

1 **Extracellular Vesicle-delivered Bone Morphogenetic Proteins: A**
2 **novel paracrine mechanism during embryonic development**

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13 **Morphogens including Wnt, Hedgehog and BMP proteins are essential**
14 **during embryonic development and early induction of organ**
15 **progenitors. Besides free diffusion to form signalling gradients,**
16 **extracellular vesicle- (EV-) mediated morphogen transport was**
17 **identified as a central mechanism for Wnt- and Hh-signalling. Here, we**
18 **investigated EVs isolated from whole zebrafish embryos as a potential**
19 **morphogen transport mechanism. Inhibition of EV-secretion during**
20 **development leads to severe dorsalization phenotypes, reminiscent of**
21 **disrupted BMP-signalling. Subsequently, we found that EVs isolated**

22 **from zebrafish embryos at bud stage contain biologically active BMP2/4**
23 **protein. Embryos with inhibited EV secretion display reduced**
24 **Smad1/5/9-phosphorylation and downstream gene expression activity.**
25 **We further show that BMP-containing EVs are secreted by endodermal**
26 **cells *in vitro*, and inhibition of endodermal-EV release *in vivo* causes**
27 **signs of BMP signalling loss. Our data provides evidence that establishes**
28 **the transport of BMP2/4 by EVs as an essential but so far undiscovered**
29 **mechanism in developmental morphogenesis.**

30 **Introduction**

31 During embryogenesis, a diverse set of cells originates from a single cell of origin. A
32 major task during development is to ensure the correct position and fate of cells. The
33 initially seemingly identical cells start to express a specific set of genes, thereby
34 differentiating into new cell types. This process follows a specific pattern and requires
35 intensive regulation and communication between cells and tissues¹. Molecules
36 mediating tissue patterning are called morphogens. A hallmark of morphogens is to
37 form a gradient² and to thereby activate concentration dependent signalling
38 responses^{3,4}.

39 The formation of morphogen gradients is facilitated by multiple components and
40 mechanisms, which are still not fully understood. Extracellular morphogens are
41 typically thought to be secreted into the extracellular space as freely diffusing,
42 unpackaged proteins^{5,6}. First models described the gradient formation by diffusion

43 and uptake of morphogens by cells either at the end ('source-sink' model⁷) or along
44 the gradient ('synthesis-diffusion-degradation' model^{7, 8}). More recent findings added
45 more complexity to the existing models. Several soluble and membrane-bound
46 proteins were found to bind morphogens. Depending on the binding factor and
47 context the binding can lead to opposing effects. Thus, binding of bone
48 morphogenetic proteins (BMPs) to Chordin inhibits receptor binding^{9, 10}, but can also
49 facilitate increased diffusion rates¹¹. BMPs also bind to plasma membrane-bound
50 heparan sulphate proteoglycans (HSPGs), which removes the ability to diffuse freely¹²,
51 ¹³. Knockout of HSPGs however also disrupts BMP-signalling^{12, 13}.

52 Recently, the morphogens Wnt, TGF- β and Hedgehog have been reported to reside in
53 extracellular vesicles (EVs)¹⁴⁻¹⁷. The term EV describes a superfamily of vesicles that
54 are characterized by their secretion from cells¹⁸. EVs include microvesicles (MV) and
55 exosomes among other subtypes. Currently the different types of EVs are mainly
56 characterized by their size, content and cellular compartment, in which they are
57 synthesized. MVs are between 100 - 1.000 nm in size and are formed and shed at the
58 plasma membrane of the secreting cell^{19, 20}. Exosomes are the smallest kind of vesicles
59 with a diameter of 50-150 nm in size^{19, 20}. They form inside multivesicular bodies
60 (MVBs), a part of the endosomal pathway, which can fuse with the plasma membrane,
61 thereby releasing the exosomes²¹. The increasing attention EVs received during recent
62 years is mostly due to their function in transporting specific sets of multiple
63 biomolecules to target cells over long distances²²⁻²⁵. The transport of multiple
64 molecules by the same vehicle allows the reliable delivery of complex and fine-tuned
65 signals, while packaging of signalling molecules inside EVs might protect them from

66 degradation and antagonists. Investigating the role of EVs in morphogenetic signalling
67 may thus reveal an additional way of fine tuning morphogenesis during development.
68 Here we report that BMP2 and/or BMP4 (BMP2/4) are secreted in exosome-like EVs
69 *in vitro* and *in vivo* during embryogenesis. This EV-associated BMP was functionally
70 active and activated BMP-dependent transcription in cells. Inhibiting EV-release
71 strongly disturbed BMP-signalling in zebrafish, leading to phenotypes similar to the
72 phenotypes caused by dorsomorphin, a BMP inhibitor. We postulate that EV-
73 association of BMP is an additional tool to shape the BMP gradient.

74

75 **Results**

76 **Zebrafish secrete EVs during development**

77 To investigate the role of EVs during embryonic development, we chose the zebrafish
78 as a well-established model in developmental research²⁶. EV isolation protocols
79 established so far are mostly optimized to purify EVs from biofluids, like blood plasma
80 or cell culture media, while only limited options exist for purifying EVs from solid
81 tissue^{27, 28}. We adapted existing EV isolation procedures to establish a protocol that
82 allowed us an enzyme-free isolation of EVs from early-stage zebrafish embryos by
83 using EDTA and mild shear forces to dissociate embryonic cells, thereby freeing EVs
84 (Figure 1A). EVs were then isolated by sequential ultracentrifugation^{14, 28}.
85 Nanoparticle tracking analysis (NTA) demonstrated an enrichment of particles with a
86 mode size of 140.3 nm in the isolates, which is within the size range of exosomes
87 (Figure 1B). Electron microscopy showed vesicles of smaller than 200 nm in size,

88 presenting the typical cup-like shape as previously described for EVs²⁸ (Figure 1C).
89 Western blot analysis additionally showed the presence of the EV-proteins TSG101,
90 Lamp1 and ALIX in both cell lysate and EV isolates, while the endoplasmic reticulum
91 (ER)-marker GP96 was only present in cell lysate, but not in EVs (Figure 1D). This data
92 indicates a pure EV-isolate with strong enrichment of exosome-like particles secreted
93 by zebrafish embryos.

94

95 **Inhibition of EV-secretion in zebrafish embryos causes a phenotype resembling** 96 **dysfunctional BMP-signalling**

97 To evaluate the relevance of EVs during embryonic development, we inhibited EV-
98 secretion by morpholino-based knock down of Rab11a and Rab35. Rab11a and Rab35
99 are known to be essential for fusion of the multi vesicular body (MVB) with the plasma
100 membrane and therefore are well-established molecular targets to block EV-release
101 into the interstitium²⁹⁻³¹. To validate the inhibition of EV secretion, EVs were isolated
102 from 130 control- or morphant embryos and compared on the basis of the amount of
103 ALIX present in the samples as determined by western blotting (Figure 2A). EV-isolates
104 of Rab11a-morphants (125 μ M MO-Rab11a) showed a non-significant reduction in
105 ALIX content (-14.3 ± 20.7 %) (mean \pm standard deviation). Rab35-morphants (250 μ M
106 MO-Rab35) however presented a significant reduction of 49.2 ± 29.2 % and were used
107 in further experiments (Figure 2B). The knockdown (KD) of Rab35 resulted in a
108 dorsalization phenotype with varying penetrance and severity (Figure 2C). Classifying
109 phenotype severity using the dorsalization categories published by Mullins et al.³²
110 indicated that 34.0 % of the morphants presented severe dorsalization (C4-5), 8.2 %

111 with weak dorsalization (C1-3) and 27.8 % were dead, while only 29.9 % presented no
112 dorsalization phenotype (Figure 2C). Dorsalization is a typical sign of dysfunctional
113 morphogen signalling during dorso-ventral-patterning, especially of reduced BMP2,
114 BMP4 or BMP7-signalling activity³³⁻³⁶. Inhibition of BMP signalling by dorsomorphin
115 during embryonic development resulted in an identical phenotype (Figure 2C).

