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Supplemental Information

3D Reconstitution of the Patterned Neural Tube from Embryonic Stem Cells

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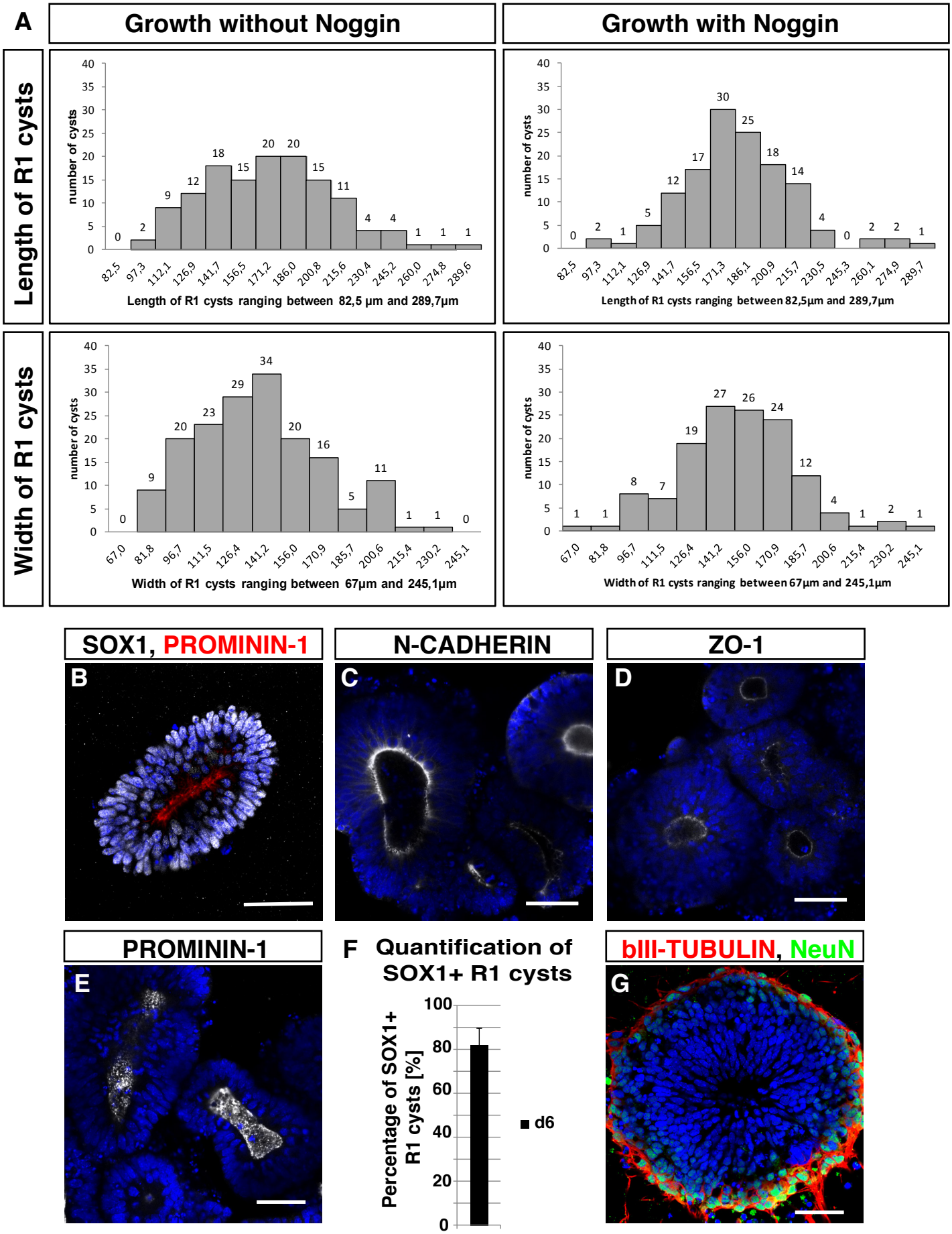


Meinhardt *et al.*, Figure S1

Figure S1: Characterization of R1 neural cysts (Related to Figure 1)

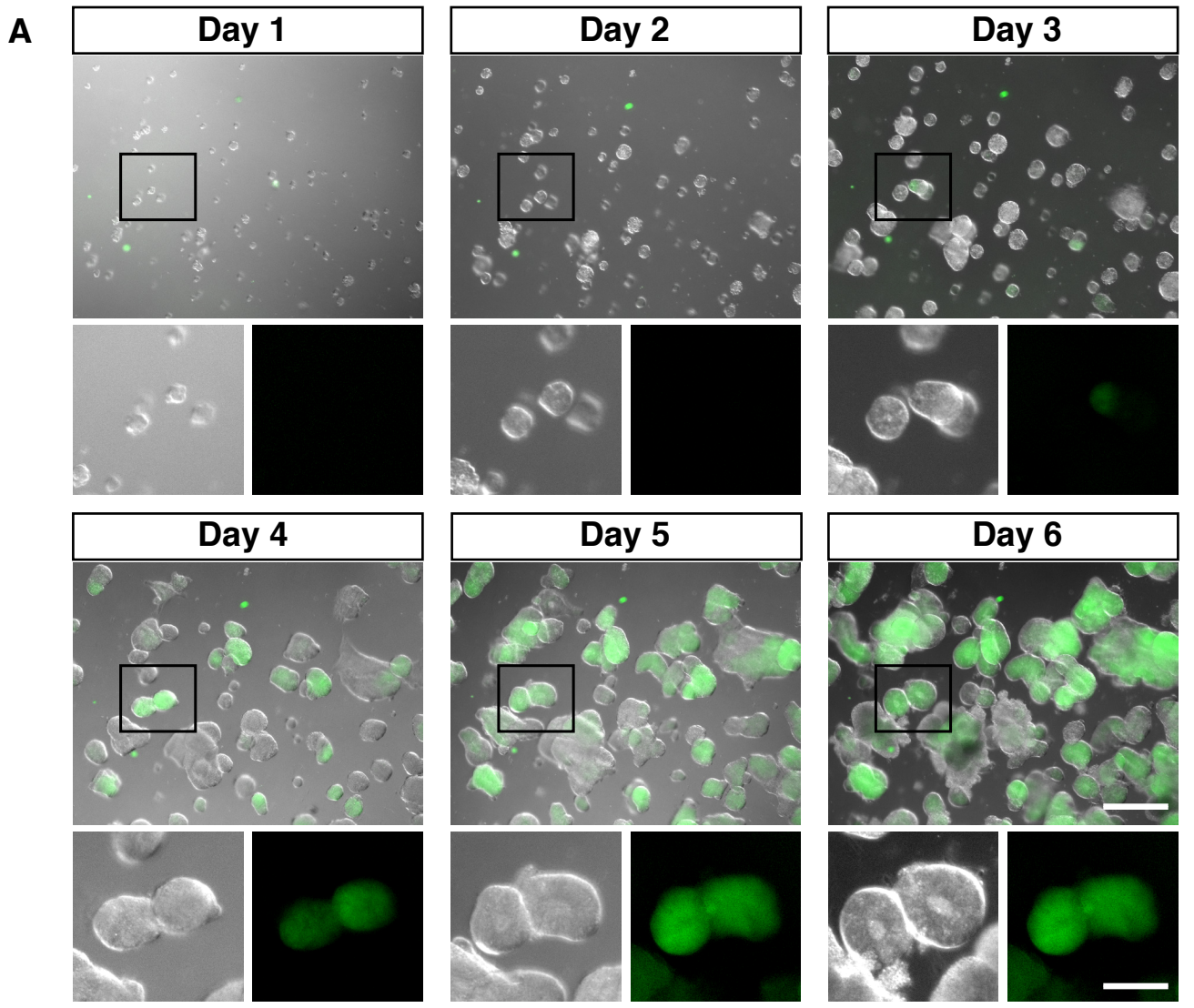
R1 ESCs were applied to the same culture protocol as 46C ESCs.

