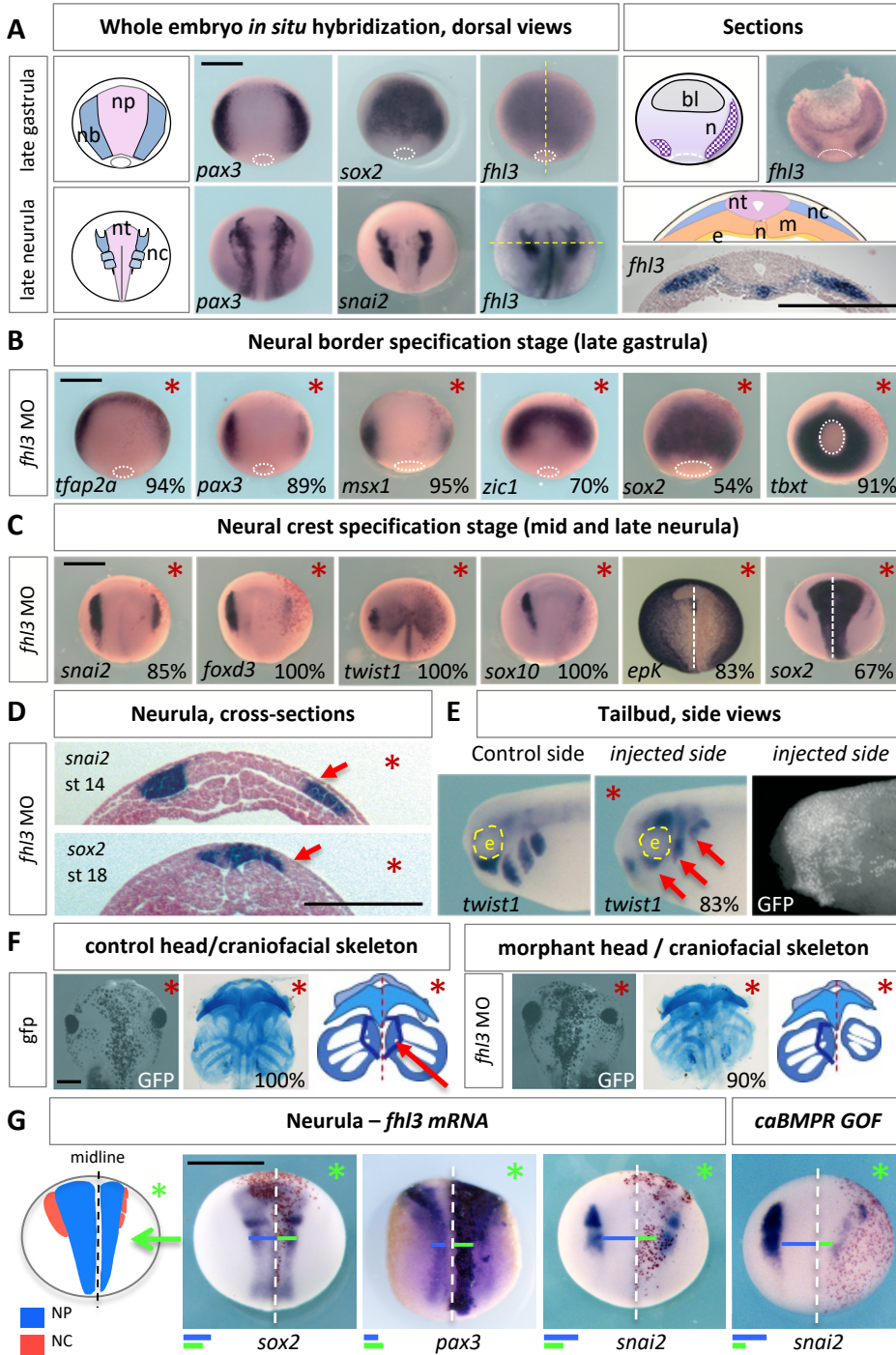
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