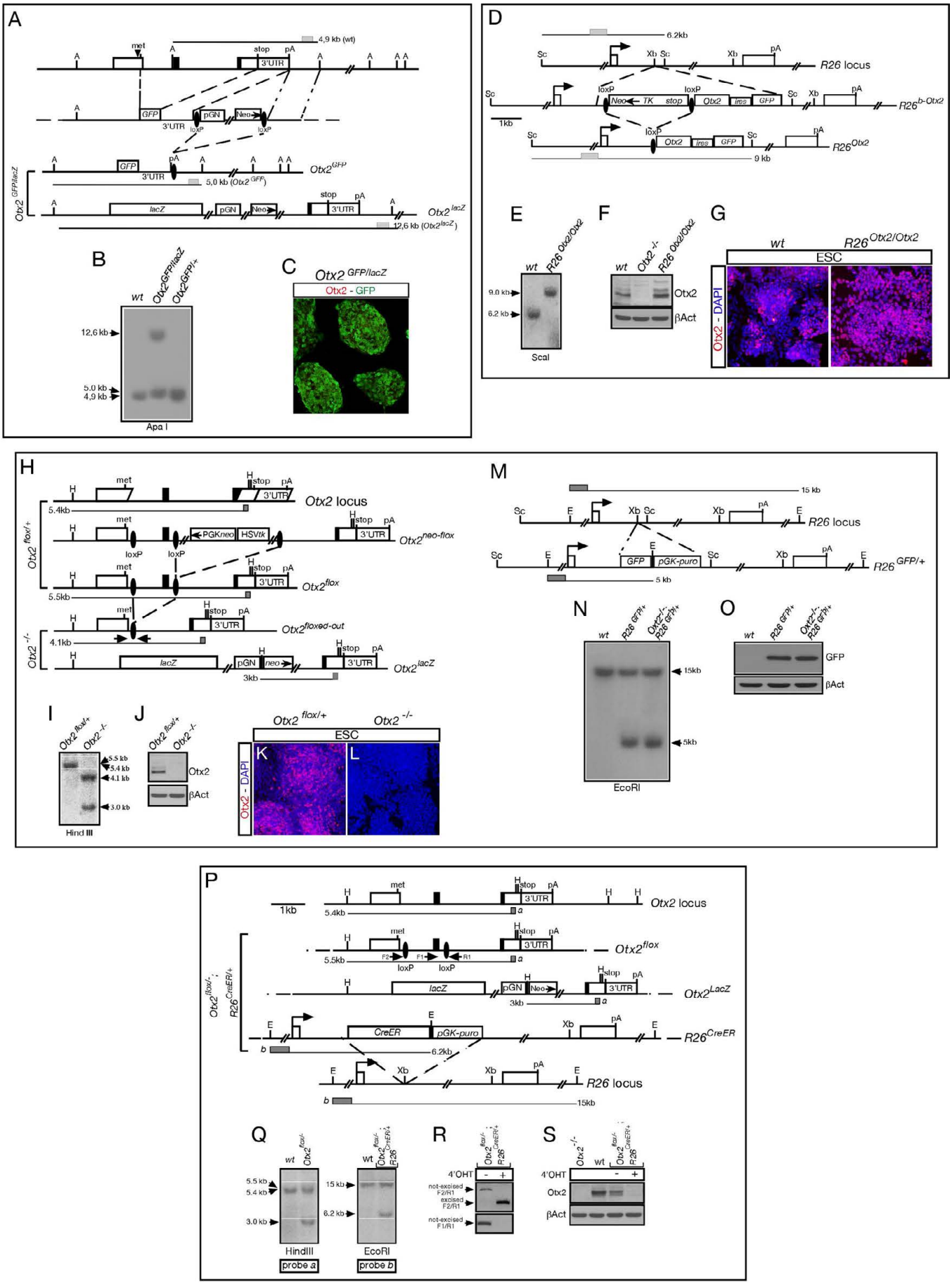
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met
stop
pA
3'UTR
4,9 kb (wt)
GFP
pGN
Neo
loxP
Otx2 GFP
5,0 kb (Otx2 GFP)
lacZ
Otx2 lacZ
12,6 kb (Otx2 lacZ)
Otx2 GFP/lacZ
B
wt
Otx2GFP/lacZ
Otx2GFP/+
12,6 kb
5.0 kb
4,9 kb
Apa I
C
Otx2 GFP/lacZ
Otx2 - GFP
D
6.2kb
R26 locus
TK
Otx2
ires
GFP
R26 b-Otx2
1kb
R26 Otx2
9 kb
E
wt
R26 Otx2/Otx2
9.0 kb
6.2 kb
ScaI
F
wt
Otx2 -/-
R26 Otx2/Otx2
Otx2
βAct
G
wt
R26 Otx2/Otx2
ESC
Otx2 - DAPI
H
Otx2 locus
5.4kb
PGKneo
HSVtk
Otx2 neo-flox
Otx2 flox
5.5kb
Otx2 floxed-out
4.1kb
Otx2 -/-
Otx2 lacZ
3kb
I
Otx2 flox/+
Otx2 -/-
5.5 kb
5.4 kb
4.1 kb
3.0 kb
Hind III
J
Otx2 flox/+
Otx2 -/-
Otx2
βAct
Otx2 flox/+
Otx2 -/-
ESC
K
L
Otx2 - DAPI
M
15 kb
R26 locus
GFP
pGK-puro
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N
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Otx2 -/- R26 GFP/+
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EcoRI
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Otx2 -/- R26 GFP/+
GFP
βAct
P
1kb
Otx2 locus
5.4kb
a
Otx2 flox
5.5kb
F2
F1
R1
loxP
Otx2 LacZ
3kb
CreER
pGK-puro
R26 CreER
b
6.2kb
R26 locus
15kb
Otx2 flox/-; R26 CreER/+
Q
wt
Otx2 flox/-
Otx2 flox/-; R26 CreER/+
5.5 kb
5.4 kb
3.0 kb
15 kb
6.2 kb
HindIII
EcoRI
probe a
probe b
R
4'OHT
- +
not-excised F2/R1
excised F2/R1
not-excised F1/R1
S
Otx2 -/-
Otx2 flox/-; R26 CreER/+
4'OHT
- +
Otx2
βAct

Fig. S1. Generation of *Otx2* mutant ESC lines. (**A**) Schematic representation of the $Otx2^{GFP/lacZ}$ targeting strategy. (**B**) Southern blot of control and mutant ESC lines with the probe corresponding to the gray box in A. (**C**) Immunohistochemistry assay with Otx2 and GFP shows lack of Otx2 and GFP immunoreactivity in $Otx2^{GFP/lacZ}$ ($Otx2^{-/-}$) ESCs. (**D**) Targeting strategy for the $R26^{Otx2}$ allele. (**E**) Southern blot hybridized with the external probe (gray box in