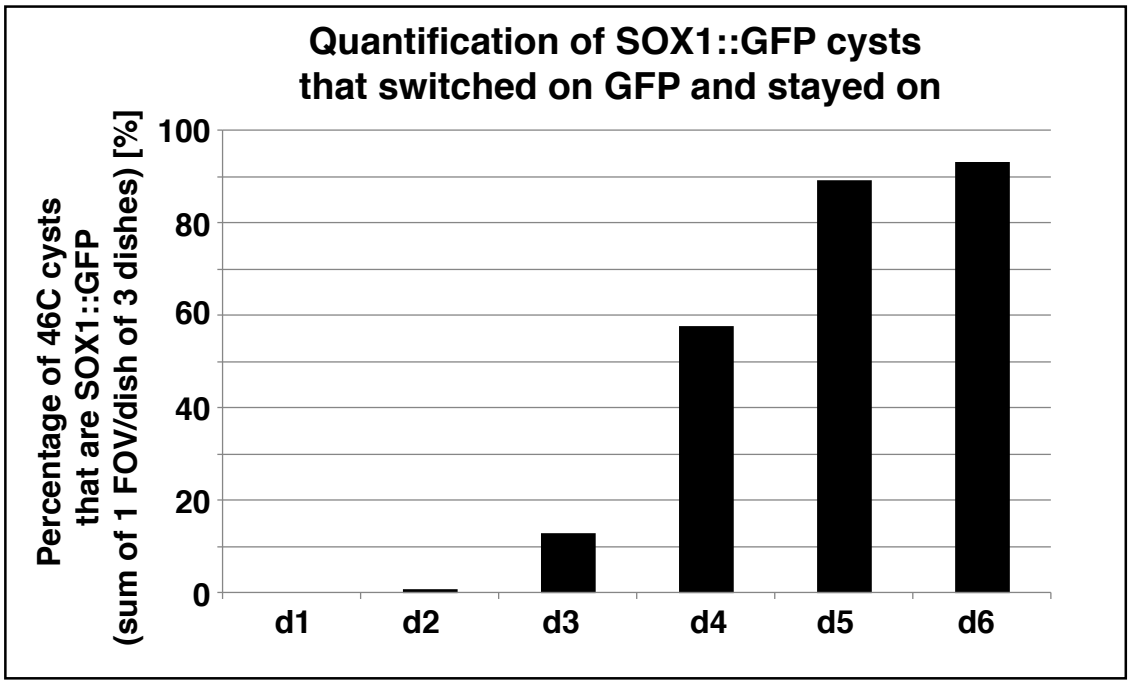
(A) Histograms of the distribution of the width and length of control R1 cysts on day 6 show that cysts grew to similar but not identical sizes in the presence or absence of Noggin.

Neural cysts formed and expressed the same apical-basal and neural stem cell markers as neural cysts derived from 46C. Neuroepithelial cysts from R1 ESCs express the apical markers PROMININ-1 (B, E) and ZO-1 (D). To show that they are neural we stained for SOX1 (B) and N-CADHERIN (C).

(F) Quantification of $SOX1^+$ R1 cysts at day 6 of differentiation based on SOX1 immunofluorescence showing that a similar percentage of R1 cysts was $SOX1^+$ compared to 46C derived cysts. Data are represented as mean ± SD (n=3 independent experiments with 100 cysts counted per experiment).

(G) Around d7-d8 postmitotic neurons grew out on the basal side as evidenced by immunofluorescence stainings for NeuN and bIII-TUBULIN. All nuclei were counterstained with Hoechst. Scale 50 µm (B-E, G).