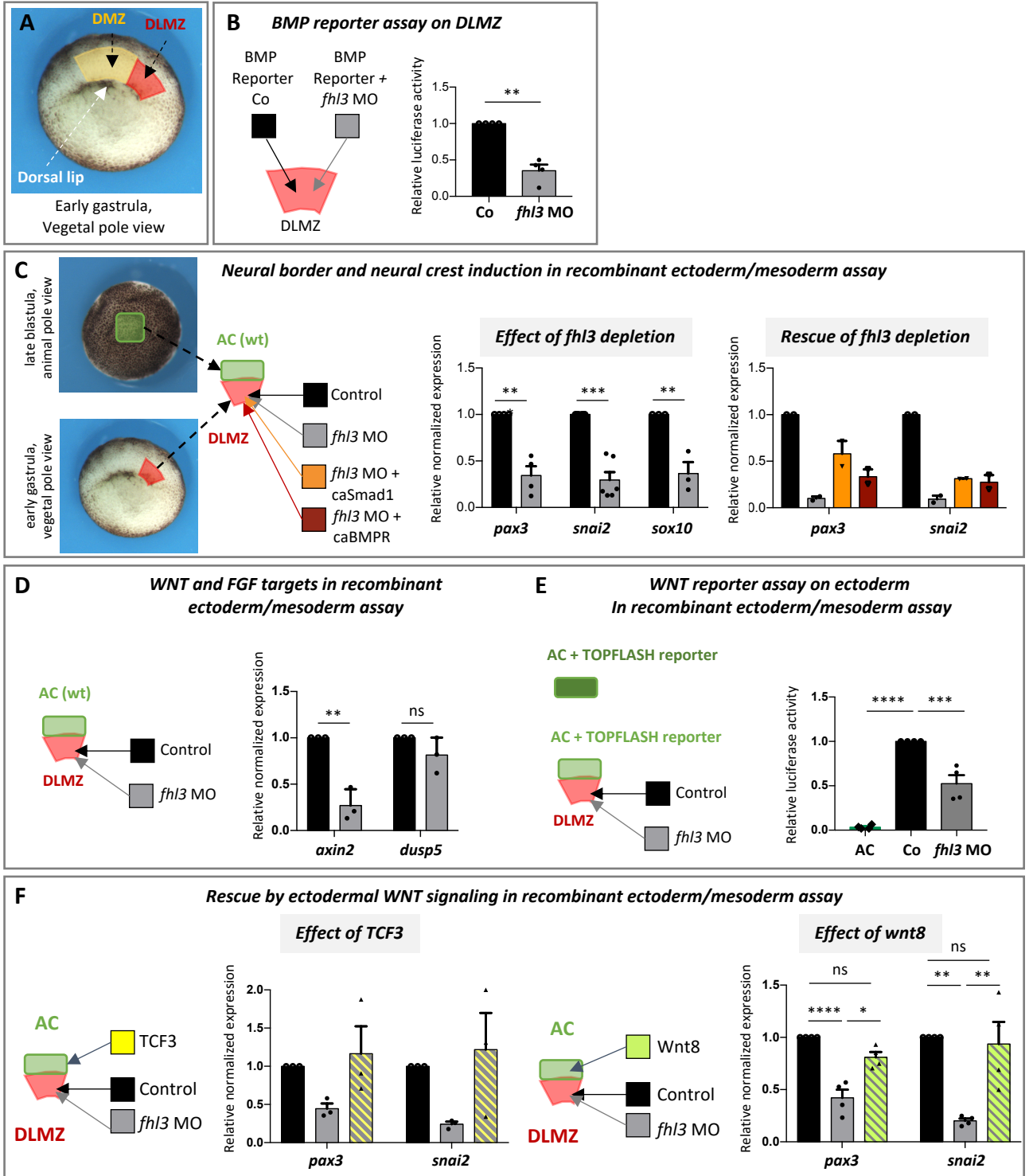
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