Supplementary Figure S1. Morpholino-mediated knockdown of zebrafish Emilin3 transcripts. (**A**) RT-PCR analysis of RNA extracted from 24 hpf non-injected embryos (WT) and embryos injected with the indicated morpholinos. β-actin was used as a loading control. (**B**) Whole mount immunofluorescence labeling for Emilin3 in 24 hpf embryos injected with 4 ng control morpholino (Ctrl MO), 2 ng/each of *emilin3a* and *emilin3b* splice-blocking morpholinos (e3a+e3b MO splice), or 2 ng/each of *emilin3a* and *emilin3b* translation-blocking morpholinos (e3a+e3b MO ATG). Scale bar, 50 μm. (**C**) Lateral views of 24 hpf embryos injected with 4 ng control morpholino (Ctrl MO) or 2 ng/each of *emilin3a* and *emilin3b* translation-blocking morpholinos (e3a+e3b MO ATG). Arrows point at notochord distortions in Emilin3 double morphants. (**D**) Lateral views of 24 hpf embryos injected with 10 ng control morpholino (Ctrl MO), 2 ng/each of *emilin3a* and *emilin3b* splice-blocking morpholinos (e3a+e3b MO), 6 ng p53 translation-blocking morpholino (p53 MO) and the combination of p53, *emilin3a* and *emilin3b* morpholinos (e3a+e3b+p53 MO). no, notochord.

Supplementary Figure S2. Quantification of the mean number of notochord cells. (**A**) Notochord cell nuclei of the same trunk region from 24 hpf control and Emilin3 double morphant embryos were stained with Hoechst. Scale bar, 50 μm. (**B**) Nuclei, stained as in (A), were counted and reported as mean number of nuclei/somite (not significant; n=15). Ctrl MO, control morpholino; e3a+e3b MO, *emilin3a* + *emilin3b* morpholinos.

Supplementary Figure S3. Notch activity is not grossly affected in the notochord of Emilin3 depleted embryos. (**A**) Notch-responsive reporter zebrafish (Tg_Hbb:EGFP) were injected with the indicated morpholinos and the fluorescence analyzed at 24 hpf. Scale bar, 50 μm. (**B**) Quantification of the mean number of GFP-positive notochord cells in the trunk region (250 μm in length) (not significant; n=10). Ctrl MO, control morpholino; e3a+e3b MO, *emilin3a* + *emilin3b* morpholinos.

Supplementary Figure S4. Analysis of collagen II and laminin expression in Emilin3 morphant embryos. (**A**, **B**) Zebrafish embryos were injected with the indicated morpholinos and probed for *col2a1* expression at 24 and 48 hpf, respectively. The fraction of embryos displaying the corresponding phenotype is provided in each panel. Note that at 24 hpf, even embryos injected with *emilin3a* or *emilin3b* morpholino alone displayed a slight increase in *col2a1* expression. (**C**) Lateral views of controls and Emilin3 morphant embryos labeled by immunohistochemistry for laminin at 24 hpf. The panels display projection of confocal sections around somite 10, showing the notochord. Scale bar, 25 μm. Ctrl MO, control morpholino; e3a MO, *emilin3a* morpholino; e3b MO, *emilin3b* morpholino; e3a+e3b MO, *emilin3a* + *emilin3b* morpholinos.

Supplementary Figure S5. Hedgehog upregulation in Emilin3 morphant embryos is dependent on Emilin3 protein depletion. (**A, B**) Embryos were injected with the indicated splice- or translation-blocking morpholinos (ATG) and probed for *ptc1* expression at 24 hpf. Lateral view of the trunk, head is on the left. (**C**) Control and Emilin3 double morphant embryos were probed for *ptc1* expression at 8-somite stage. (**D**) Dorsal view of wild-type embryos immunostained for Emilin3 and β-catenin at at 8-somite stage. Note the absence of Emilin3 in the notochord (marked by the asterisks) at this stage of development. Scale bar, 100 μm. The fraction of embryos displaying the corresponding phenotype is provided in each panel. Ctrl MO, control morpholino; e3a+e3b MO, *emilin3a* + *emilin3b* morpholinos; p53 MO, p53 morpholino.

Supplementary Figure S6. Upregulation of Hedgehog target genes in Emilin3 morphant embryos. (**A**) Lateral views of 24 hpf embryos injected with the indicated morpholinos and probed for *olig2*, *vegfa* and *eng2a* expression. (**B**) Expression of *fgf8* is similar between control and Emilin3 double morphant embryos, thus excluding a generalized developmental delay. Head is on the left. The fraction of embryos displaying the corresponding phenotype is provided in each panel. Ctrl MO, control morpholino; e3a+e3b MO, *emilin3a* + *emilin3b* morpholinos.

Supplementary Figure S7. Hedgehog upregulation in Emilin3 morphant embryos is not dependent on BMP or TGF-β signaling. **(A**) Lateral views of 24 hpf embryos injected with the indicated morpholinos and treated at 8 hpf with 5 μM cyclopamine (lower panels) or with the corresponding volume of ethanol as a control (upper panels). Embryos were then stained with the monoclonal 4D9 antibody (green) and the polyclonal pSMAD1/5/8 antibody (purple). The panels show magnifications around somite 10. Scale bar, 50 μm. (**B**) Wild-type embryos were injected with the indicated morpholinos. Embryos were then left untreated or treated at 8 hpf with 30 μM of the selective TGF-β receptor inhibitor LY364947 from 8 hpf and probed for *ptc1* expression at 24 hpf. The fraction of embryos displaying the corresponding phenotype is provided in each panel. Ctrl MO, control morpholino; e3a+e3b MO, *emilin3a* + *emilin3b* morpholinos.