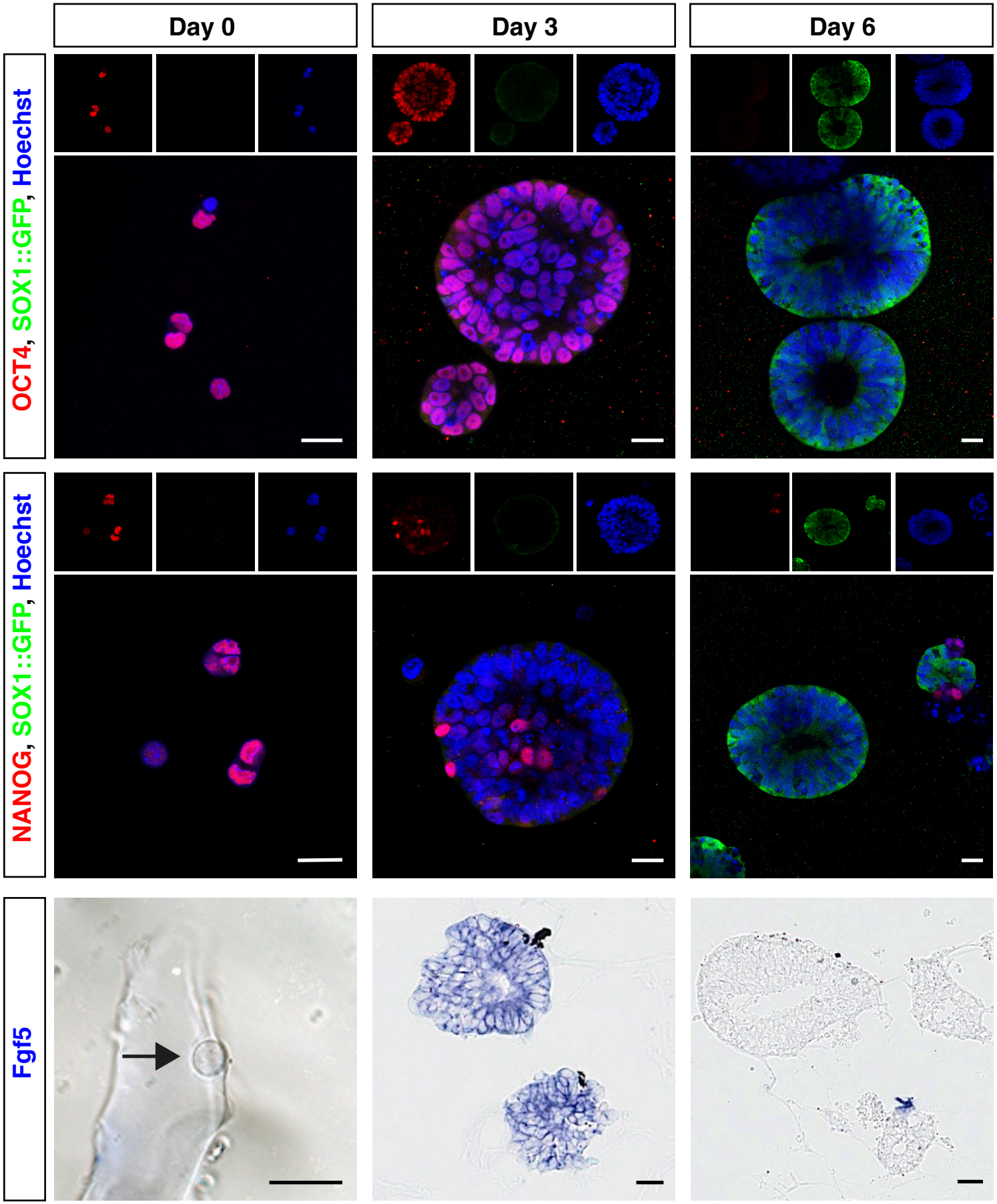


Meinhardt *et al.*, Figure S2

Figure S2: Tracing of cyst growth over 6 days (Related to Figure 2)

(A) The same field of growing 46C cysts was followed on a daily basis by DIC and immunofluorescence analysis over a time course of 6 days showing that a stable number of cysts differentiated over time without significant cell death in the culture. Scale 200 µm.

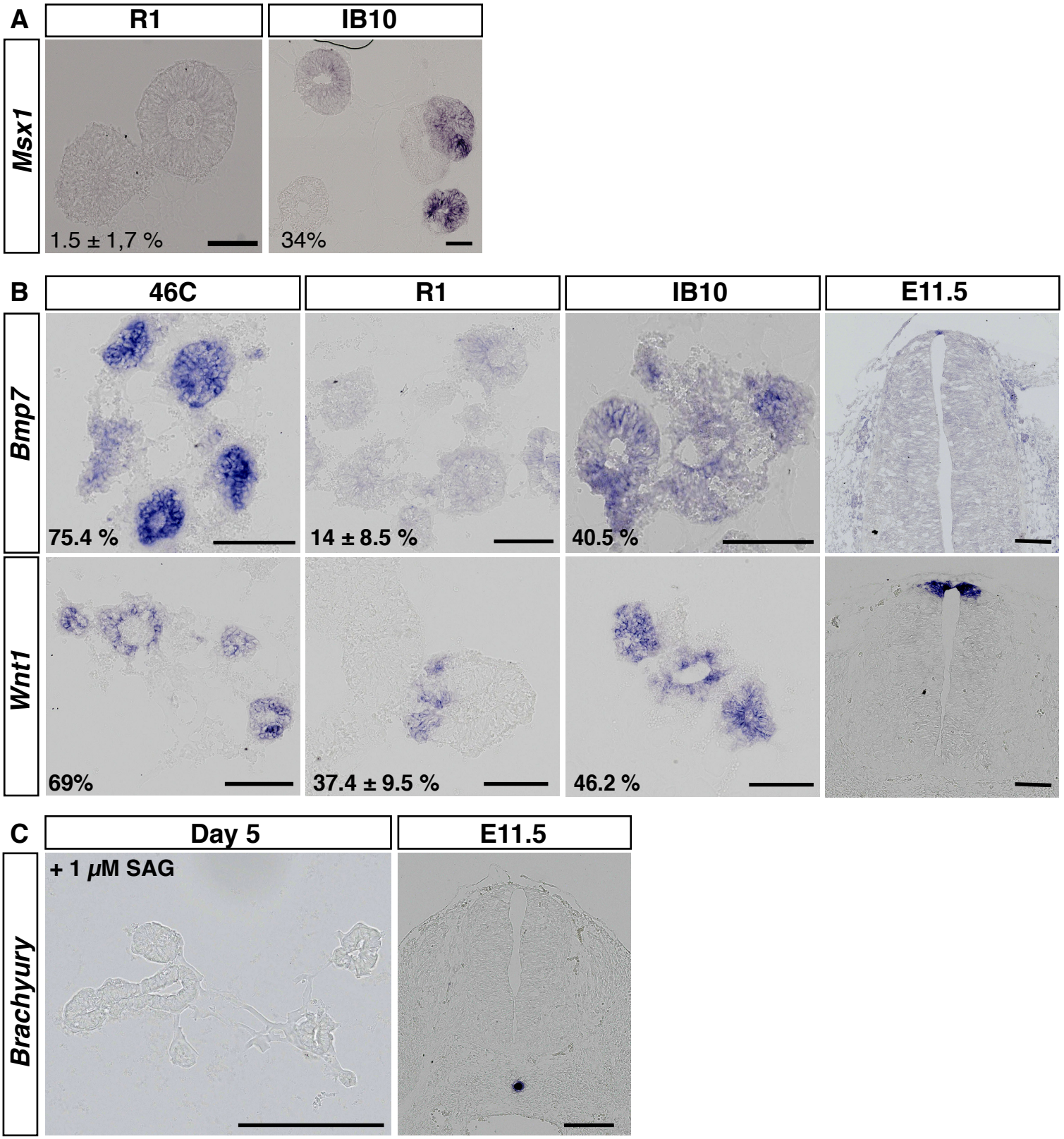
(B) Determination of the onset of SOX1::GFP expression showing that on day 3 SOX1::GFP expression started. On day 6 approximately 93 % of the cysts are SOX1::GFP^{+}.