D). (**F**) Western blot probed with Otx2 and β-actin antibodies shows that, compared with wt, in $R26^{Otx2/Otx2}$ ESCs the Otx2 total level is approximately doubled. (**G**) Immunohistochemistry assays showing ubiquitous expression of Otx2 in $R26^{Otx2/Otx2}$ ESCs. (**H**) To generate the $Otx2^{-/-};R26^{GFP/+}$ ESC line for chimerism studies, we first inactivated Otx2 through sequential steps required to obtain a new $Otx2^{-/-}$ ESC line without GFP. (**I**) Southern blot hybridized with the external probe (hatched box in H). (**J-L**) Western blots probed with Otx2 and β-actin antibodies (J) and immunohistochemistry assays showing the lack of Otx2 (K,L). (**M**) The $Otx2^{-/-}$ ESC line in H was retransfected to insert the *GFP* gene into one *Rosa26* allele using a GFP, pGK-puro targeting vector; in parallel, E14Tg2a ESCs were transfected only with the GFP targeting construct to generate an $R26^{GFP/+}$ ESC line to be used as control in chimerism studies. (**N**,**O**) Southern and western blot assays of the $R26^{GFP/+}$ and $Otx2^{-/-};R26^{GFP/+}$ mutant ESC lines hybridized with the external probe (hatched box in M) (N), and probed with GFP and β-actin antibodies (O). (**P**) Schematic representation of targeted alleles and sequential steps required to obtain the $Otx2^{flox/-};R26^{CreER/+}$ ESC line. (**Q**) Southern blots hybridized with the *Otx2*-specific external probe *a* and with the *Rosa26*-specific external probe *b* (gray boxes in P). (**R**) Representative PCRs with the indicated primers (horizontal arrows in P) to check *Otx2* DNA excision after 36 hours of exposure to 4-OHT. (**S**) Western blot probed with the Otx2 antibody to show full Otx2 inactivation. β-actin is employed to normalize cell extracts. A, S, X, H, E indicate *Apa*I, *Sca*I, *Xba*I, *Hin*dIII and *Eco*RI, respectively; pA indicates the poly(A) addition site.