Supplementary Figure S8. Injection of Emilin3 cDNA rescues the phenotype of Emilin3 morphants. (A) Lateral views of 24 hpf embryos co-injected with the indicated morpholinos (4 ng/embryo) and with the indicated cDNA constructs (25 ng/μl). Arrows point at notochord distortions in Emilin3 double morphants, which are largely rescued by co-injection with the pCS-twhh-mEmilin3 construct. The fraction of embryos displaying the corresponding phenotype is provided in each panel. (**B**) Whole mount immunofluorescence labeling for engrailed and Emilin3 in 24 hpf embryos injected as in (A). Scale bar, 25 μm. (**C**) Quantification of engrailed-positive cells in 24 hpf co-injected embryos (*, $P < 0.05$; n.s., not significant; $n = 10$). Ctrl MO, control morpholino; e3a+e3b MO, *emilin3a* + *emilin3b* morpholinos; Trunk MPs, trunk muscle pioneers; Trunk MFFs, trunk medial fast fibers.

Supplementary Figure S9. **Conditioned medium from HEK293T cells can stimulate Shh release**. 293-Shh cells were incubated for 6 hr with fresh medium (DMEM) or with conditioned media derived from HEK293T transfected with the indicated plasmids. The release rate of Shh was assessed by immunoblot of media and cell lysates. CM, conditioned media; E3, murine full-length Emilin3; ev, empty vector; WB, western blot.

Supplementary Figure S10. Emilin3 and Scube2 functionally interact. (**A**) Cell lysates of HEK293T transfected with the indicated plasmids were either analyzed directly by western blot or subjected to immunoprecipitation with an anti-HA affinity gel followed by western blot for Scube2 (anti-FLAG). (**B**) *In vitro* binding between Emilin3 and the EGF fragment of Scube2. HEK293T were transiently co-transfected with Emilin3 or Scube2-EGF constructs. Media were then harvested, and subjected to western blot and immunoprecipitation with the indicated antibodies. (**C**) HEK293T were co-transfected with the Shh plasmid and the indicated constructs and left untreated or treated overnight with 50 μg/ml of soluble heparin in serum free medium. Cell lysates and conditioned media were then analyzed by immunoblotting. (**D**,**E**) HEK293T were co-transfected with the Shh plasmid and the indicated constructs (D) or transfected and then treated with increasing concentration of Scube2-conditioned medium (E). Shh release was then studied by western blot. E3, murine full-length Emilin3 cDNA; E3-HA, murine full-length HA-tagged Emilin3 cDNA; ev, empty vector; IP, immunoprecipitation; Scube2-FLAG, murine full-length FLAG-tagged Scube2 cDNA; WB, western blot.

Supplementary Figure S11. Morpholino-mediated knockdown of zebrafish Emilin3 and Scube2. (**A**) Lateral views of 24 hpf embryos injected with 6 ng of control morpholino (Ctrl MO) or 2 ng/each of the indicated morpholinos. (**B**) qRT-PCR for *scube2* transcript in control and Emilin3 double morphant embryos (not significant; $n = 30$). Ctrl MO, control morpholino; e3a+e3b MO, *emilin3a* + *emilin3b* morpholinos; Scube2 MO, Scube2 morpholino.

Supplementary Table S1. Summary of the phenotypic defects detected after injection of morpholino oligonucleotides. Wild-type zebrafish embryos were injected with the indicated morpholinos and examined at 24 hpf. The dose for each morpholino, the total number of injected embryos, and the number and percentage of embryos displaying the indicated phenotypic defects is provided. Embryos were assigned to one of the following three classes based on their phenotype: *a)* normal (no evidence of morphological defect); *b)* notochord defects; *c)* radialized embryos. Ctrl, control morpholino; e3a splice, *emilin3a* splicing-blocking morpholino; e3b splice, *emilin3b* splicing-blocking morpholino; e3a ATG, *emilin3a* translation-blocking morpholino; e3b ATG, *emilin3b* translation-blocking morpholino.

Supplementary Table S2. Sequences of morpholino oligonucleotides used in the study. The table describes the sequences of morpholinos targeting the donor splice site between the first exon and the first intron of *emilin3a* or *emilin3b* (e3a MO splice and e3b MO splice), the splice site between the first exon and the first intron of *col2a1* (col2a1 MO splice; Mangos et al., 2010), the translation initiation codon of *emilin3a* or *emilin3b* (e3a MO ATG and e3b MO ATG), the translational initiation codon of *scube2* (scube2 MO ATG; Woods et al., 2005), the translation initiation codon of p53 (p53 MO ATG; Robu et al., 2007) and a standard control morpholino (Ctrl MO). All sequences were provided by Gene tools, LLC.