Meinhardt *et al.*, Figure S3

Figure S3: Progressive expression of developmental markers on a cellular level (Related to Figure 2)

On day 0, 3 and 6 cultures were fixed and analyzed by immunofluorescence for OCT4, NANOG and GFP to look for the progression of the pluripotency markers and the induction of the earliest neuroepithelial marker SOX1. On day 0 almost all cells expressed OCT4 and NANOG. By day 3 almost all structures analyzed were still $OCT4^+$ and not yet $SOX1^+$. NANOG expression already started to decline on day 3 and was almost completely absent on day 6 when most of the structures analyzed were $SOX1^+$. Nuclei were counterstained with Hoechst. By *in situ* hybridization 20 µm thick cyrosections were analyzed for *Fgf5* expression. Only on day 3 uniformly $Fgf5^+$ structures were detected. Scale 20 µm.



Meinhardt *et al.*, Figure S4

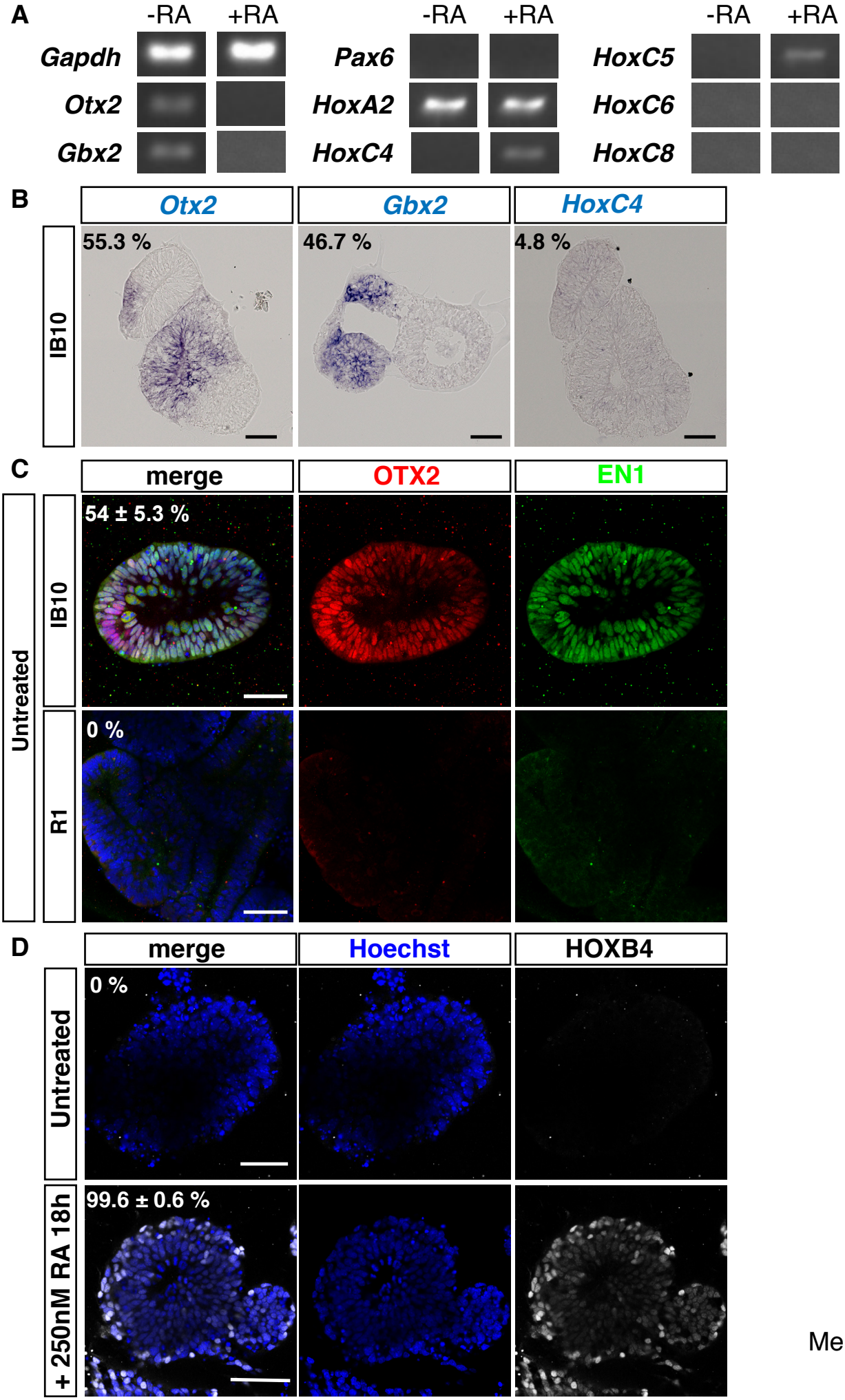
Figure S4: Expression of molecular markers in neuroepithelial cyst cultures (Related to Figure 3)

(A) Characterization of the DV identity of R1 and IB10 neuroepithelial cysts by *in situ* hybridization using an *Msx1* probe showing that R1 cysts are *Msx1* negative and on average 34% of IB10 cysts express *Msx1*.

(B) Untreated control cysts derived from 46C, R1 and IB10 were analyzed on day 7 for their expression of *Wnt1* and *Bmp7* by *in situ* hybridization showing expression with significant differences among the 3 cell lines. As corresponding positive control we used a cross section of a murine E11.5 spinal cord that shows labeling only in roof plate.