116 **EVs in zebrafish embryos at bud stage contain BMPs**

117 To assess whether BMPs are transported by embryonic zebrafish EVs, we performed
118 western blotting of zebrafish EVs secreted at bud stage (10 hpf). The antibody was
119 chosen to recognize the mature domain of BMP2, allowing the detection of both
120 processed and unprocessed BMP2. Since the mature domain of BMP2 and BMP4 are
121 nearly identical, the antibody detected both BMPs. Thus, we will refer to the detected
122 BMPs as BMP2 and/or BMP4 (BMP2/4). Surprisingly, we detected the BMP2/4-
123 precursor as well as the mature BMP2/4 ligand (Figure 2D). To confirm that BMP2/4
124 is associated to EVs and not purified as a contaminant, the crude EV-suspension was
125 separated on an OptiPrep[®]-gradient (Figure 2E). BMP2/4 mainly co-purified within the
126 same fractions as TSG101 at a density of 1.13 - 1.18 g/mL, which was previously
127 described for EVs^{19, 28}. In Rab35-morphants the amount of BMP2/4 in EV-isolates was
128 significantly reduced as compared to EV-isolates obtained from control zebrafish,
129 while BMP2/4-levels remained constant in the cell lysate of the same embryos (Figure
130 2F,G). Rab11-KD on the other hand did not reduce the amount of BMP2/4 in EV-
131 isolates.

132 To demonstrate the biological activity of EV-associated BMP, we performed a dual
133 luciferase reporter assay in HEK293A cells using a construct expressing Firefly

134 Luciferase under the control of BMP-responsive elements (BRE)³⁷. Since zebrafish EVs
135 might not actually be endocytosed by HEK293A-cells, we verified the uptake by
136 labelling the EVs using PKH26 and imaged EV-treated cells after 5 h (Figure 2H). The
137 cells showed a large number of fluorescent punctae, resembling endosomes, inside
138 their cytosol, thereby indicating EV uptake. HEK293A cells were transfected with
139 pGL3-BRE:Luciferase and pIS2-Renilla and subjected to a 16 h-treatment with either
140 PBS or EVs isolated from zebrafish at bud stage. The EV-treatment resulted in a
141 significant, dose-dependent increase in luciferase activity indicating that EV-delivered
142 BMP indeed is able to activate BMP-dependent transcription (Figure 2I).

143 **EV-transport of BMP2/4 is required for BMP-signalling during development**

144 To address the hypothesis that BMP signalling is reduced by inhibition of EV-secretion,
145 we used *Nkx2.5*, a transcriptional target of BMP2/4, as a readout to measure BMP-
146 activated transcriptional activity in Rab35-morphants. Whole mount *in situ*
147 hybridisation (WISH)-stainings of *nkx2.5* at the 7-somite stage (7SS) resulted in a
148 typical staining with two parallel stripes along the midline, representing cardiac
149 progenitor cells, in wildtype zebrafish embryos and control morphants³⁸ (Figure 3A).
150 In Rab35-morphants we found the *nkx2.5*-positive areas to be smaller and situated
151 more laterally (Figure 3A). Quantification of the *nkx2.5*-staining showed a significant
152 reduction of the *nkx2.5*-expression to a median of 36.9 % (Quantiles (25 % - 75 %):
153 12.4 – 87.8 %) in Rab35-morphants as compared to wildtype zebrafish embryos. These
154 results suggested that inhibition of EV-secretion results in reduced BMP-mediated
155 transcriptional activity.

156 A well-observable and strong BMP-signalling event during early zebrafish
157 development happens in the tail bud during tail development³⁹. We used whole
158 mount immunofluorescence stainings to detect phosphorylated Smad1/5/9
159 (pSmad1/5/9) in the tail bud of 7SS zebrafish embryos as an additional measure of
160 BMP-signalling activity (Figure 3C). Again, while levels of pSmad1/5/9 were
161 indistinguishable in wildtype and MO-Cntr-injected zebrafish, Rab35-KD significantly
162 reduced the overall Smad1/5/9-phosphorylation in the tail bud to 45.4 % (Quantiles
163 (25 % - 75 %): 28.4 – 77.0 %) (Figure 3D).

164 Taken together, these experiments strongly suggest that EVs play a crucial role in
165 mediating BMP signalling activity and that EV-mediated BMP signalling is significantly
166 involved in early cardiac progenitor cell induction.

167 **Endoderm cells secrete EVs containing BMP2/4**

168 BMP2 responsible for activating the transcription of Nkx2.5 in cardiac mesoderm cells
169 is known to originate from the endoderm⁴⁰⁻⁴². Since the EV isolation protocol for
170 zebrafish embryos does not allow purification of EVs originating from a specific tissue
171 or cell type, we used the mouse endodermal cell line End2 as an endodermal *in vitro*
172 model⁴³. Using western blotting, we verified the purity of the End2-EVs purified from
173 cell culture medium (Figure 4A). Western blotting also confirmed that BMP2/4 was
174 present in EVs isolated from End2-cells. The purity of End2-EV isolates was further
175 asserted by electron microscopy (Figure 4B) and NTA measurements (Figure 4C),
176 confirming an enrichment of particles smaller than 200 nm with a mode diameter of
177 140.6 nm. On an OptiPrep[®]-gradient BMP2/4 largely co-purified in the same fractions
178 as the EV-markers Flot1 and ALIX, suggesting their association (Figure 4D).

179 PKH26-labeling again confirmed the uptake of End2-derived EVs by HEK293A-cells
180 (Figure 4E), providing the basis for dual luciferase assays to test BMP-activity. As
181 expected, the BMP-activity assay verified the ability of End2-EVs to activate BMP-
182 dependent transcription (Figure 4F). Further, fluorescently labelled BMP2 (Venus-
183 BMP2) expressed in End2-cells was detected in EV-isolates. After treatment of
184 HEK293A-cells with PKH26-labelled EVs from Venus-BMP2-expressing End2-cells,
185 overlapping fluorescent signals for PKH26 and Venus were found in the HEK293A-cells,
186 again confirming EV and BMP 2/4 association (Figure 4G).

187 **Endoderm-specific inhibition of EV-secretion dampens Smad1/5/9-phosphorylation**
188 **in the zebrafish tail bud *in vivo***

189 To assess the significance of the endoderm as a source of EV-associated BMP2/4 *in*
190 *vivo* we injected zebrafish embryos with a plasmid construct expressing a dominant
191 negative variant of Rab35 (Rab35^{N120I})²⁹ tagged with an N-terminal GFP (GFP-Rab35^{dn}).
192 An endoderm-specific expression was achieved by using the *sox17*-promoter⁴⁴. Since
193 zebrafish stably expressing GFP-Rab35^{dn} were not viable, transient expression was
194 used instead, leading to a mosaic expression pattern only. Nevertheless, the
195 expression of Rab35^{dn} in the endoderm resulted in a similar phenotype as the
196 ubiquitous Rab35-KD (Figure 5A, S1A), indicating that the endoderm is an important
197 source for EVs involved in BMP-signalling. Surprisingly, overexpressing the wildtype
198 form of Rab35 in the endoderm also increased the mortality and number of dorsalized
199 embryos, although to a lesser extent than Rab35^{dn}.
200 Immune fluorescence stainings against pSmad1/5/9 showed that the expression of
201 GFP-Rab35^{dn} in contrast to expression of GFP or GFP-Rab35 resulted in morphological

202 changes and reduced fluorescence in the tail bud (Figure 5B). Quantification of the
203 pSmad1/5/9 distribution and intensity in the tail bud validated these initial
204 observations (Figure 5C). Interestingly, no overlap of the transgene-expressing cells
205 with cells positive for pSmad1/5/9 was needed to cause the observed phenotype,
206 indicating that a paracrine effect might be the cause for the reduced Smad1/5/9-
207 phosphorylation (Figure S1C). Thus, our results suggest that endoderm-derived EVs
208 are required for BMP-signalling during zebrafish development.