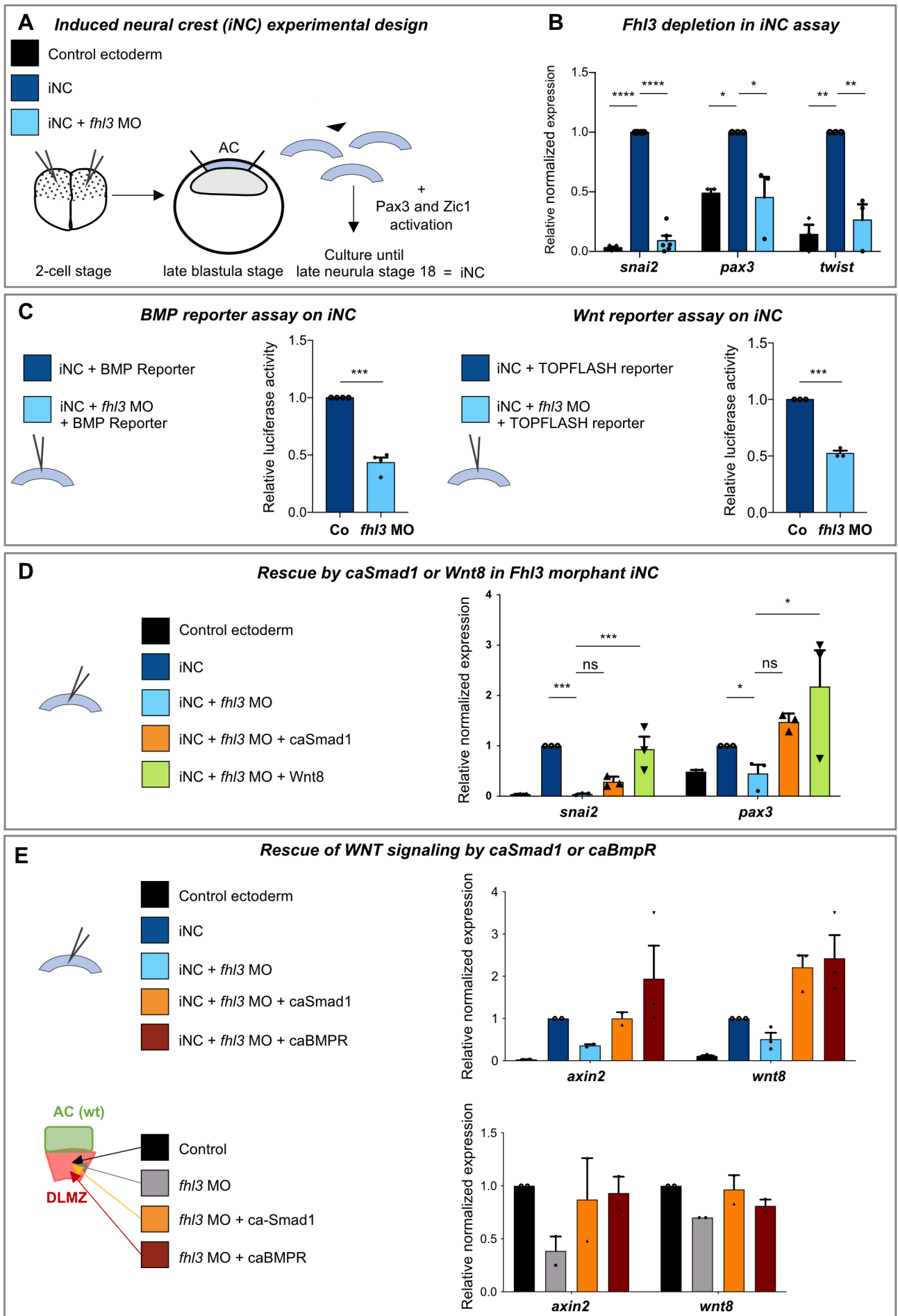
Alkobtawi et al., Figure 1