(C) Characterization of SAG-induced floor plate (FP) formation. To exclude that FP induction occurred via an intermediate mesodermal differentiation of the cells we performed *in situ* hybridization for *Brachyury* on day 5 using the cell line 46C. As corresponding positive control we used a cross section of a murine E11.5 spinal cord that shows labeling only in notochord.

Scale (A) 50µm; (B) 100 µm; (C) 200 µm.



Meinhardt *et al.*,
Figure S5

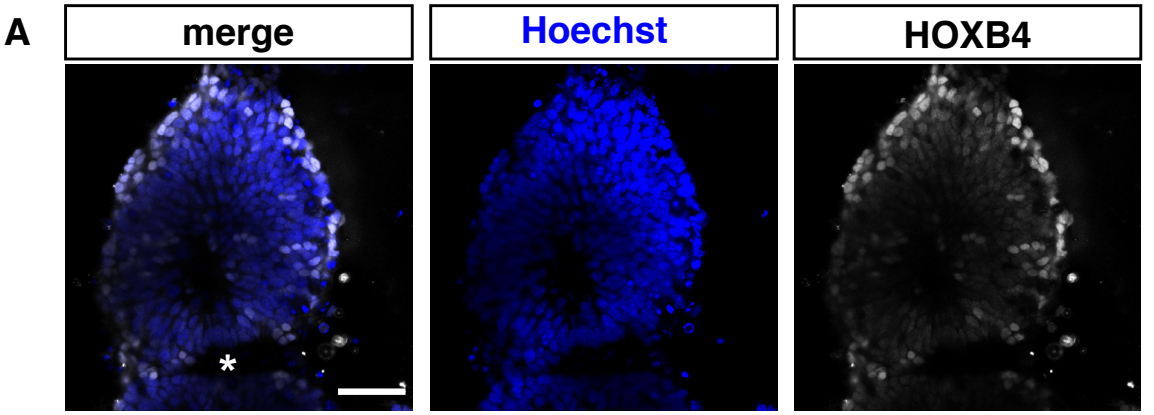
Figure S5: A/P positional identity of R1 and IB10 neural cysts and posteriorization to the cervical level of spinal cord (Related to Figure 4)

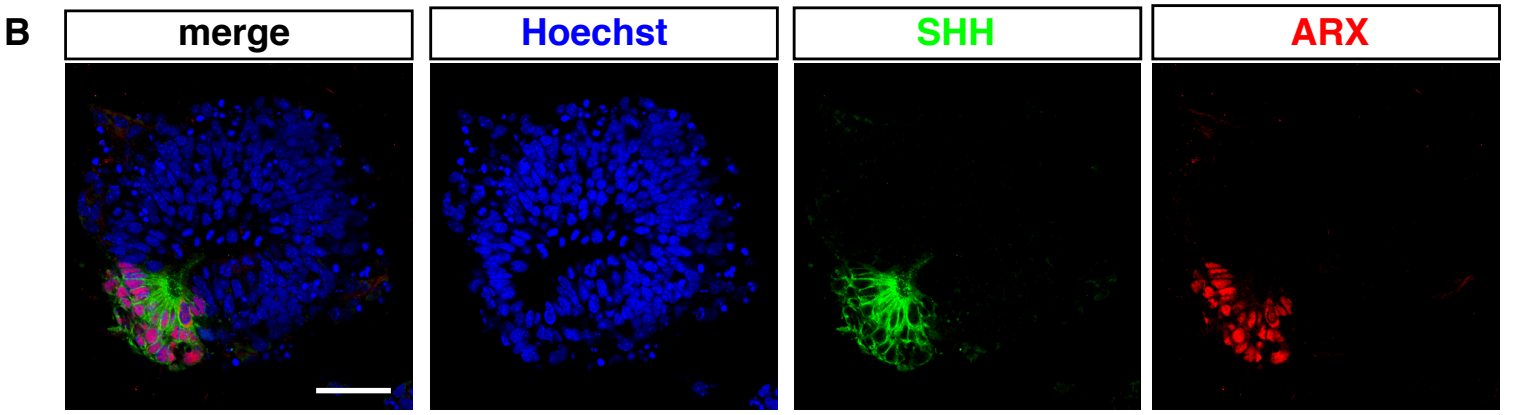
(A) RT-PCR analyses for genes involved in A/P positional identity along the anterior-posterior axis on untreated cysts (-RA) as well as posteriorized cysts (+RA) on day 7 of differentiation using line R1.

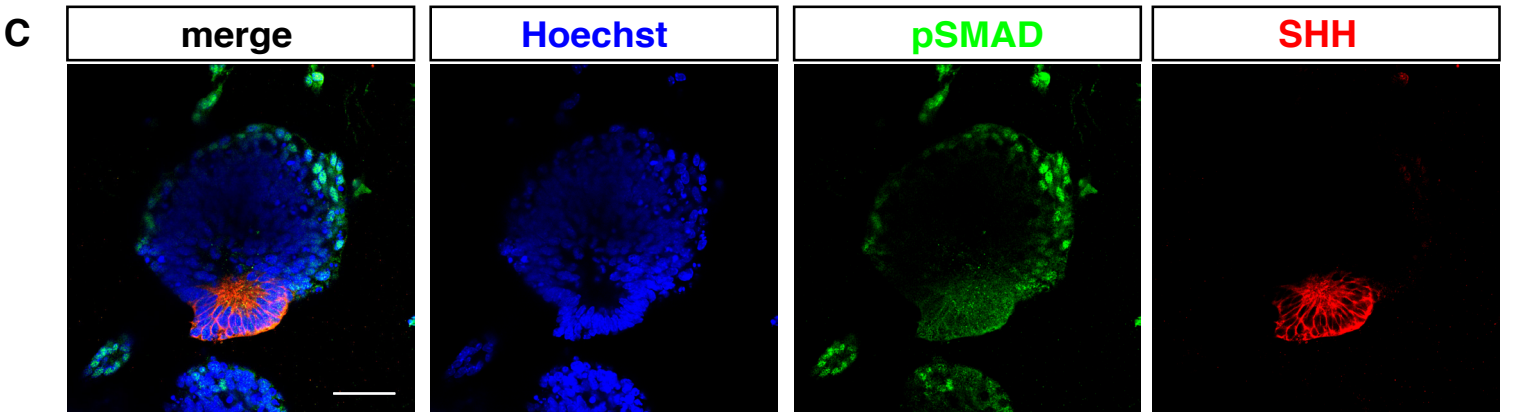
(B) *In situ* hybridization on cryosectioned day 6 IB10 cysts to examine localization of *Otx2*, *Gbx2* and *HoxC4* mRNAs. Subsets of day 6 cysts express *Otx2* or *Gbx2*. Almost all cysts were negative for *HoxC4*.