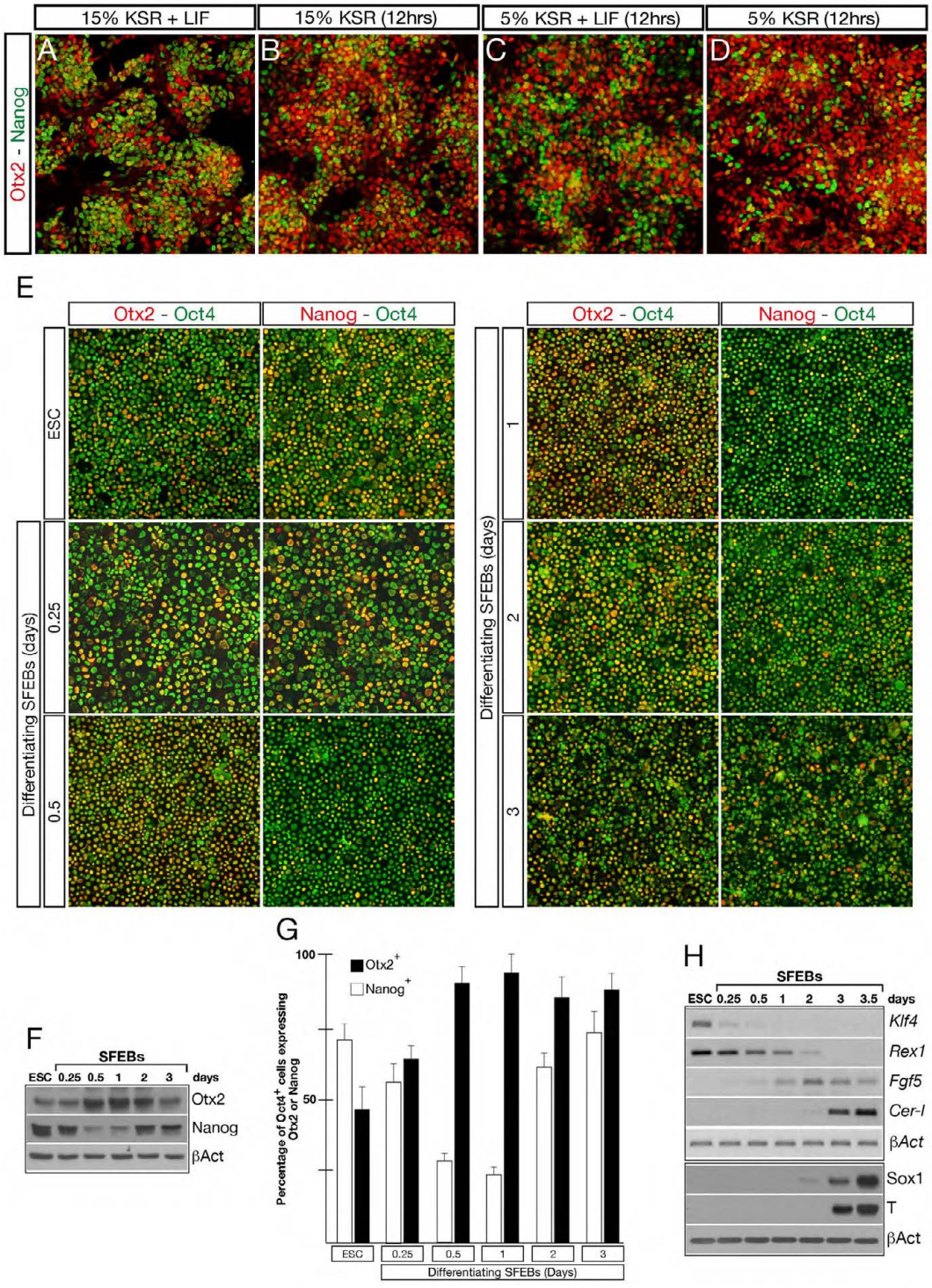

Fig. S2. Otx2 expression is activated by culture conditions favoring differentiation. (**A-D**) Otx2 and Nanog immunohistochemistry assays show that, compared with normal ESC culture conditions (A), LIF withdrawal (B), or diminished concentration of KSR (C) or both LIF withdrawal and reduced concentration in KSR (D) generate a rapid increase in the number of ESCs expressing Otx2 and a corresponding decrease of those expressing Nanog. (**E-G**) Immunohistochemistry experiments (E), western blots (F), and cell-counting analysis (G) show the expression profile (E,F) and the percentage of Oct4^{+} cells co-expressing Otx2 or Nanog at the indicated days (d) of differentiation (G), and reveal