Figure S1

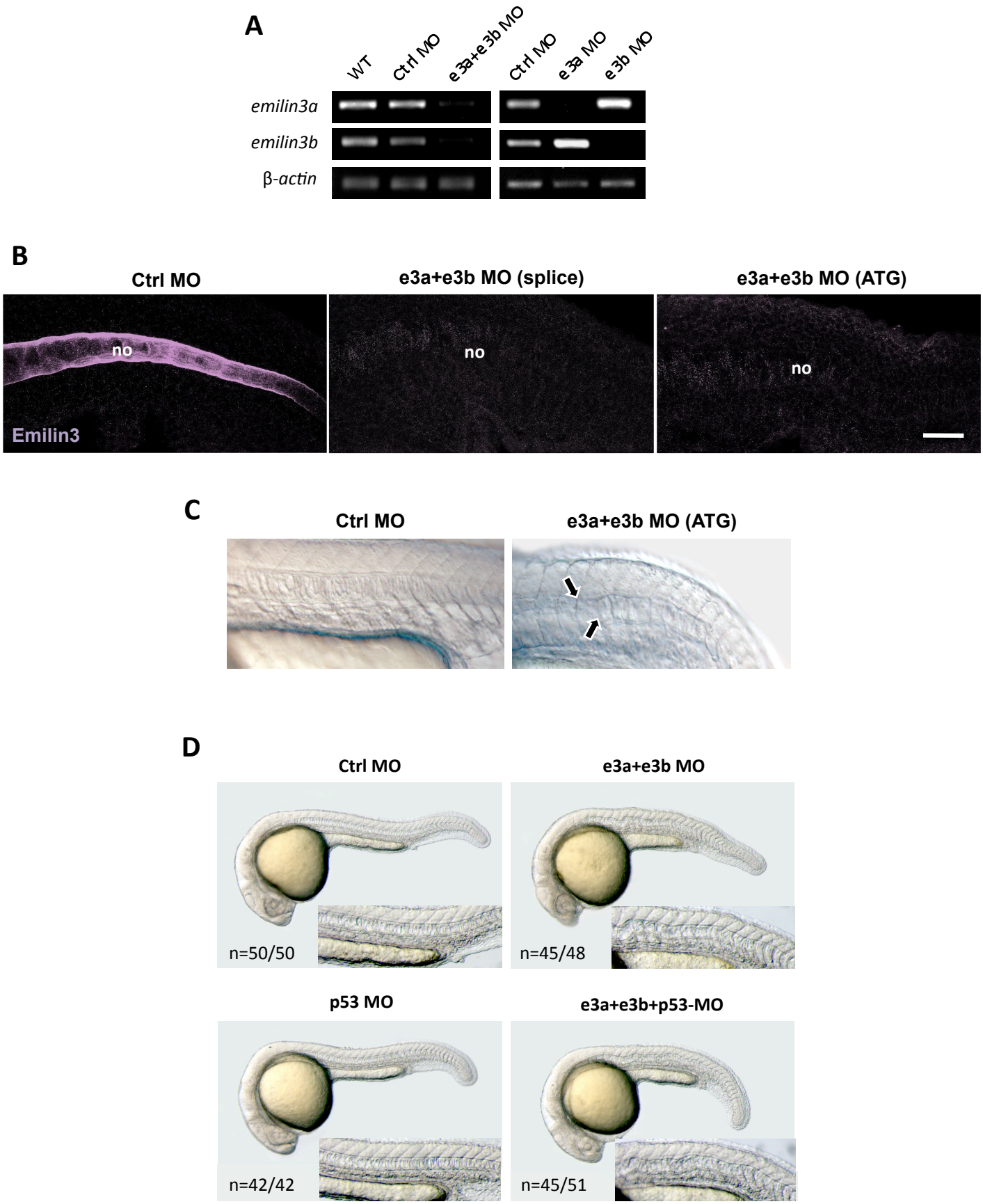

A
WT
Ctrl MO
e3a+e3b MO
Ctrl MO
e3a MO
e3b MO
emilin3a
emilin3b
β-actin
B
Ctrl MO
e3a+e3b MO (splice)
e3a+e3b MO (ATG)
no
no
no
Emilin3
C
Ctrl MO
e3a+e3b MO (ATG)
D
Ctrl MO
e3a+e3b MO
n=50/50
n=45/48
p53 MO
e3a+e3b+p53-MO
n=42/42
n=45/51