209 **BMP2/4 is tethered to EV surfaces by binding to HSPGs**

210 BMPs, other than Wnts or Hh, do not possess a lipid anchor, with which they can bind
211 to EV-membranes⁴⁵⁻⁴⁷. However, BMPs are known to bind to heparan sulphate
212 proteoglycans (HSPGs) on cell surfaces^{13, 48, 49}. Since HSPGs are present on EV-surfaces
213 as well⁵⁰, we investigated whether BMPs are tethered to EVs by HSPGs. We divided
214 End2-EV isolates into two equal fractions. Fraction I was treated with Heparinase 3,
215 which cleaves off the sugar chains of HSPGs to which BMPs are bound⁵¹. Fraction II
216 was used as a control. If HSPGs are present and are responsible in linking BMPs to EVs,
217 Heparinase 3 treatment should result in the decrease in BMP signal using western
218 blot. Heparinase 3-treatment indeed led to a decrease in BMP2/4 in the EV-isolate
219 (Figure 6A,B), indicating that BMP2/4 is tethered to the EV surface in part by binding
220 to HSPGs.

221

222 Discussion

223 Here, we report that BMP2 and/or BMP4 are transported by EVs during zebrafish
224 development, a mechanism that was essential for BMP-dependent morphogenetic
225 signalling during zebrafish development. We further provide a mechanism of
226 tethering morphogens without lipid-modification to EVs.

227 Morphogenetic signalling has been investigated for over a century and while it is
228 commonly accepted that diffusion gradients are a hallmark feature of morphogen
229 signalling, the mechanisms behind the formation of morphogen gradients are still not
230 definitely resolved^{8, 52}. Differences in morphogen gradient formation do not only
231 occur between different morphogens, but also between morphogenetic signalling
232 events facilitated by the same morphogen. The Dpp-gradient during the dorso-
233 ventral-patterning in drosophila, for example, is formed within 30 min by an active
234 shuttling mechanism involving Sog^{53, 54}. In the imaginal wing disc on the other hand,
235 the Dpp-gradient needs 4 h to form, which is thought to happen by a different unaided
236 mechanism⁵⁵. Several additional models were proposed based on experimental
237 observations. This includes the Source-Sink-model^{7, 56, 57}, the Counter-Gradient-
238 model⁵⁶⁻⁵⁸, restricted diffusion^{11, 12}, transcytosis⁵⁹, cytoneme transport⁶⁰ and more
239 recently also vesicular transport^{14-16, 61}. This role of EVs in morphogenetic signalling is
240 by now a well-established mechanism after Wnt-proteins and Hh were found in EVs,
241 a mechanism that allows these lipid-modified proteins to diffuse efficiently without
242 unspecifically binding to membranes¹⁴⁻¹⁶. The observation that BMPs, that do not
243 possess a lipid anchor, are transported by EVs as well, indicates that masking lipid
244 groups is not the only function of EVs in morphogenetic signalling. EVs might be

245 needed to mobilize BMP that is bound to HSPGs on cell surfaces and thus restricted in
246 their diffusion.

247 Multiple proteomics studies detected BMP in EVs but did not investigate this finding
248 in detail⁶²⁻⁶⁹. Furthermore, the secretion of BMPs in matrix vesicles, a specialized type
249 of EV that is involved in bone formation was reported earlier by Nahar et al.⁶¹, further
250 supporting our findings.

251 The phenotypical changes observed in zebrafish embryos after Rab35-KD suggest that
252 EV-transport is essential for BMP-signalling during embryonic development. The
253 severity of the phenotypes is surprising, considering that BMP is thought to diffuse
254 unaided. But whether the importance of EVs in BMP-signalling is due to their ability
255 to mobilize BMPs or due to other reasons, remains to be investigated. A limitation of
256 this study is that known factors involved in EV-secretion, that can be manipulated to
257 inhibit the release of EVs, are also involved in essential cellular processes, especially
258 endosomal trafficking, including receptor recycling^{29, 70, 71}. Thus, unspecific effects of
259 changes in Rab35-activity might add to the observed phenotype. Our experiments
260 however show that inhibition of Rab35 in the endoderm affected BMP-signalling
261 activity in cells of the zebrafish tailbud, which are mesoderm cells⁷². Processes that
262 take part in computing the BMP-signal response in the mesodermal target cells, as for
263 example BMP-receptor recycling, cannot be influenced by Rab35^{dn} directly, that is
264 exclusively expressed in the endoderm. This indicates, that a paracrine effect is likely
265 responsible for the reduced Smad1/5/9-phosphorylation.

266 The finding, that EV-associated BMP2/4 is secreted by the endoderm, is intuitive, since
267 the endoderm was already described to be responsible for secreting freely diffusing

268 BMP2/4 during early development⁴⁰⁻⁴². Nevertheless, since BMP2/4 associate to EVs
269 by binding to HSPGs on their surface should theoretically be able to catch free BMP2/4
270 in the extracellular space. This would mean that EVs from cells, which are not secreting
271 BMP2/4, could be able to influence BMP2/4-signaling by secreting BMP-tethering EVs,
272 thereby modulating BMP gradient formation and thus BMP signalling. Whether this
273 hypothesis is true, remains to be investigated in future studies.

274 In summary, we describe an additional mode of transport for BMPs during embryonic
275 development. While future studies will have to show how EV-associated BMPs
276 contribute to BMP-signalling as compared to previously known mechanisms, the
277 findings presented here provide additional information, that will help to understand
278 the formation of BMP-gradients.

279

280 **Experimental Procedures**

281 **Zebrafish care and maintenance**

282 All animal experiments have been performed in accordance with the guidelines of the
283 state of Baden-Wuerttemberg and all experimental protocols. Wildtype zebrafish
284 were used as basis for all experiments. For imaging, zebrafish embryos were mounted
285 in 2% methyl-cellulose and analysed under the microscope.

286 **Inhibition of EV secretion**

287 Morpholinos were obtained from Gene Tools LLC. The morpholinos against Rab11 and
288 Rab35 were designed to bind at the translation start site. The sequences were as
289 follows: Rab11⁷³: 5'-GTATTCGTCGTCTCGTGTCCCCATC-3'; Rab35⁷⁴: 5'-
290 AGAGGTGATCGTAGTCGCGGGCCAT-3'. MO-Cntr: non-targeting standard control
291 (Gene Tools LLC). Morpholinos were solubilized in water at a stock-concentration of
292 1 mM. Stock solutions were diluted with 200 mM KCl to working concentrations of
293 125 µM–250 µM. The injection volume was 2 – 4 nL/embryo. Effective doses were
294 determined for every morpholino separately.

295 Inhibition of EV secretion was verified by western blot analysis using an antibody
296 targeting ALIX (see below). EVs of uninjected and MO-Cntr-injected zebrafish embryos
297 were used as controls. Experiments showing large differences in ALIX-content of EVs
298 between uninjected and MO-Cntr-injected zebrafish embryos were excluded.

299 **Cell Culture**

300 HEK293A-cells were maintained in Dulbecco's Modified Eagle Medium (DMEM)
301 (Gibco) containing 10 % Foetal Calf Serum (FCS) (Gibco), 1 % L-Glutamine (Gibco) and
302 1 % Penicillin/Streptavidin (Gibco). End2 cells⁴³ were maintained in DMEM/F-12

303 medium (Gibco) containing 7.5 % FCS and 1 % Penicillin/Streptavidin. All cells were
304 cultured at 37 °C and 5 % CO₂ in a humid atmosphere. Transient transfections of
305 plasmids were performed using Lipofectamine 2000 (Invitrogen) following the
306 manufacturer's instructions.