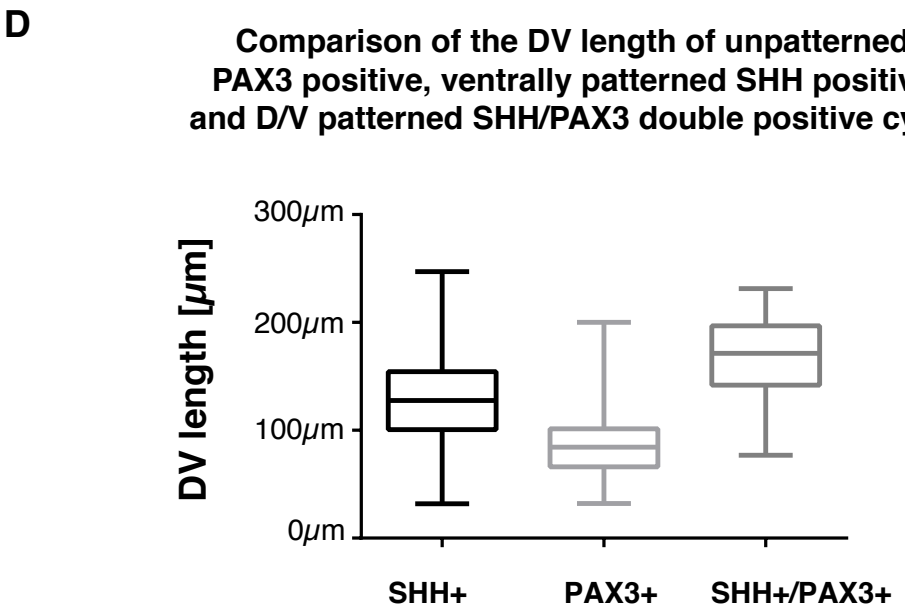
(C) Immunofluorescence staining for OTX2 and EN1 showing that IB10 cysts co-express OTX2 (red) and EN1 (green) whereas R1 cysts do not express any OTX2 or EN1.

(D) Confocal analysis of immunostainings for HOXB4 in ctrl R1 cysts (-RA, upper panel) and posteriorized cysts (+RA, lower panel) showing that HOXB4 is only expressed after posteriorization. Nuclei were counterstained with Hoechst in (C) and (D). Scale 50 µm.









Meinhardt *et al.*, Figure S6

Figure S6: Characterization of cervical DV neural cysts (Related to Figure 5)

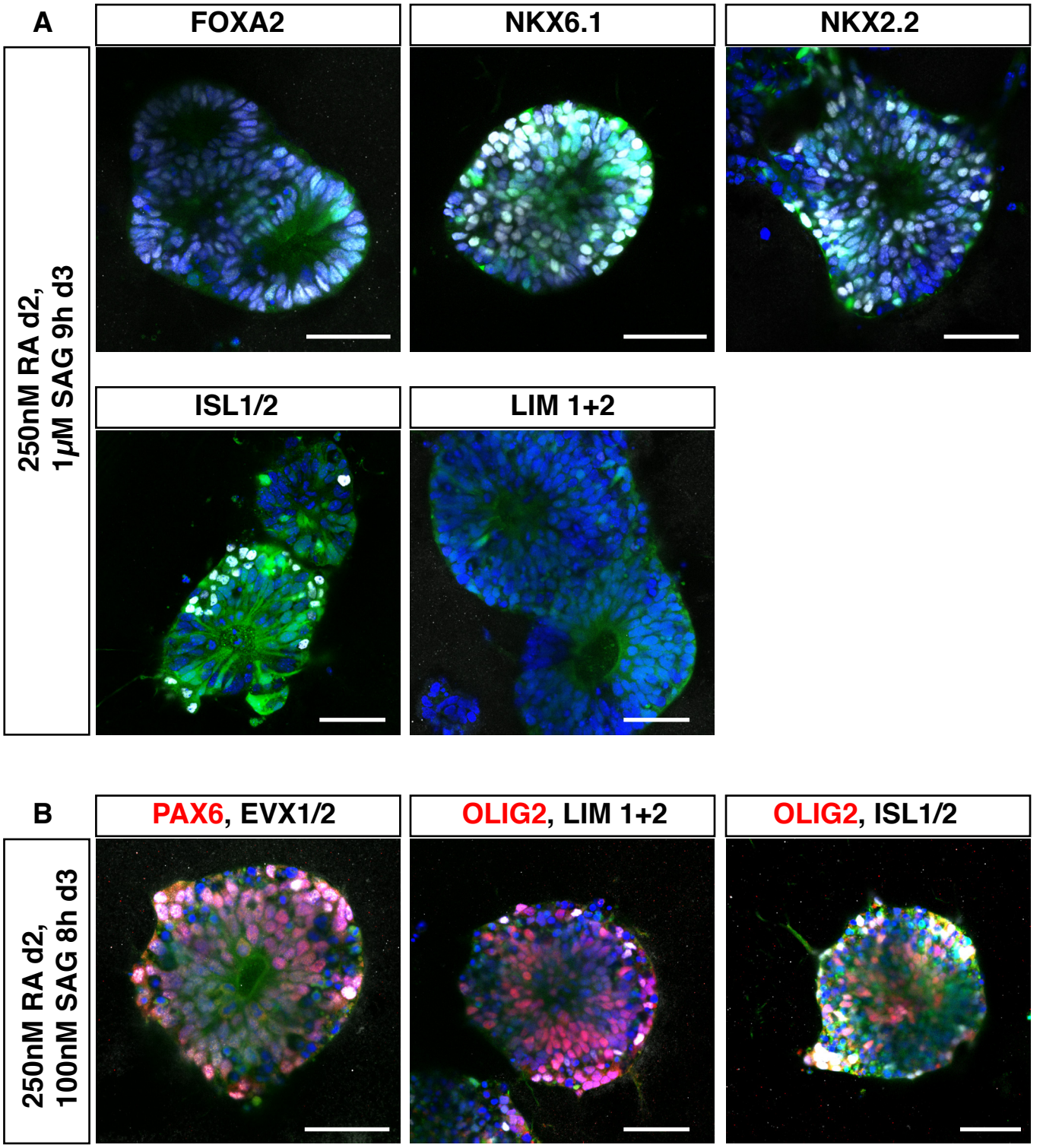
(A) Immunofluorescence analysis for HOXB4 (white) showing that self-patterned R1 cysts are posteriorized. The asterisk marks the FP.