Figure S2

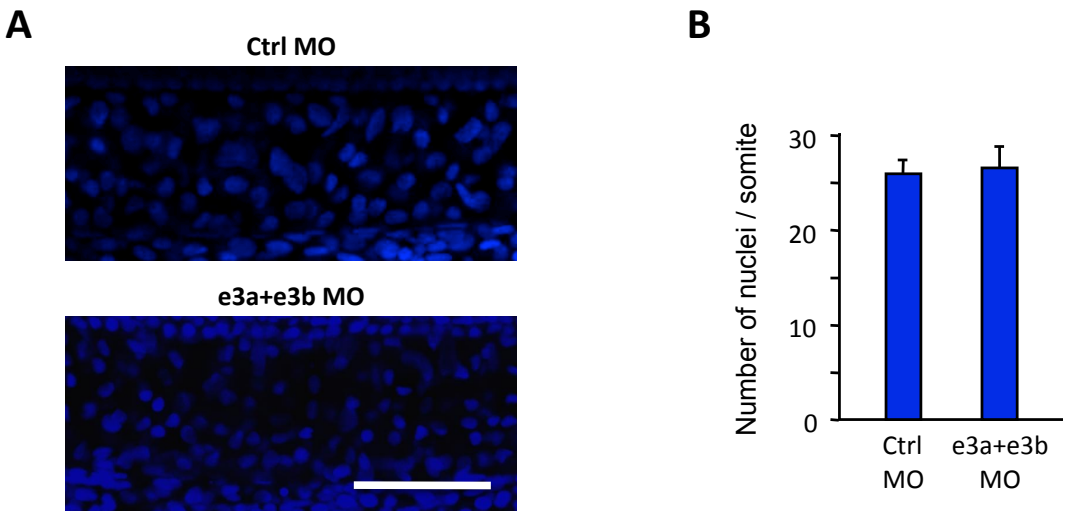

A
Ctrl MO
e3a+e3b MO
B
Number of nuclei / somite
30
20
10
0
Ctrl MO
e3a+e3b MO

Figure S3

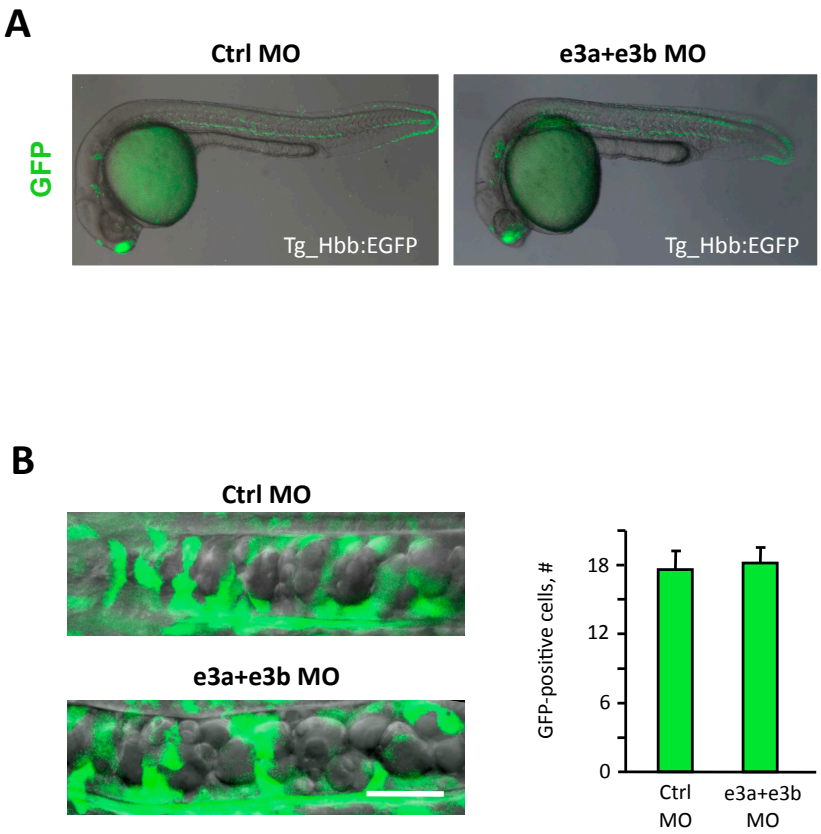
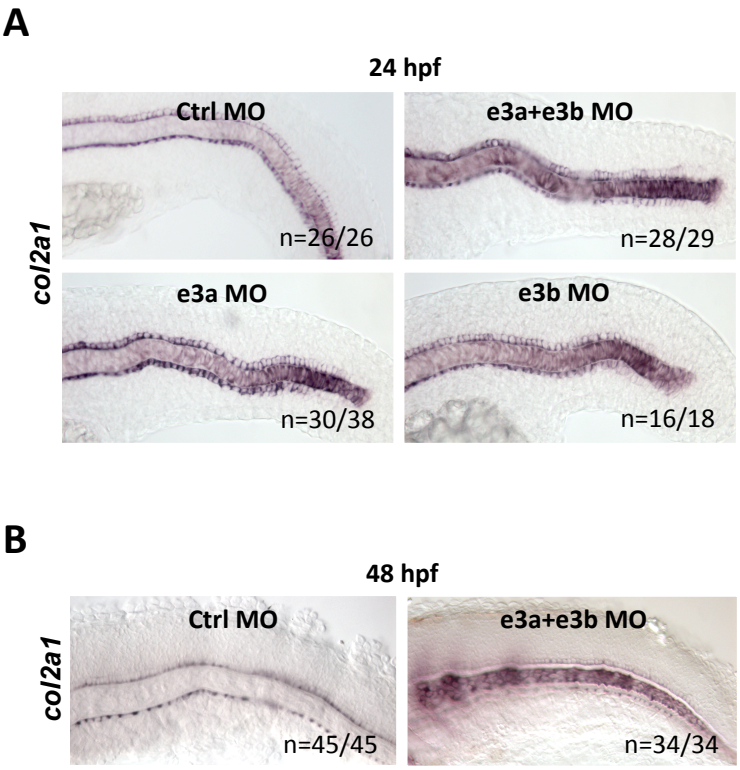

A
Ctrl MO
e3a+e3b MO
GFP
Tg_Hbb:EGFP
Tg_Hbb:EGFP
B
Ctrl MO
e3a+e3b MO
GFP-positive cells, #
18
12
6
0
Ctrl MO
e3a+e3b MO

Figure S4


A
24 hpf
col2a1
Ctrl MO
n=26/26
e3a+e3b MO
n=28/29
e3a MO
n=30/38
e3b MO
n=16/18
B
48 hpf
col2a1
Ctrl MO
n=45/45
e3a+e3b MO
n=34/34