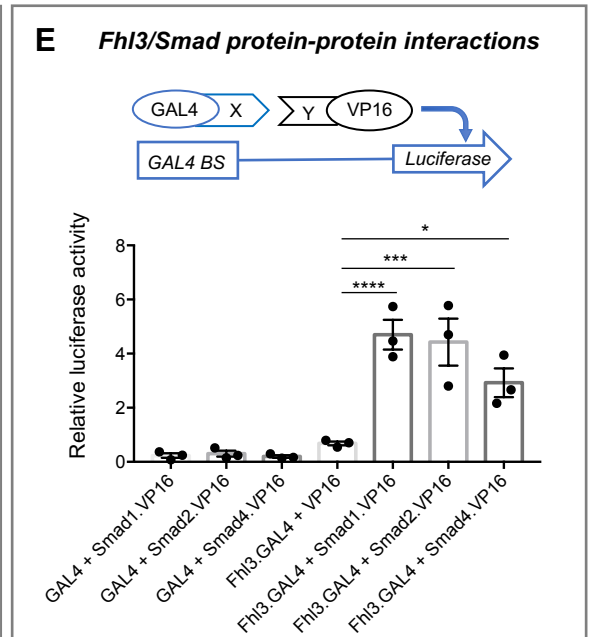
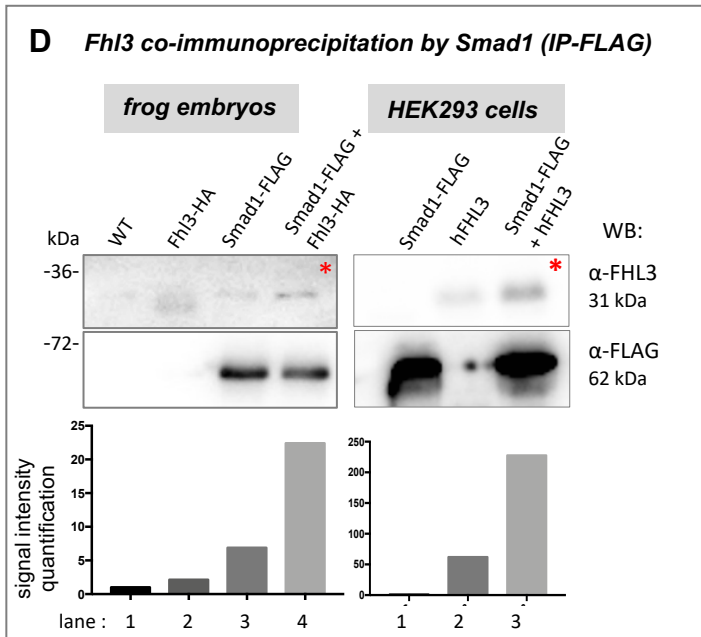
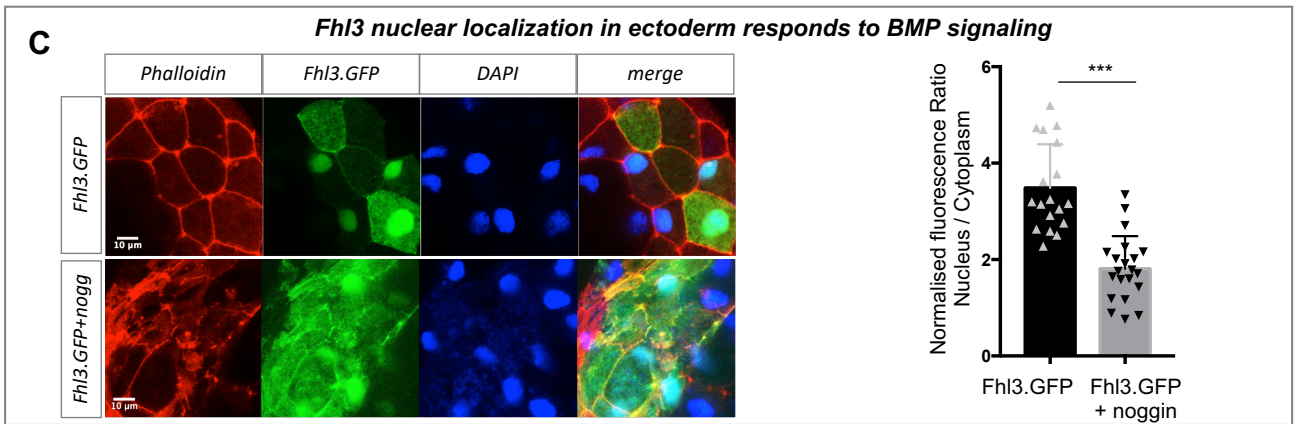