307 **EV Purification of Cell Culture Medium**

308 Cells were grown to 70 % confluency. The medium was exchanged with fresh medium
309 containing FCS, that was depleted from EVs by centrifugation at 100,000 g for 18 h
310 and successively filtered through a 0.2 µm filter. After 24 h the conditioned medium
311 was collected and EVs were isolated by ultracentrifugation following the protocol
312 described by Gross et al.¹⁴.

313 **Nanoparticle Tracking Analysis**

314 The EV pellet were resuspended in PBS. Particle counts and size distribution were
315 measured using a NanoSight LM10 (Malvern Instruments) equipped with a 405 nm
316 laser.

317 **Electron Microscopy**

318 EVs resuspended in PBS were prepared for electron microscopy following the protocol
319 described by Théry et al.²⁸

320 **OptiPrep Gradient Centrifugation**

321 EV pellets were resuspended in 500 µL Homogenization Media (0.25 M sucrose, 10
322 mM Tris-HCL, (pH 7.4)). 40 %, 20 %, 10 % and 5 % OptiPrep/Homogenization Media
323 solutions were prepared. The gradient was created by layering 3 mL of the 40 %, 20 %
324 and 10 % OptiPrep solutions and 2.5 mL of the 5 % OptiPrep solution in order. The EV
325 sample was layered on top of the gradient. The gradients were ultracentrifuged at

326 100,000 g for 18 h at 4 °C. Fractions of 1 mL were collected beginning from the top of
327 the gradient and diluted with PBS. EVs in each fraction were isolated by
328 ultracentrifugation at 100,000 g for 3 h at 4 °C.

329 **Imaging EV uptake**

330 For labelling with PKH26 (Sigma Aldrich) the EV pellet was resuspended in 1 mL
331 Diluent C. 2 µL of PKH26 was added and the samples were incubated for 30 min at
332 room temperature. Afterwards the sample was diluted by adding 11 mL of PBS. The
333 labelled EVs were collected by ultracentrifugation at 100,000 g for 3 h at 4 °C. The EV
334 pellet was resuspended in PBS. Cells were treated with 50 µg/mL of PKH26-labelled
335 EVs. Life-cell imaging was performed 5 h after treatment.

336 ***In vitro* BMP activity assay**

337 HEK293A cells seeded in 96-well plates were transfected with 10 ng pIS2-Renilla
338 Control Vector and 100 ng pGL3-BRE:Luciferase³⁷. The medium was exchanged to
339 serum-free medium 1 h before treatment. Cells were either treated with EVs or PBS.
340 16 h post treatment cells were analysed using the Dual Luciferase Reporter Assay
341 system (Promega, Cat.-No. E1960) following the manufacturer's instructions. Renilla
342 Luciferase activity was used to normalize Firefly Luciferase activity.

343 **Whole mount in situ hybridization**

344 The whole mount in situ hybridization was performed as previously described by
345 Thisse et al.⁷⁵ The probes to detect nkx2.5 were generated using the primer pair
346 5'-CGGGACATACTGAACCTGGA-3', 5'-TCTCCCAGACACGGTTTACC-3'. Staining intensity
347 was quantified using Fiji (ImageJ). Images were contrasted consistently in each
348 experiment. Each stained area (both heart fields) was segmented manually. The area

349 of the staining was normalized to the total area of the embryo. The background
350 intensity was measured close to the stained area and used for background
351 subtraction. The *nkx2.5*-expression was defined as the product of the background-
352 corrected mean staining intensity and the normalized staining area.

353 **Whole mount immunofluorescence**

354 Zebrafish embryos fixed in 4 % PFA were dehydrated with an ascending methanol
355 series. Before staining, the embryos were rehydrated with a descending methanol
356 series and permeabilized by incubation with ice cold acetone for 8 min at -20 °C.
357 Embryos were blocked in PBS with 1 % Triton-X100, 2 % BSA, 10 % sheep serum and
358 1 % DMSO for 30 min before incubation with the primary antibody for at least 20 h at
359 4 °C. Incubation with the secondary antibody was performed overnight at 4 °C. The
360 staining intensity in the tail bud of the embryos was analysed in the same way as the
361 *nkx2.5*-whole mount *in situ* stainings.

362 **Heparinase 3-treatment**

363 EVs resuspended in PBS were divided into two equal parts of which one was treated
364 with 2 mIU/mL Heparinase 3 in reaction buffer (20 mM TrisHCl, 0.1 mg/mL BSA, 4 mM
365 CaCl₂ (pH 7.5)) and the other with reaction buffer for 3 h at 37 °C. EVs were isolated
366 from samples by ultracentrifugation with 100,000 g for 3 h at 4 °C before further
367 analysis.

368 **Antibodies**

369 For western blotting experiments the following antibodies were used: GP96 (CST,
370 #2104), TSG101 (Sigma Aldrich, AV38773), ALIX (Sigma Aldrich, sab4200476), Flotillin-

371 1 (BD Biosciences, 610821), LAMP1 (Sigma Aldrich, sab3500285), beta-Actin (Sigma
372 Aldrich, A5441), pSmad1/5/9 (CST, #13820) and BMP2/4 (RnD Systems, MAB1128).

373 **Statistics**

374 Statistical tests used for analysis are indicated in the respective figure captions.

375 Significance levels are indicated as follows: ns: not significant; *: $p \leq 0.05$; **: $p \leq 0.01$;

376 ***: $p \leq 0.001$. If not indicated otherwise error bars represent the standard deviation.

377

378 **Acknowledgements**

379 We thank Prof. Dr. Michael Boutros for allowing us to perform NTA-measurements at

380 his lab, Ms. Hosser for taking the electron microscopy images, Prof. Dr. Mummery for

381 providing us with the End2-cell line, Prof. Dr. Mikael Simons for sharing Rab35-

382 constructs and Prof. Dr. De Robertis for the Venus-BMP2 construct.

383

384 **Author Contributions**

385 D.H. and T.D. designed the experiments and wrote the article. T.D., J.H. and L.J.

386 performed the experiments. T.D. analysed the data. H.A.K. and D.H. provided the

387 material, read and revised the article.