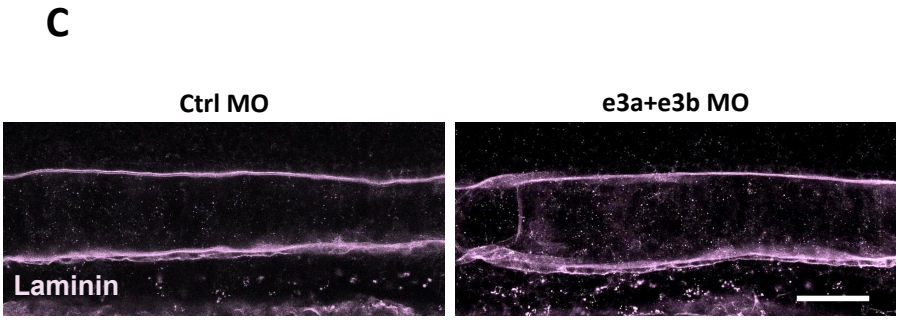

C
Ctrl MO
e3a+e3b MO
Laminin

Figure S5

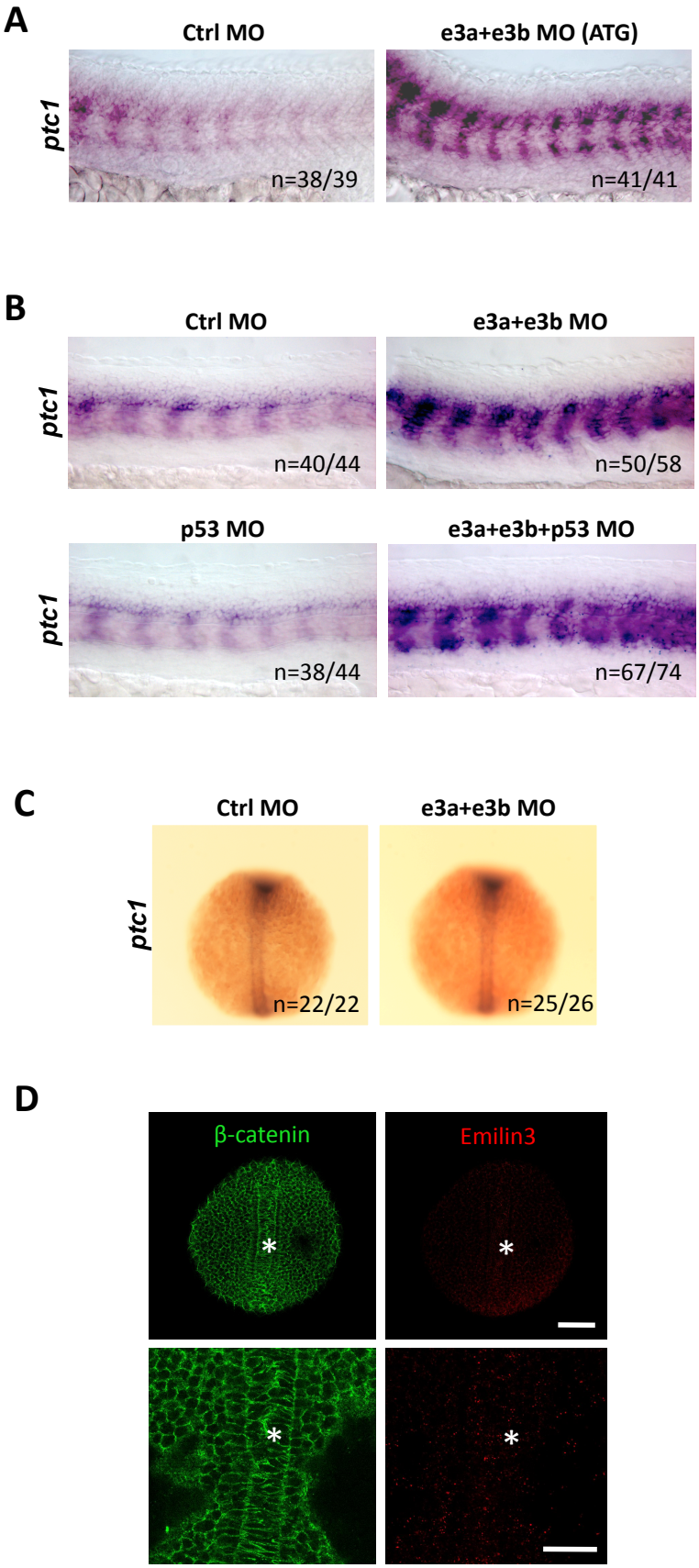

A
Ctrl MO
e3a+e3b MO (ATG)
ptc1
n=38/39
n=41/41
B
Ctrl MO
e3a+e3b MO
ptc1
n=40/44
n=50/58
p53 MO
e3a+e3b+p53 MO
ptc1
n=38/44
n=67/74
C
Ctrl MO
e3a+e3b MO
ptc1
n=22/22
n=25/26
D
β-catenin
Emilin3

Figure S6

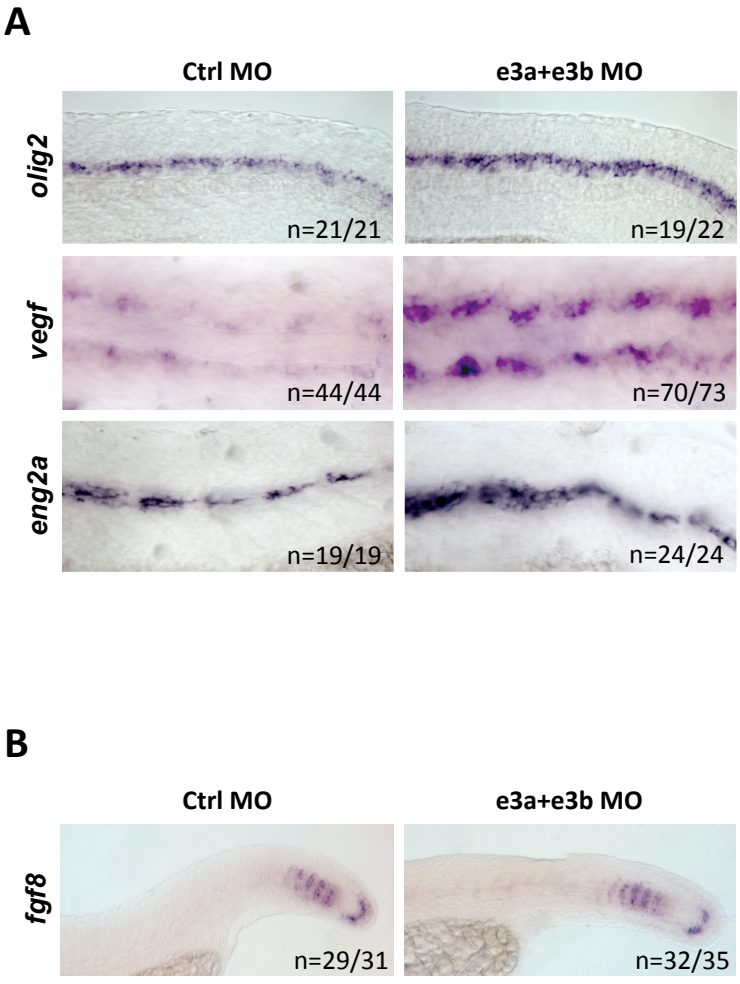

A
Ctrl MO
e3a+e3b MO
olig2
n=21/21
n=19/22
vegf
n=44/44
n=70/73
eng2a
n=19/19
n=24/24
B
Ctrl MO
e3a+e3b MO
fgf8
n=29/31
n=32/35