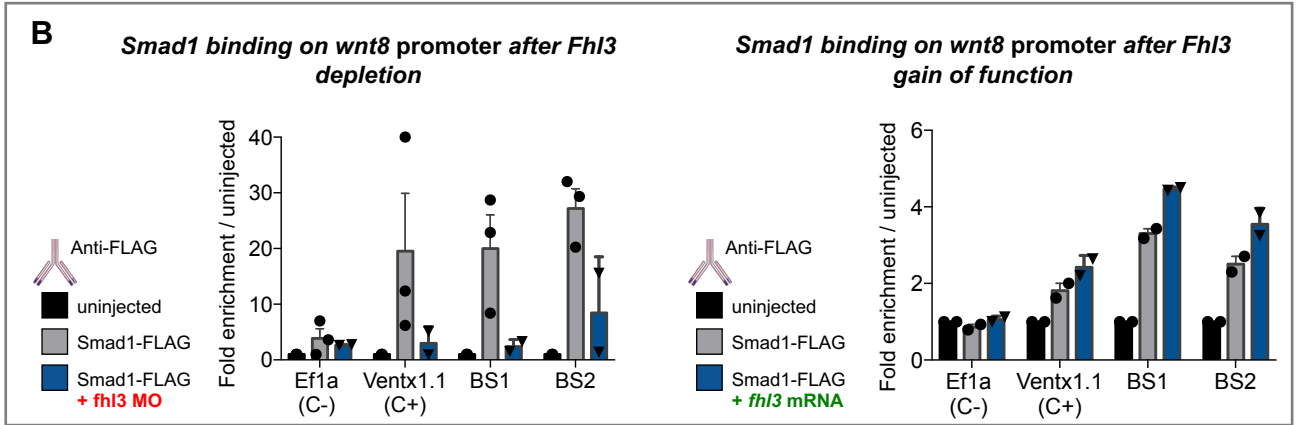
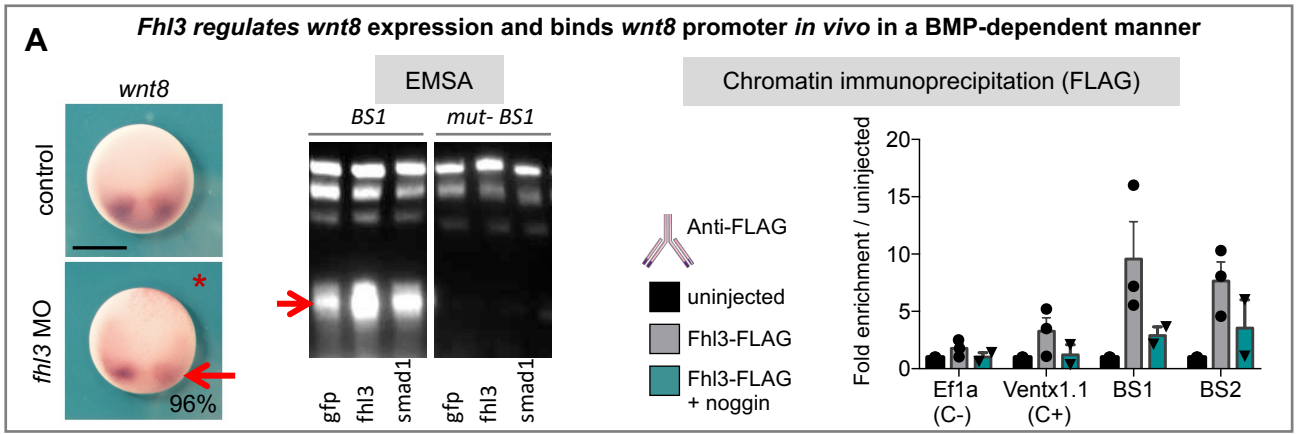