that Otx2 expression expands to virtually all Oct4^{+} cells within d1 and mirrors the early reduction in Nanog^{+} cells. (**H**) Western blots and RT-PCR assays show that Otx2 activation mirrors also the extinction of *Klf4* and *Rex1* expression and anticipates the maximal activation of the epiblast markers *Fgf5* and *Cer-l* before differentiation into Sox1^{+} neural and T^{+} mesendoderm cells occurs. β-actin is used to normalize western blots and RT-PCRs.

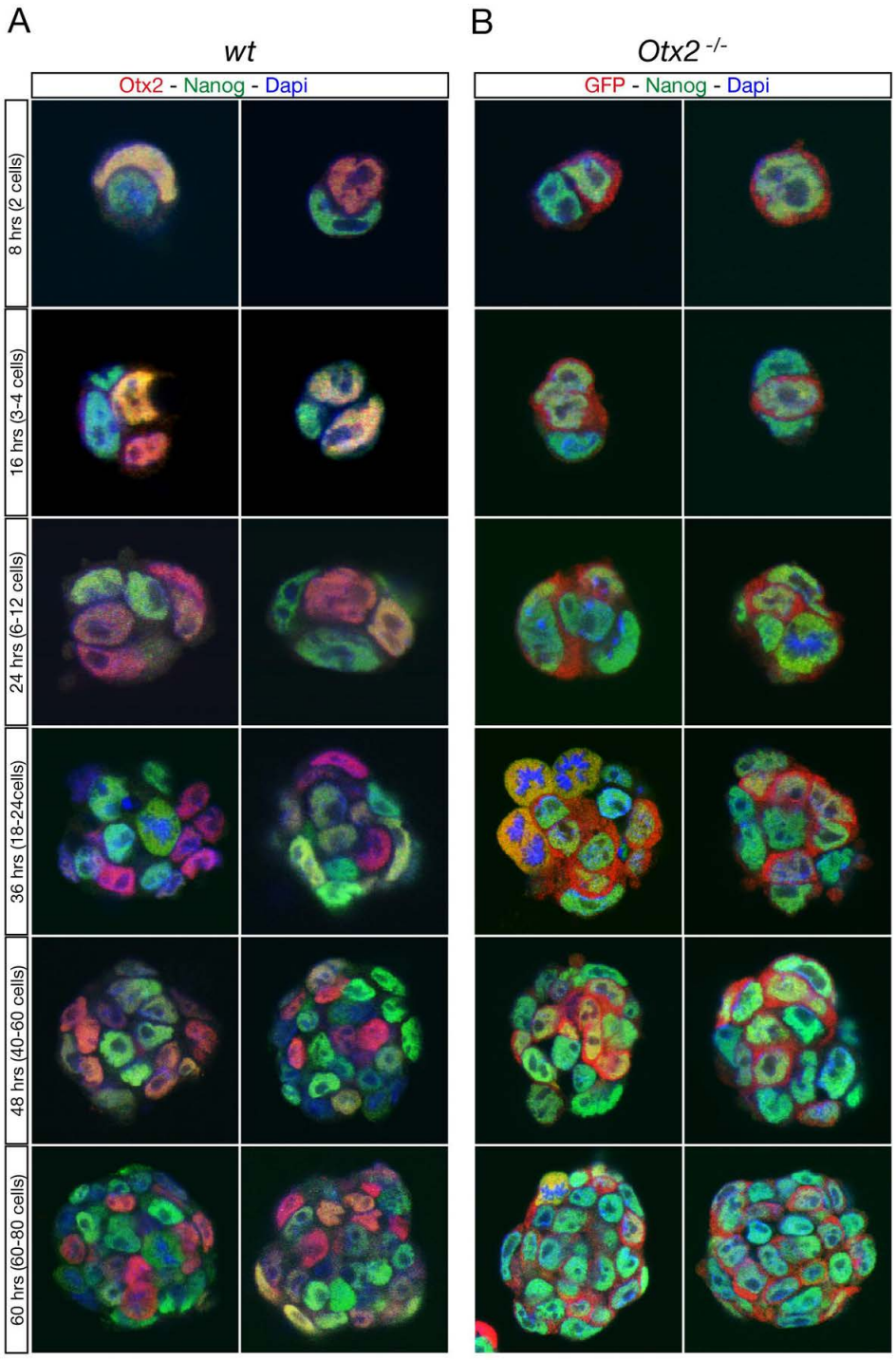

Fig. S3. Lack of Otx2 abolishes cell-to-cell variations of Nanog expression during formation of ESC colonies. Co-immunohistochemistry assays with Otx2 and Nanog, and GFP and Nanog, show that during the early formation of wt ESC