Figure S7

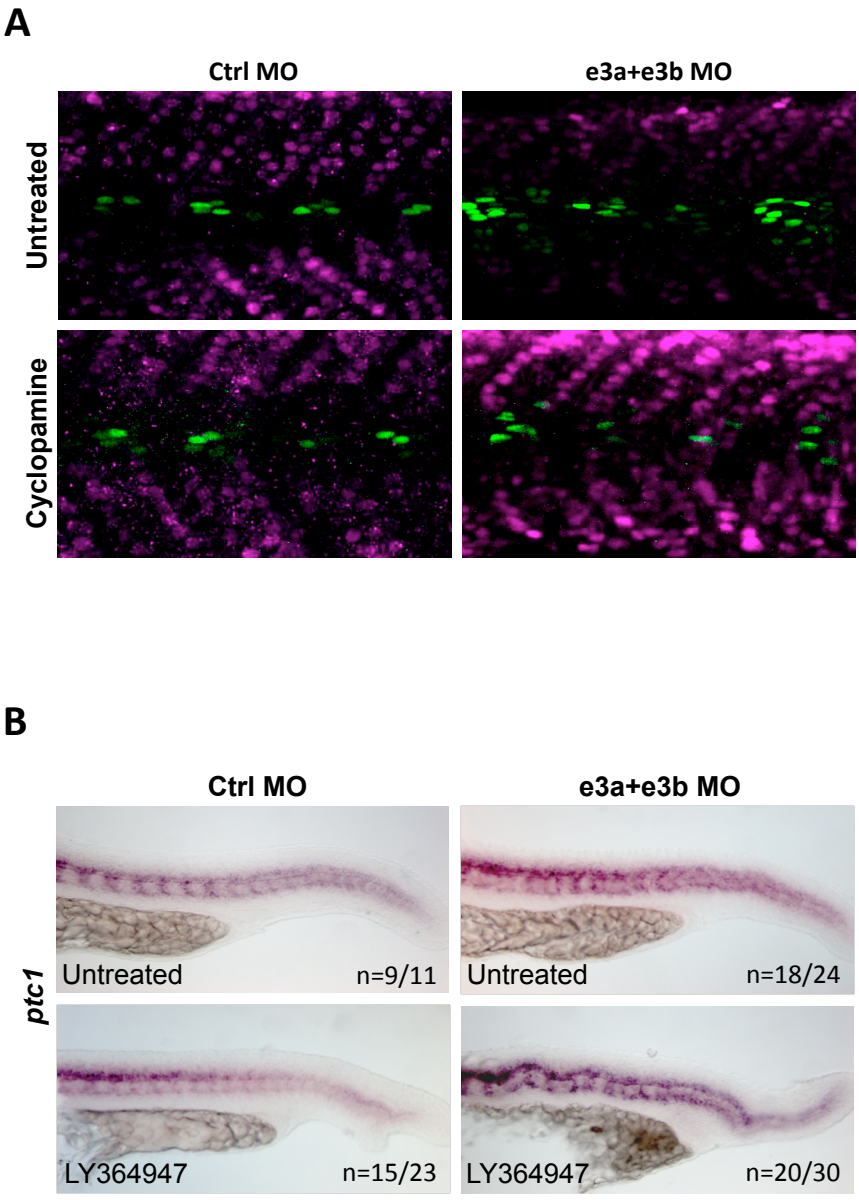

A
Ctrl MO
e3a+e3b MO
Untreated
Cyclopamine
B
Ctrl MO
e3a+e3b MO
ptc1
Untreated
n=9/11
Untreated
n=18/24
LY364947
n=15/23
LY364947
n=20/30