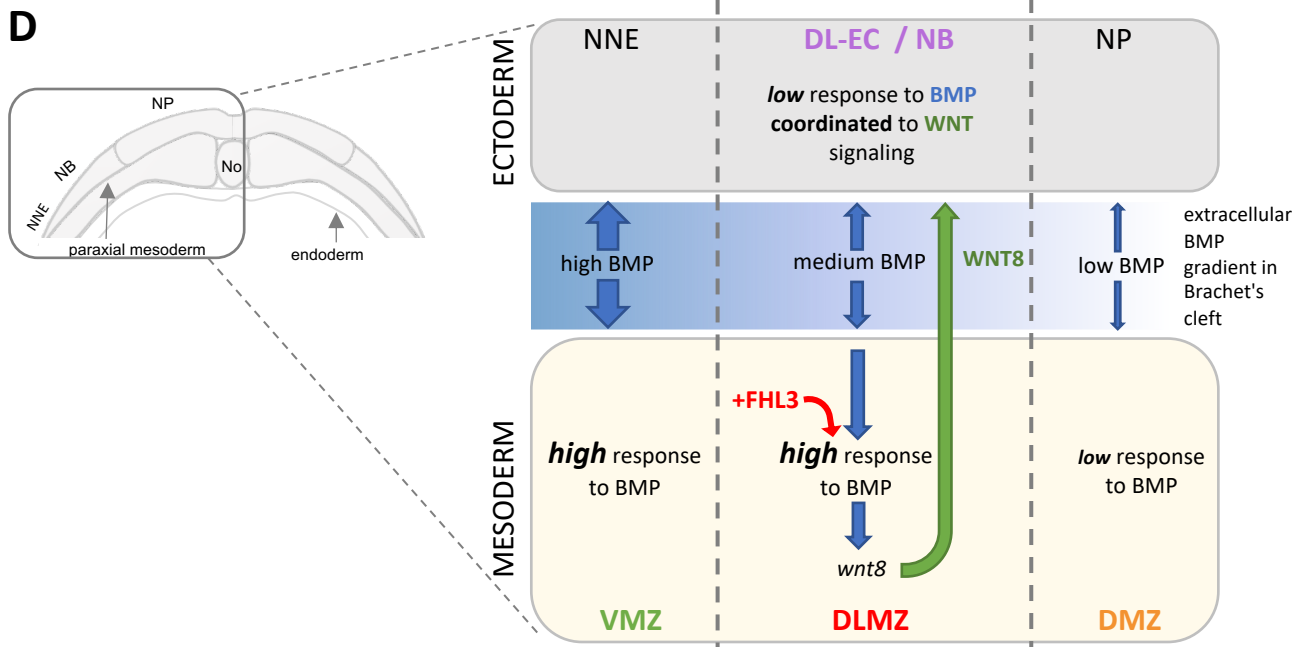
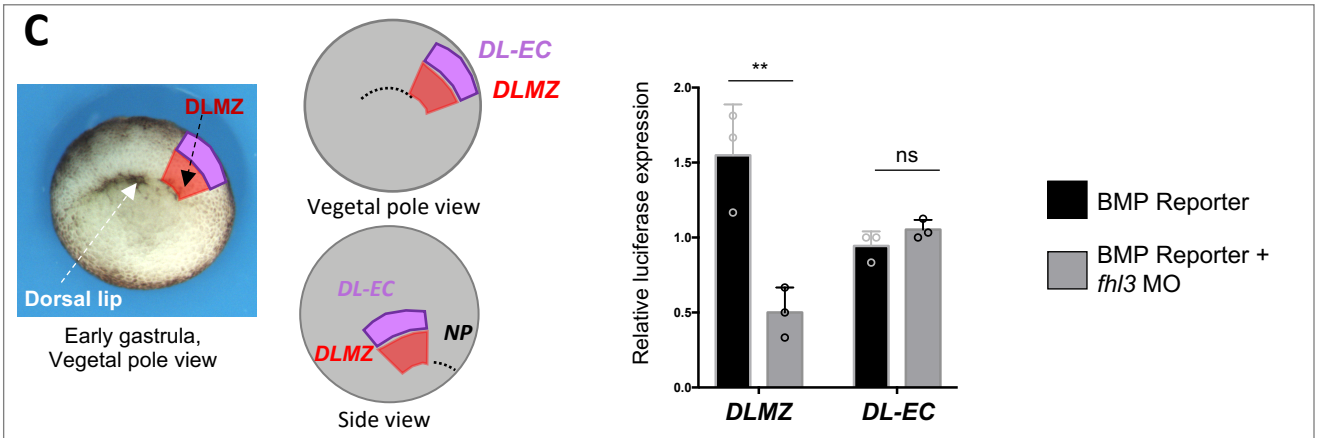
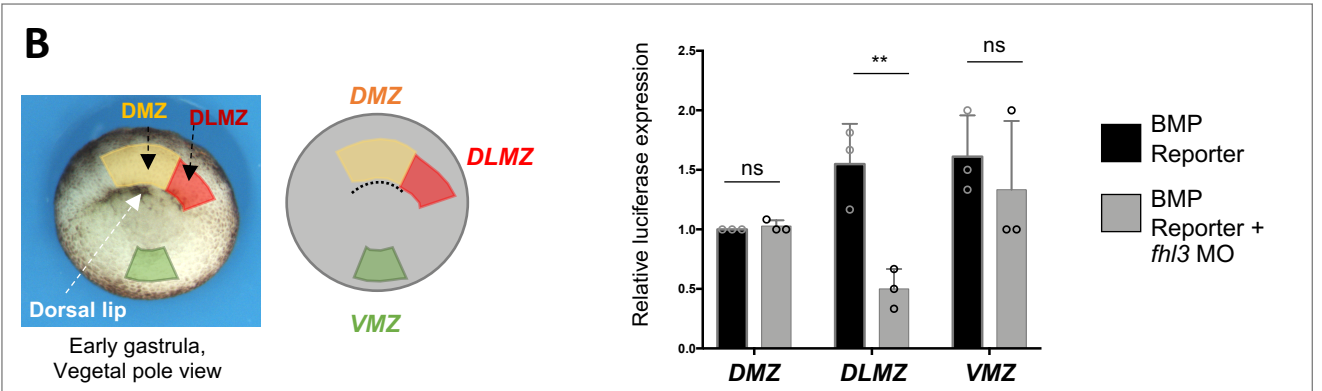
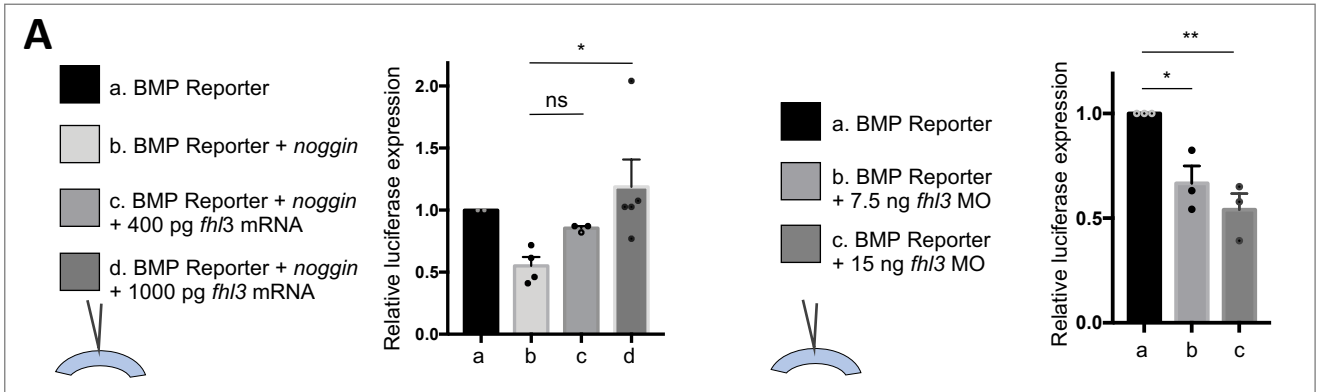
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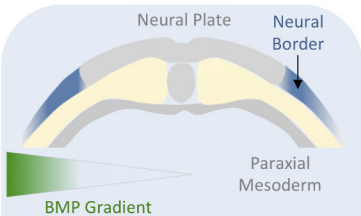
Alkobtawi et al., Figure 3







Gastrula



Neural Border

low BMP response

HIGH WNT response

low BMP

WNT8

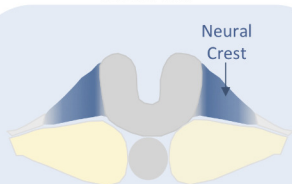
FHL3

HIGH BMP response

WNT8

Paraxial Mesoderm

Neurula



Neural Border

Neural Border genes

FHL3

FHL3

HIGH BMP response

HIGH WNT response

Neural crest