388

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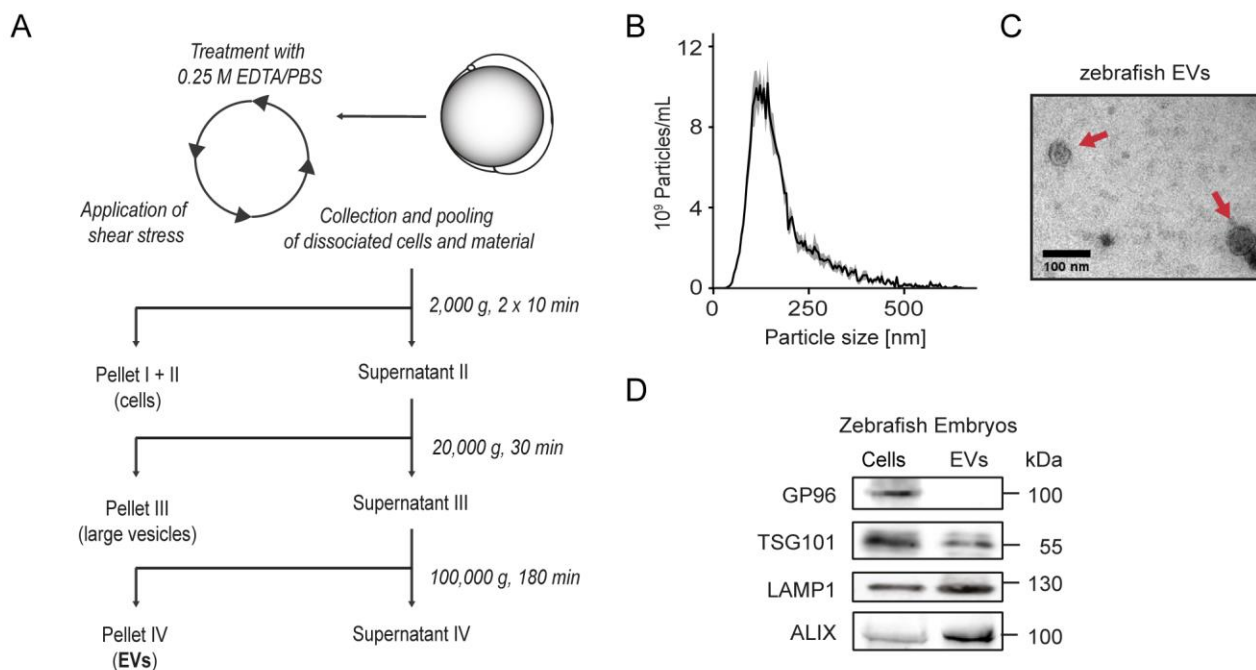
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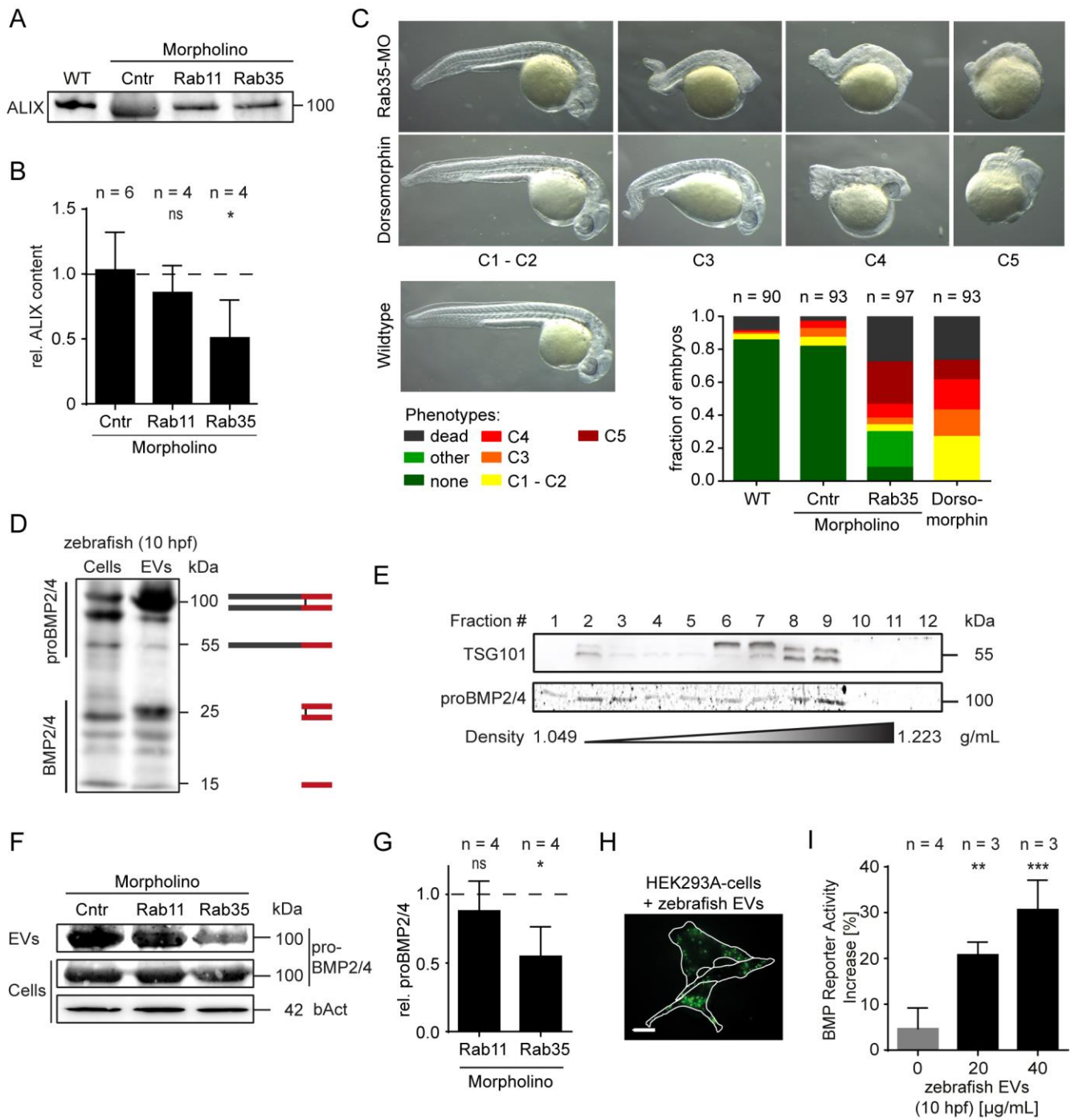
583

584 Figures



585 **Figure 1 | Isolation of EVs from zebrafish embryos.** (A) Schematic representation of
 586 the protocol used to isolate EVs from zebrafish embryos. Experiments to verify the
 587 purity of the EV-isolates were performed with EVs isolated from zebrafish embryos at
 588 bud stage (10 hpf): (B) The particle size distribution in the EV-isolates was determined
 589 by NTA measurements. The mean of five measurements of the same sample is

590 depicted (black line) with the standard deviation (grey area). (C) The morphology and
591 size of EVs was observed with transmission electron microscopy. Scale bar: 100 nm.
592 (D) Cell lysate (Pellet I/II) and EVs (Pellet IV) from zebrafish embryos were
593 characterized using immunoblots with antibodies detecting GP96, TSG101, LAMP1
594 and ALIX.
595



596

597 **Figure 2 | BMP2 and/or BMP4 are transported by EVs.** (A) Zebrafish embryos were
598 injected with either 250 μ M Cntr-MO, 125 μ M Rab11a-MO or 250 μ M Rab35-MO at
599 the one-cell stage. EVs were isolated from these zebrafish embryos at bud stage
600 (10 hpf). Western blot analysis with antibodies against ALIX were used to estimate the
601 EV-content of the isolates. The quantification of the western blot signals is plotted in
602 (B). The dashed line represents the measured signal intensity of the wildtype sample,
603 which was used as a normalizer. The samples were compared to the Cntr-MO sample
604 using ANOVA with Tukey's post hoc test. (C) Images of Rab35-morphants at 24 hpf
605 displaying the spectrum of phenotype severities, resembling dorsalization. For
606 comparison, zebrafish treated with the BMP-signalling inhibitor 10 μ M dorsomorphin
607 are shown. The embryos were classified by phenotype severity following the
608 suggestions made by Mullins et al.³². The quantification of the fraction of embryos in
609 the respective classes is plotted below the images. (D) EVs were isolated from
610 zebrafish embryos at bud stage (10 hpf) and used in western blot experiments under
611 non-reducing conditions to detect BMP2/4. Multiple preprocessing intermediates of
612 BMP2/4, which are depicted schematically next to the respective protein bands, were
613 detected (dark grey: prodomain; red: ligand domain). (E) EV isolates from zebrafish
614 embryos were further separated on an OptiPrep™-gradient. The gradient was split
615 into 12 fractions, which were investigated by immunoblotting with antibodies
616 targeting TSG101 and BMP2/4. (F) Cell lysate and EVs from Cntr-, Rab11- and Rab35-
617 morphants were investigated for differences in BMP2/4-content by western blot
618 analysis. The quantification of BMP2/4-band intensities in EV isolates is shown in (G).
619 The samples were compared to the normalized mean of the Cntr-morphants (dashed

620 line) using one-sample t-tests with Bonferroni correction for multiple testing. (H)

621 HEK293A-cells were treated with PKH26-labeled EVs isolated from zebrafish embryos

622 and imaged after 5 h. White lines depict cell borders. The scale bar represents 20 μm .