(B) Day 7 R1 cysts were stained for the late FP marker ARX (red) as well as SHH (green) to show co-localization.

(C) Dorsal signaling was evidenced in patterned R1 cysts by immunostainings for pSMAD (green) and SHH (red). Nuclear pSMAD staining is observed opposite to the FP region marked with SHH.

Nuclei in A-C were counterstained with Hoechst. Scale 50µm.

(D) Quantification of the DV length of day 7 R1 cysts after a RA pulse on day 2 through manual measurement of a total of 5 dishes with 5-7 FOVs/dish. Data are shown as box and whisker diagram (median, interquartile range and full range of variation) and correspond to one representative experiment. Biggest cysts are fully patterned ($PAX3^+$ and SHH^+). Ventrally patterned cysts are smaller than fully patterned cyst (SHH^+) and the smallest cysts are unpatterned ($PAX3^+$).



Meinhardt *et al.*, Figure S7

Figure S7: Responsiveness of posteriorized cysts on additional SAG treatment (Related to Figure 6)

(A) Treatment of posteriorized 46C cysts with 1 µM SAG for 9h lead to a low expression level of FOXA2 and maintenance of SOX1. Expression of NKX6.1, NKX2.2 as well as ISL1/2 in the absence of LIM1+2 suggested that unpatterned cysts differentiated relatively uniformly to visceral motor neurons.

(B) Shortening the pulse and lowering the SAG concentration to 100 nM for 8h lead to the mixture of cysts expressing PAX6, EVX1/2, OLIG2, LIM1+2 and ISL1/2, thus markers for interneurons as well as motor neurons.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES:

List of antibodies used for immunocytochemistry

Primary antibodies used:

rat anti-ZO-1 (R26.4C, DSHB 1:50), mouse anti-NESTIN (Rat-401, DSHB 1:50), mouse anti-FOXA2 (4C7, DSHB 1:50), anti-MUSASHI (gift of H. Okano, Japan), PROMININ-1 (13A4, gift of W. Huttner, Max Planck Institute of Molecular Cell Biology and Genetics, Dresden, Germany), goat anti-SOX1 (R&D, 1:5000), rabbit anti-pH3 (Chemicon, 1:200), mouse anti-Map2a (Sigma, 1:200), mouse anti-βIII-TUBULIN (Chemicon, 1:200), rabbit anti-SHH (Santa Cruz Biotechnology, 1:200), goat anti-OLIG2 (R&D, 1:200), rabbit anti-OLIG2(Millipore, 1:200), rabbit anti-OTX2 (Abcam, 1:200), mouse anti-EN1 (4G11, DSHB, 1:50), mouse anti-PAX3 (Pax3, DSHB, 1:50), mouse anti-PAX7 (PAX7, DSHB, 1:50), mouse anti-E-CADHERIN (BD Biosciences, 1:200), rat anti-N-CADHERIN (MNCD2, DSHB, 1:50), rat anti-HOXB4 (I12 anti-Hoxb4, DSHB, 1:50), mouse anti-ISL1/2 (39.4D5, DSHB, 1:100), mouse anti-LIM1+2 (4F2, DSHB, 1:100), goat anti-BRN3a (Santa Cruz Biotechnology, 1:100), mouse anti-NeuN (Millipore, 1:200), rabbit anti-OCT4 (Abcam, 1:500), rabbit anti-NANOG (NEB, 1:500), chicken anti-GFP (Abcam, 1:500), sheep anti-ARX (R&D, 1:200), rabbit anti-pSMAD (Cell Signaling, 1:200)

Secondary antibodies used:

Alexa Fluor 488 donkey anti-rabbit IgG, Alexa Fluor 647 donkey anti-rabbit IgG, Alexa Fluor 555 donkey anti-mouse IgG, Alexa Fluor 647 donkey anti-mouse,

Alexa Fluor 647 donkey anti-goat IgG, Alexa Fluor 488 goat anti-chicken IgG, Alexa Fluor 488 goat anti-mouse IgG1, Alexa Fluor 555 goat anti-mouse IgG_{2a}, Alexa Fluor 555 donkey anti-sheep (all used at 1:200, Invitrogen); Cy3-conjugated goat anti-rat (1:200, Jackson), Alexa Fluor 647 conjugated goat anti-mouse IgG1 (1:200, Jackson), Alexa Fluor 647 conjugated donkey anti-goat (1:200, Jackson)

In situ hybridization on cryosections of neural cysts

Neural cysts were fixed at day 7 in fresh 4% PFA for 20 min at RT, washed with PBS, equilibrated in 30 % sucrose (Sigma) and embedded in Tissue-Tek O.C.T.™ Compound (Sakura). 12 µm thick cryosections were washed in PBS/0,1 % (v/v) Tween-20 and hybridized with 500 ng/ml DIG-labeled probe in hybridization buffer (50 % formamide (Merck), 10 % dextran, 5x SSC, 0.1 % Tween-20, 1 mg/ml yeast RNA, 100 µg/ml heparin, 1x Denhardt's, 0.1 % CHAPS, 5 mM EDTA) o/n at 70°C. Slides were washed three times 1 hour each and then o/n at 70°C in 5x SSC buffer (50 % (v/v) formamide, 5x SSC, 0.1 % (v/v) Tween-20), followed by two one hour washes at 70°C in post-hybridization buffer (50 % (v/v) formamide, 2x SSC, 0.1 % (v/v) Tween-20). Slides were further washed twice 5 min and once 20 min at RT in maleic acid buffer (MAB; 100 mM maleic acid, 150 mM NaCl, 0.1% Tween, pH 7.5), blocked in MAB plus 1% blocking reagent (Roche) for one hour at RT, and then incubated o/n at 4°C with anti-DIG antibody (1:5000, Roche) in blocking solution. Slides were washed 5x 10 min with MAB and 2x 10 min with alkaline phosphatase buffer (100 mM Tris pH 9.5, 50

mM $MgCl_2$, 100 mM NaCl, 0.1% Tween). Each slide was overlaid with BM purple (Roche) for the chromogenic reaction for 6-48 hours at 37°C. The reaction was stopped in cold PBS/1 mM EDTA and the slides were mounted in 50 % glycerol.