Figure S8

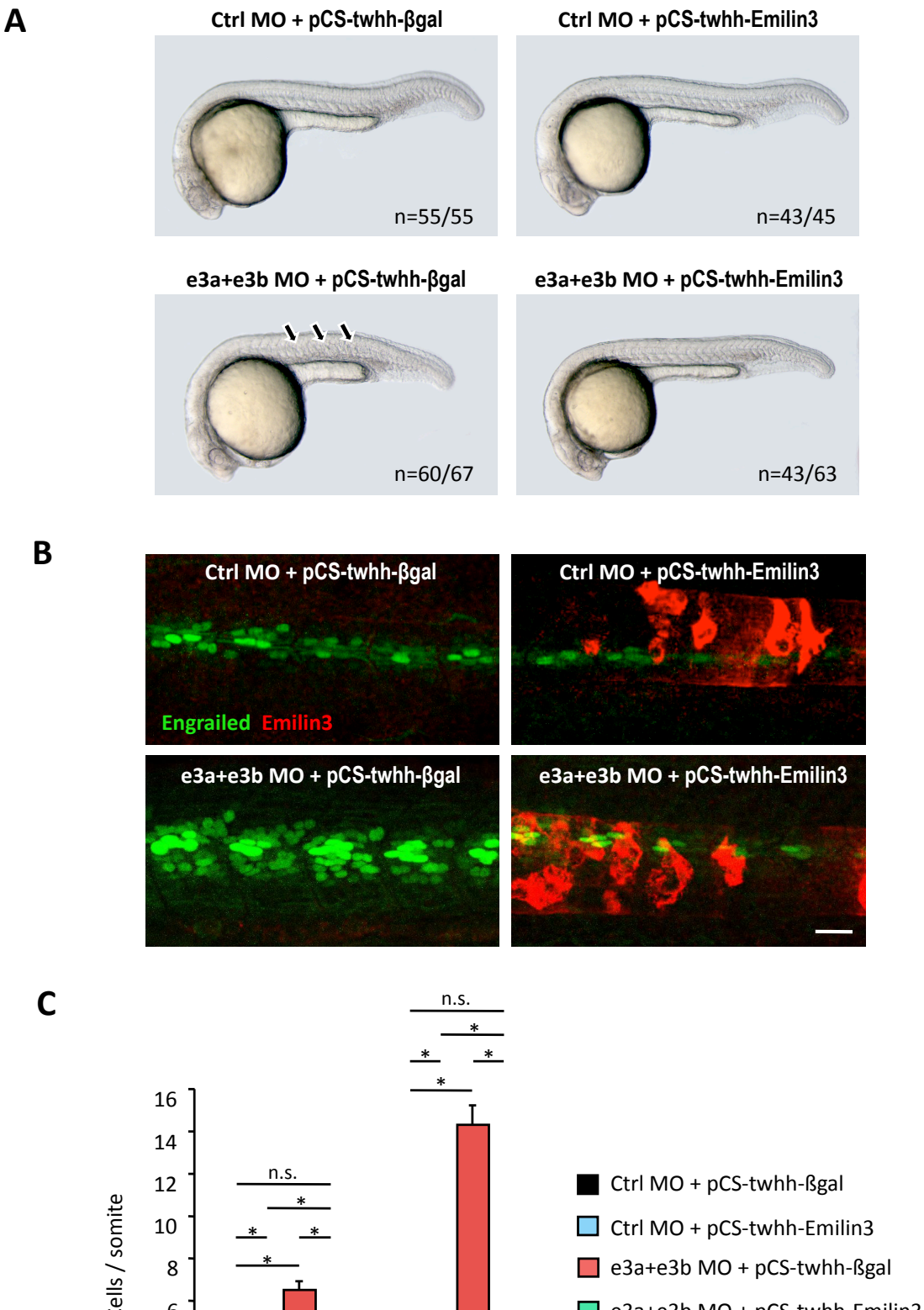

A
Ctrl MO + pCS-twhh-βgal
n=55/55
Ctrl MO + pCS-twhh-Emilin3
n=43/45
e3a+e3b MO + pCS-twhh-βgal
n=60/67
e3a+e3b MO + pCS-twhh-Emilin3
n=43/63
B
Ctrl MO + pCS-twhh-βgal
Engrailed Emilin3
Ctrl MO + pCS-twhh-Emilin3
e3a+e3b MO + pCS-twhh-βgal
e3a+e3b MO + pCS-twhh-Emilin3
C
Cells / somite
16
14
12
10
8
6
4
2
0
n.s.
*
*
*
*
n.s.
*
*
*
*
Trunk MPs
Trunk MFFs
Ctrl MO + pCS-twhh-ßgal
Ctrl MO + pCS-twhh-Emilin3
e3a+e3b MO + pCS-twhh-ßgal
e3a+e3b MO + pCS-twhh-Emilin3

Figure S9

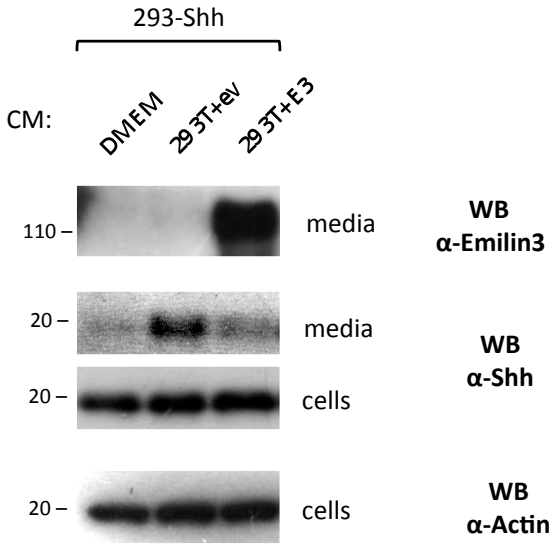

293-Shh
CM:
DMEM
293T+ev
293T+E3
110 –
media
WB
α-Emilin3
20 –
media
20 –
cells
WB
α-Shh
20 –
cells
WB
α-Actin