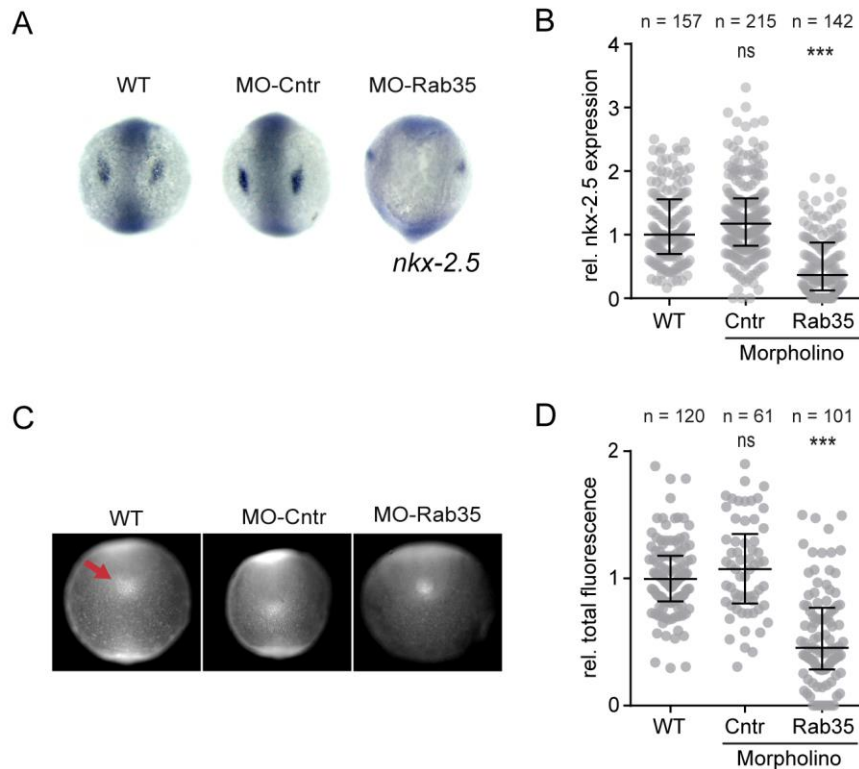
623 (I) HEK293A-cells transiently expressing pGL3-BRE:Luciferase and pIS2-Renilla were

624 treated with unlabelled zebrafish EVs and used in dual luciferase assay 16 h after

625 treatment. Values were normalized to an independent untreated control sample.

626 Samples were compared using ANOVA with Tukey's post hoc test.

627



628

629 **Figure 3 | Rab35-KD reduces BMP2/4-dependent signalling activity.** Wildtype

630 zebrafish embryos, Cntr- and Rab35-morphants were collected at the 7-somite stage.

631 (A-B) Whole mount *in situ* hybridizations was performed to detect *nkx-2.5*. Image

632 analysis-based quantification was used to measure the *nkx-2.5* expression in the

633 embryos. (C) Typical whole mount immunofluorescence stainings detecting

634 phosphorylated Smad1/5/9 are shown. The tail bud is indicated by the red arrow. (D)

635 The Smad1/5/9-phosphorylation was quantified in the tail bud. The total fluorescence

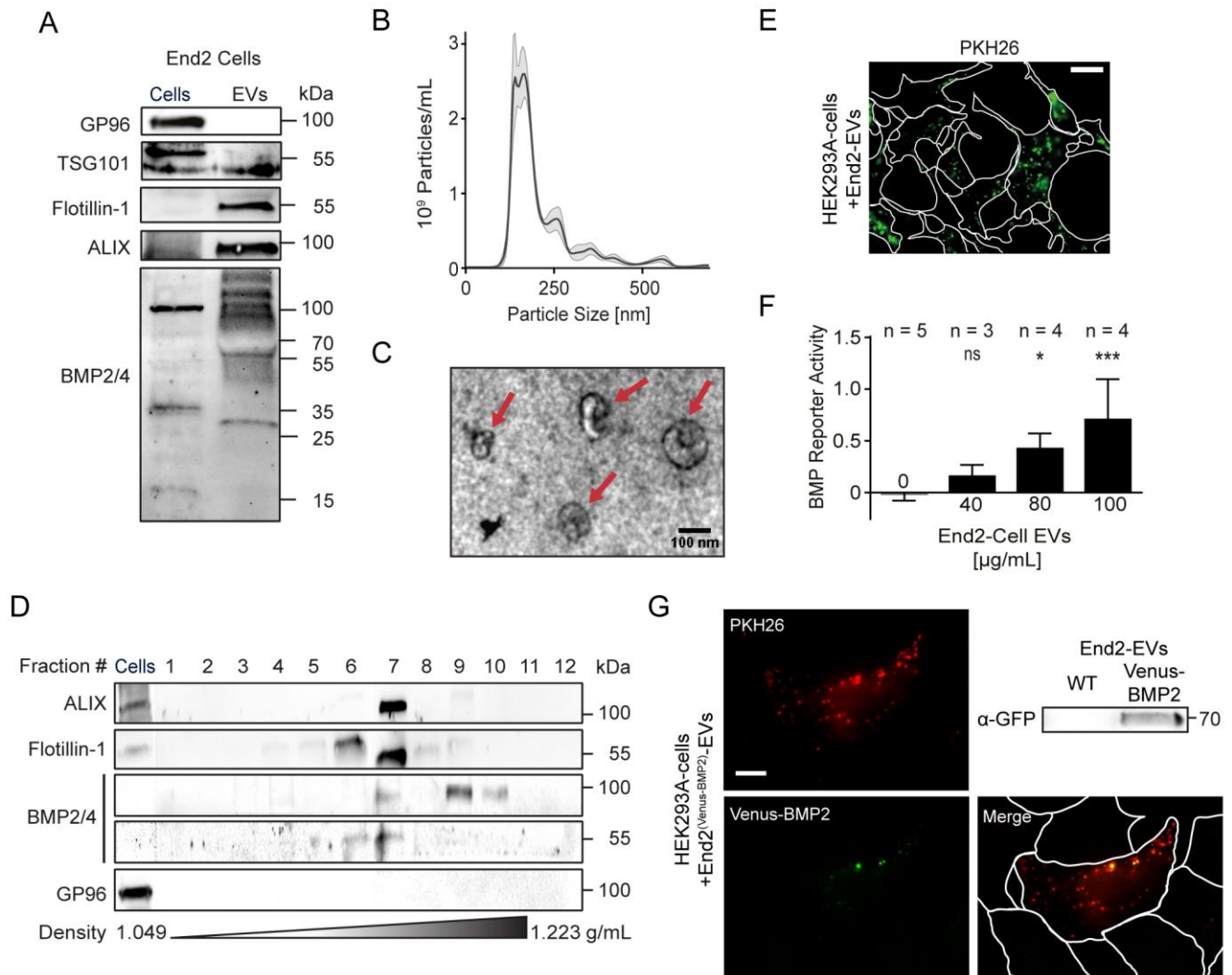
636 is defined by the product of the stained area, which was normalized to the area of the

637 embryo, and the background corrected fluorescence intensity. All statistical

638 comparisons were performed by using the Kruskal-Wallis-Test with Dunn's post hoc

639 test. In the plots (C, D) the middle lines represent the median and whiskers represent

640 the 25 % and 75 %-quantiles.