Preparation of human Noggin-Fc

The construct encoding for human Noggin-Fc was a kind gift from David Drechsel (Max Planck Institute of Molecular Cell Biology and Genetics, Dresden, Germany). The recombinant protein was expressed by transient transfection of suspension cultures of HEK293 cells and secreted into the medium. Shaking cultures were maintained at 37°C, 8% CO_2 in Freestyle293 serum-free medium (Invitrogen). For transfection, plasmid-DNA:PEI complexes, preformed at 10 µg/ml DNA and 100 µg/ml PEI (Polysciences) in 150 mM NaCl were diluted 1:10 into cells adjusted to $2x10^6$ cells/ml. After shaking incubation for 4 h, the medium was replaced and the cultures were diluted to $1x10^6$ cells/ml. After shaking for 4 days, conditioned medium was harvested by centrifugation (500 g, 5 min), sterile filtered (0.2 µm), concentrated using Ultra-15 centrifugal filter units (Amicon), and dialyzed into 1X PBS.

Electron Microscopy

Day 5.5 46C cysts embedded in Matrigel were prefixed in 4% PFA, glutaraldehyde was added to a 2 % final contentration in 0.1 M phosphate buffer and fixation continued at 4 °C o/n. The Matrigel patches were cut into small

pieces and the samples were postfixed in aqueous 2% OsO_4 for 1h at RT. They were dehydrated through a graded series of ethanol for standard embedding in EMBed-812 (Science Services). 70 nm-ultrathin sections were cut on a Leica UCT ultramicrotome (Leica Microsystems), poststained with uranyl acetate and lead citrate and viewed in a Morgagni electron microscope (FEI). Images were taken with a Morada camera (Olympus).

Sequences of primers used for RT-PCR

	Gene		Primer
RT-PCR	*Oct4*	Forward	5'- TGGAGGAAGCCGACAACAATGAGA-3'
		Reverse	5'- TGGTGCCTCAGTTTGAATGCATGG-3'
	Fgf5	Forward	5'- TCTCCTTTTATCTGCCCCCT-3'
		Reverse	5'- GAGCAGATGCACTCATTCCA-3'
	Sox1	Forward	5'- CGAGCCCTTCTCACTTGTT-3'
		Reverse	5'- TTGATGTTGGGGGTAT-3'
	Otx2	Forward	5'- GCAGAGGTCCTATCCCATGA-3'
		Reverse	5'- CTGGGTGGAAAGAGAAGCTG-3'
	Gbx2	Forward	5'- ATTTGCCTGGTCAGACTGCT-3'
		Reverse	5'- ACTTGCACCTCGGTGAGTTT-3'
	Pax6	Forward	5'- CTACCAGCCAATCCCACAGC-3'
		Reverse	5'- TTCGGCCCAACATGGAAC-3'
	HoxA2	Forward	5'- CCCGCAGGGTGGAAATCGCC-3'
		Reverse	5'- GCAGGCCTCCTGTTTTGTTTTTCAGG-3'
	HoxC4	Forward	5'- AGCAAGCAACCCATAGTCTACC-3'
		Reverse	5'- ATAACCTGGTGATGTCCTCTGC-3'
	HoxC5	Forward	5'- GACGGATGCAAGCGGTCCC-3'
		Reverse	5'- ACCTCCGGGGTTACAGGCCC-3'
	HoxC6	Forward	5´- CCGTCCCTATAACCATCTAGTTCC-3´
		Reverse	5´- GGAACTGAACACGACATTCTCC-3´
	HoxC8	Forward	5´- CCTATTACGACTGCCGGTTC-3´
		Reverse	5´- TTGGCGGAGGATTTACAGTC-3´
	Brachyury	Forward	5'- GACTTTGAAATCCTGGAATTCGTCC-3‘
		Reverse	5'- ACACTTTCTGCAGATTGTCTTTGGC-3‘
	Gapdh	Forward	5'- GCACAGTCAAGGCCGAGAAT-3'
		Reverse	5'- GTGGTTCACACCCATCACAA -3'

Sequences of primers used for *in situ* hybridization

In situ hybridization	*Otx2*	Forward	5'- AAGAATCGAAGAGCTAAGTGCCGC-3'
		Reverse	5'- GGTAATACGACTCACTATAGGGCGCAATCAGT GGTTGAGTTAAAAC-3'
	Gbx2	Forward	5'- AATCGTCCCCATTCCGAACG-3'
		Reverse	5'- TGGTCATCCTTTTCCCAAGTCG-3'
	HoxA2	Forward	5'- GAAGTCACTCTTTGAGCAAGCCC-3'
		Reverse	5'- GCAGGCCTCCTGTTTTGTTTCAGGA-3'
	HoxC4	Forward	5'- GGAGCAGCAACGGGCAGAGG-3'
		Reverse	5'- CCACGATGAGGGTAGGGGGAGC-3'
	HoxC6	Forward	5'- ACTCTCAGGGGGTGGCGGAG
		Reverse	5'- CGAGAACGGATCCAGAGGTCGG
	Shh	Forward	5'- GGAGCAGCAACGGGCAGAGG-3'
		Reverse	5'- TCGTGGGCTCGCTGCTAGGT-3'
	Msx1	Forward	5'- ATGGCCCCGGCTGCTGCTATGACTTCTTTGC-3'
		Reverse	5'- CTAAGTCAGGTGGTACATGCTGTAGCCTACATG GGC-3'
	Brachyury	Forward	5'- CCTATGCGGACAATTCATCTGCTTG-3'
		Reverse	5'- ACACTTTCTGCAGATTGTCTTTGGC-3'
	Bmp7	Forward	5'- GTGGTCCGGGCCTGTGGC-3'
		Reverse	5'- ACAGTGGCTTCTGCTTGGTTTCCC-3'
	Wnt1	Forward	5'- ATAGCCTCCTCCACGAACCT-3'
		Reverse	5'- GGGCGATTTCTCGAAGTAGA-3'
	Fgf-5	Forward	5'- GAAAAGACAGGCCGAGAGTG-3'
		Reverse	5'- AAACGCAAAGGAGATCCAGA-3'