Figure S10

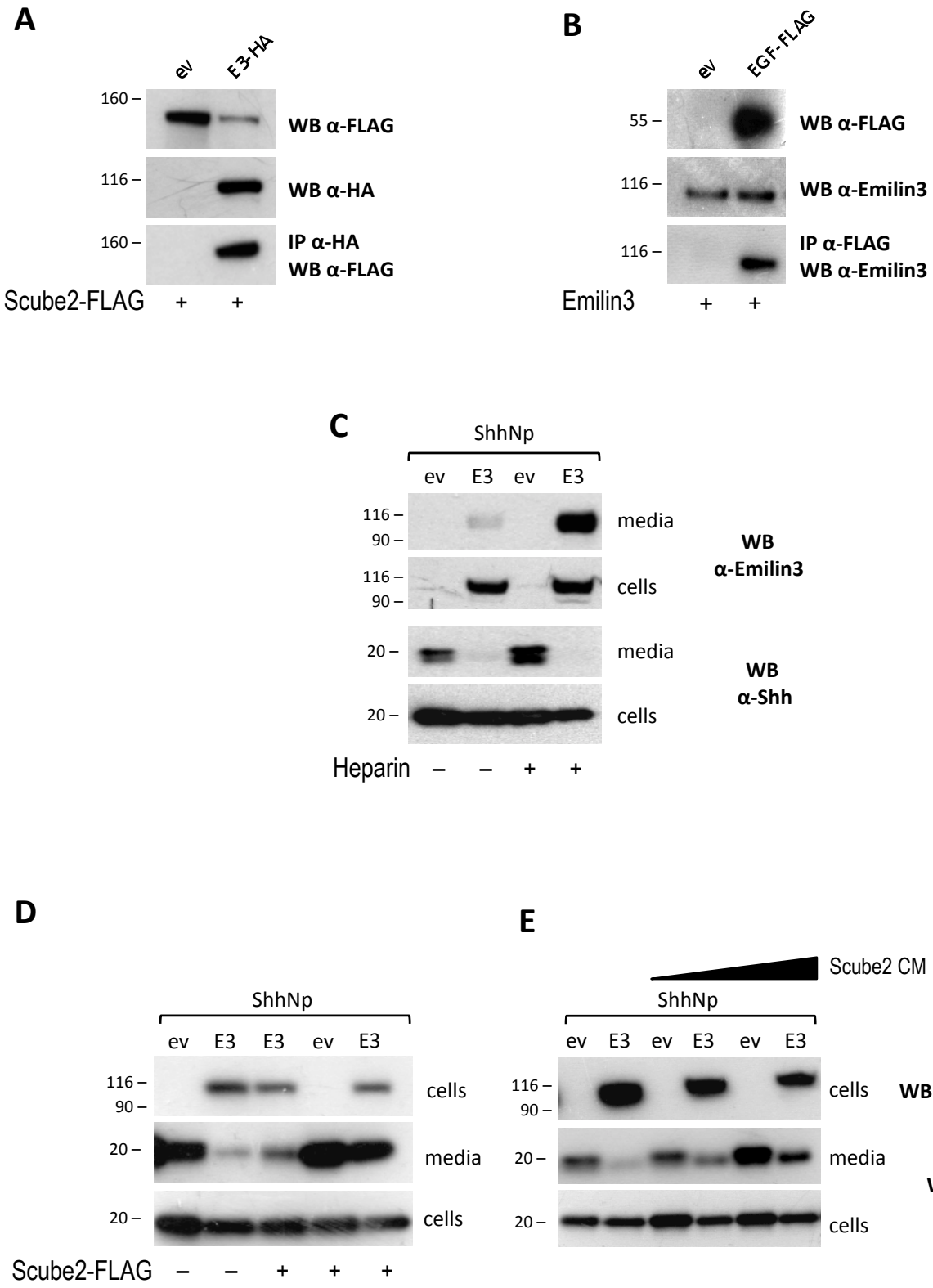

A
ev
E3-HA
160 –
WB α-FLAG
116 –
WB α-HA
160 –
IP α-HA
WB α-FLAG
Scube2-FLAG + +
B
ev
EGF-FLAG
55 –
WB α-FLAG
116 –
WB α-Emilin3
116 –
IP α-FLAG
WB α-Emilin3
Emilin3 + +
C
ShhNp
ev E3 ev E3
116 –
90 –
media
116 –
90 –
cells
WB
α-Emilin3
20 –
media
20 –
cells
WB
α-Shh
Heparin – – + +
D
ShhNp
ev E3 E3 ev E3
116 –
90 –
cells
20 –
media
20 –
cells
Scube2-FLAG – – + + +
E
Scube2 CM
ShhNp
ev E3 ev E3 ev E3
116 –
90 –
cells
WB α-Emilin3
20 –
media
WB α-Shh
20 –
cells

Figure S11

A

B

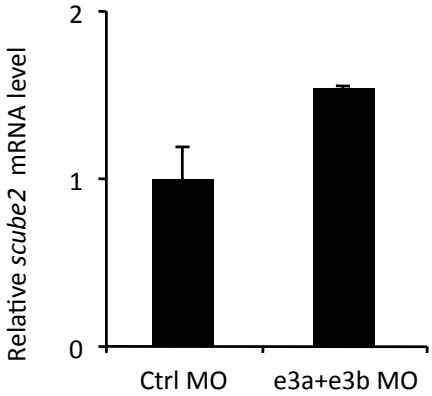


Table S1

Type of morpholino	Dose (ng)	Embryos examined	Phenotype		
			Normal	Notochord defects	Radialized embryos
Ctrl	4	153	151 (99%)	0 (0%)	2 (1%)
e3a (splice)	2	63	61 (97%)	1 (1.5%)	1 (1.5%)
e3a (splice)	4	68	50 (74%)	17 (25%)	1 (1%)
e3b (splice)	2	63	58 (92%)	3 (5%)	2 (3%)
e3b (splice)	4	63	54 (86%)	7 (11%)	2 (3%)
e3a (splice) + e3b (splice)	4 + 4	80	3 (5%)	58 (92%)	2 (3%)
	2 + 2	231	33 (14%)	195 (84%)	3 (2%)
	0.5 + 0.5	55	61 (97%)	1 (1.5%)	1 (1.5%)
e3a (ATG)	2	69	51 (74%)	17 (24%)	1 (2%)
e3b (ATG)	2	51	38 (75%)	13 (25%)	0 (0%)
e3a (ATG) + e3b (ATG)	2 + 2	106	12 (11%)	94 (89%)	0 (0%)
Ctrl	3.5	40	37 (92%)	0 (0%)	3 (8%)
e3a (splice)	2	63	61 (97%)	1 (1.5%)	1 (1.5%)
e3b (ATG)	1.5	42	42 (100%)	0 (0%)	0 (0%)
e3a (splice) + e3b (ATG)	2 + 1.5	62	27 (44%)	33 (53%)	2 (3%)

Table S2

Type of morpholino	Sequence
e3a MO (splice)	5’ –TTACTCATGGATACTTACTTGTGCC– 3’
e3b MO (splice)	5’ –TAGCGTTTACTTACTTATGATGCCC– 3’
col2a1 MO (splice)	5’ –TGAAAAACTCCAACTTACGGTCATC– 3’
e3a MO (ATG)	5’ –TGCAAAATCTTCTCC AGTAGCATGA– 3’
e3b MO (ATG)	5’ –ACGGAAATGCAAGAATCCACCTCAT– 3’
scube2 MO (ATG)	5’ –GCCGTACAGTCCAAACAGCTCCCAT– 3’
p53 MO (ATG)	5’ –GCGCCATTGCTTTGCAAGAATTG– 3’
Ctrl MO	5’ –CCTCTTACCCTAGTTACAATTTATA– 3’