641

642 **Figure 4 | The endoderm is a source for EV-associated BMP2/4.** EVs were isolated

643 from End2-cells following the protocol described by Gross et al.¹⁴. (A) Western blot

644 analysis of End2 cell lysates and End2 EVs using antibodies targeting GP96, TSG101,

645 Flotillin-1, ALIX and BMP2/4. (B) NTA-measurement of a representative End2 EV-

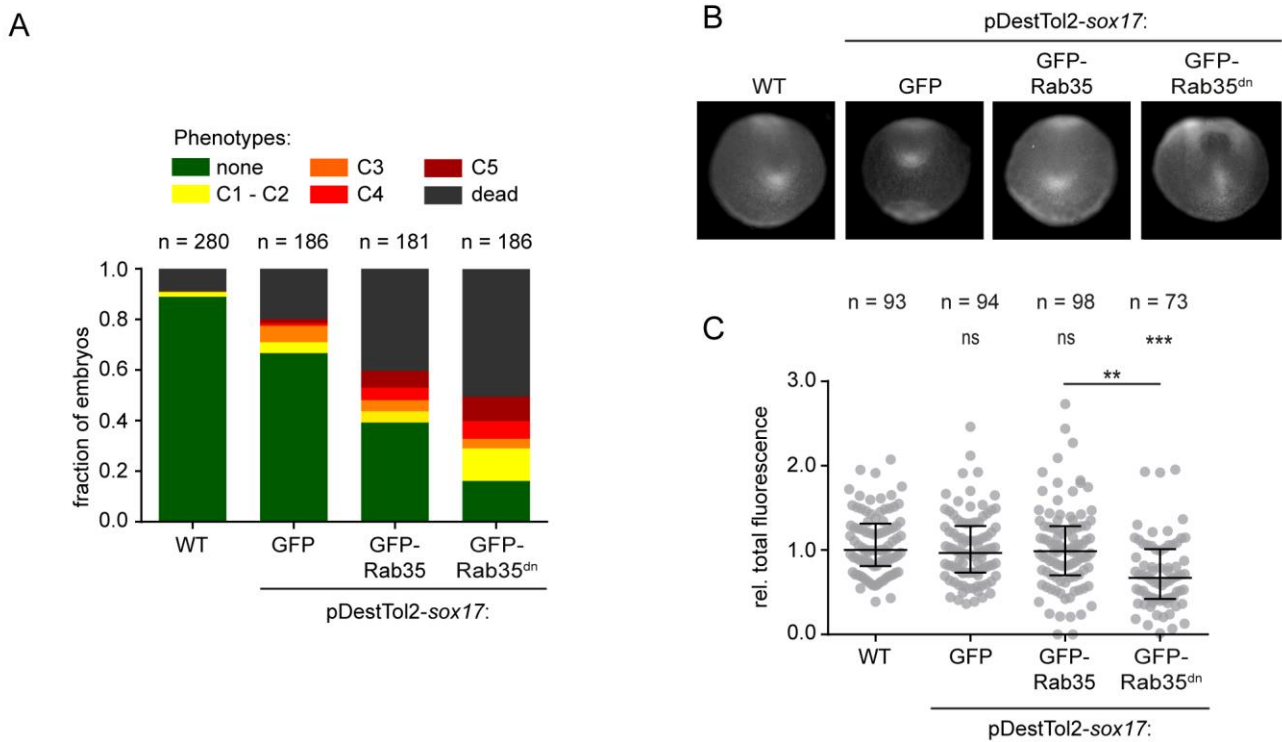
646 isolate. The black line represents the mean of five consecutive measurements. The

647 grey area depicts the standard deviation of these measurements. (C) Representative

648 electron microscopy image of End2-EVs. Red arrows mark exosome-sized EVs. The

649 scale bar represents 100 nm. (D) OptiPrep™-gradient centrifugation was performed

650 on End2-EV isolates. Gradients were separated into 12 fractions and analysed by
651 western blotting. (E) HEK293A-cells were treated with PKH26-labeled End2-EVs for 5 h
652 before live-cell imaging. The scale represents 20 μm . White lines represent cell
653 borders. (F) HEK293A-cells transfected with pGL3-BRE:Luciferase and pIS2-Renilla
654 were treated with End2-EVs for 16 h. BMP-signalling activity was measured using a
655 dual luciferase assay. Values were normalized to an independent untreated control
656 sample. Samples were compared using ANOVA with Tukey's post hoc test. (G) EVs
657 were isolated from End2-cells transfected with a plasmid encoding Venus-BMP.
658 Western blotting was used to show the presence of Venus-BMP in EV-isolates.
659 HEK293A-cells treated with PKH26-labeled End2^{Venus BMP}-EVs for 5 h. The scale bar
660 represents 20 μm . The white lines represent cell borders. Merged image shows co-
661 localization of Venus-BMP and PKH26, indicating association of BMP with EVs.
662



663

664 **Figure 5 | EV-secretion from the endoderm is needed for BMP-signalling during early**

665 **development.** Zebrafish embryos were injected with a pDestTol2 vector encoding

666 either GFP, GFP-Rab35 or the dominant negative GFP-Rab35^{N120I} (dn) under the

667 transcriptional control of the *sox17*-promoter. (A) Phenotypes were assessed at

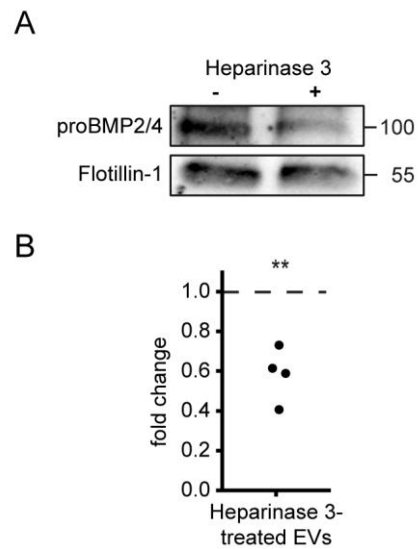
668 24 hpf. (B) Whole mount immunofluorescence stainings were used to label

669 pSmad1/5/9. Embryos were imaged in the posterior view. (C) The pSmad1/5/9

670 labelling intensity in the tail bud was quantified. The distributions were compared

671 using the Kruskal-Wallis-test in combination with Dunn's post hoc test.

672



673

674 **Figure 6 | BMP2/4 is tethered to EV-surfaces by binding to HSPGs.** End2-EV isolates

675 were split into two equal fractions and either treated with Heparinase 3 or with

676 Heparinase 3 reaction buffer. (A) Representative immunoblot of Heparinase 3-treated

677 and control EV-samples. (B) proBMP2/4-band intensities were normalized with the

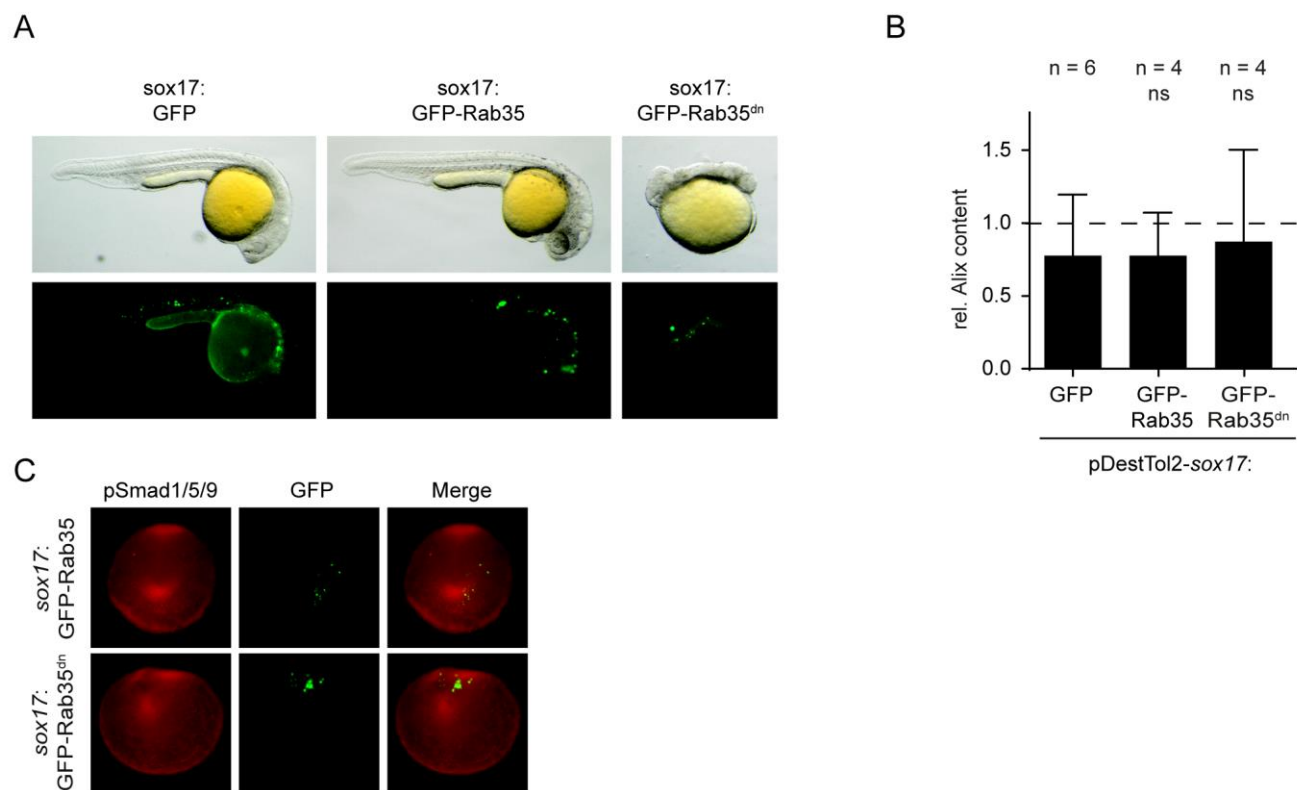
678 respective Flotillin-1-band intensities. The Heparinase 3-treated sample was further

679 normalized to the control and a one-sample t-test was used to determine whether the

680 amount of proBMP2/4 in EVs was significantly reduced after Heparinase 3-treatment.

681

682 Supplementary Information



683

684 **Figure S1 | Mosaic expression of dominant negative Rab35 in the endoderm does**

685 **not result in a measurable reduction in secreted EVs.** (A) Images of zebrafish embryos

686 at 24 hpf expressing either pDestTol2-sox17:GFP, pDestTol2-sox17:GFP-Rab35,

687 pDestTol2-sox17:GFP-Rab35^{dn}. (B) The amount of secreted EVs in transgene-

688 expressing zebrafish was measured by determining the ALIX-content in EV-isolates of

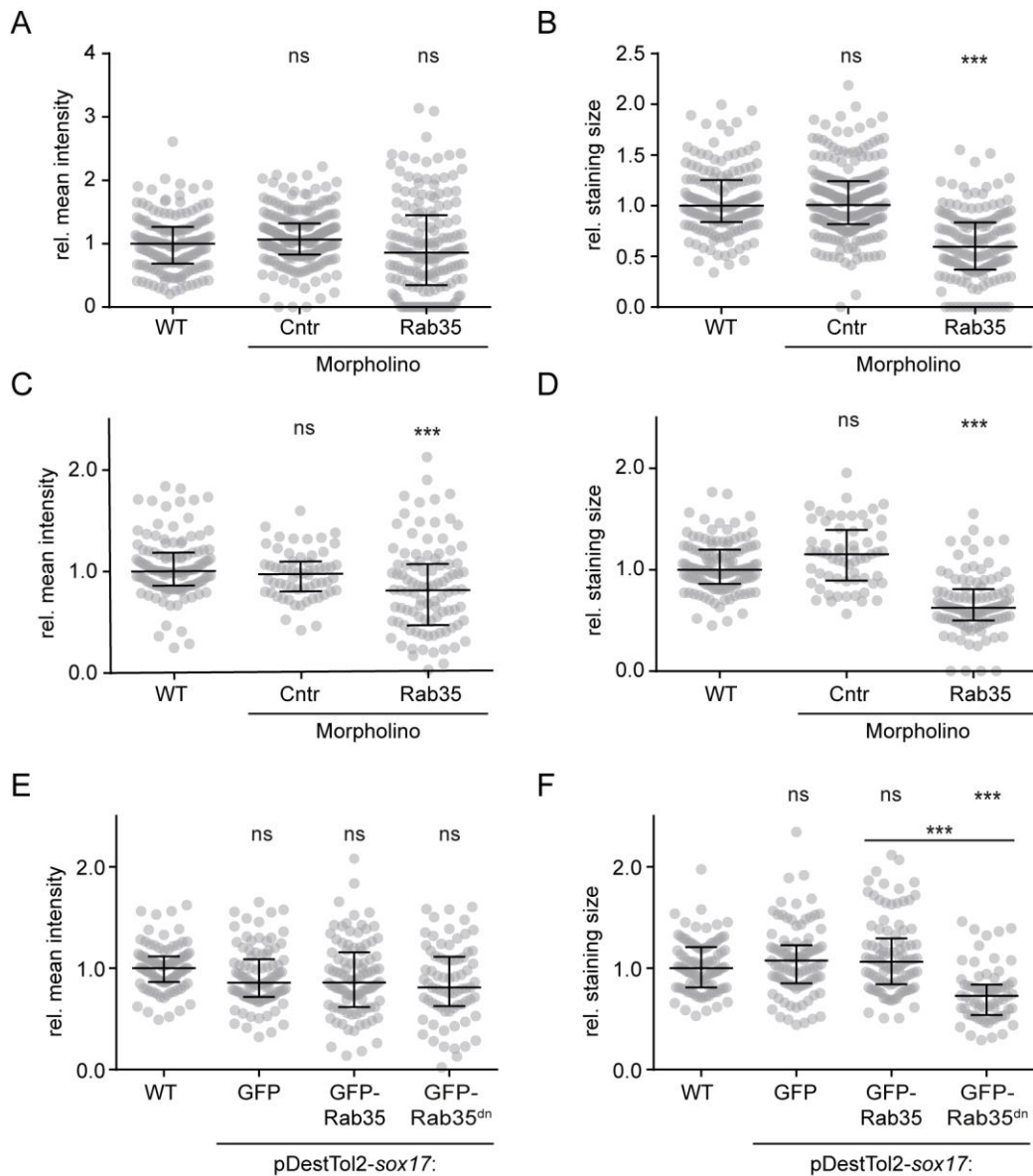
689 10 hpf-zebrafish embryos using western blotting. The values were normalized to the

690 wildtype-control and the means were compared using ANOVA and Tukey's post hoc

691 test. (C) Representative images showing the merge of the GFP-fluorescence

692 representing the transgene and pSmad1/5/9-immuno fluorescence staining.

693



694

695 **Figure S2 | The staining area is the predominant factor determining the reduced *in***

696 ***in vivo* BMP-signalling response after Inhibition of EV-secretion.** Scatter-whisker-charts

697 plotting the parameters used to calculate the *nkx2.5*-expression (A, B; Figure 3B) and

698 total fluorescence in the tail buds of pSmad1/5/9 immunofluorescence stainings (C,

699 D; Figure 3D) in Rab35-morphants as well as the total fluorescence in pSmad1/5/9-

700 immunofluorescence stainings of zebrafish embryos expressing GFP-Rab35^{dn} in the

701 endoderm (E, F; Figure 5D). (A, C, E) The background-corrected mean intensity
702 measured in the labelled regions of interest. (B, D, F) The area of the regions of interest
703 normalized with the area of the embryo. Sample distributions were compared using
704 the Kruskal-Wallis-test with Dunn's post hoc test.