colonies since the first cell duplication, Otx2 and Nanog exhibit variable degrees of complementarity and co-expression (**A**); conversely, in *Otx2*$^{-/-}$ ESCs, the expression profile generally observed is characterized by the constitutive high expression of Nanog regardless of the GFP expression (**B**). For each time point, two examples per genotype are shown.

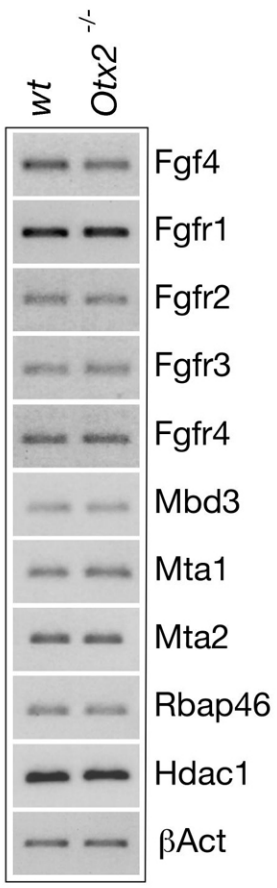

Fig. S4. Expression analysis of Fgf4, Fgf receptors and components of the NuRD complex. Compared with wt, only the expression level of *Fgf4* shows a mild reduction in *Otx2*$^{-/-}$ ESCs, whereas that of *Fgfr1*, *Fgfr2*, *Fgfr3*, *Fgfr4*, *Mbd3*, *Mta1*, *Mta2*, *Rbap46* and *Hdac1* appears unaltered in *Otx2*$^{-/-}$ ESCs.

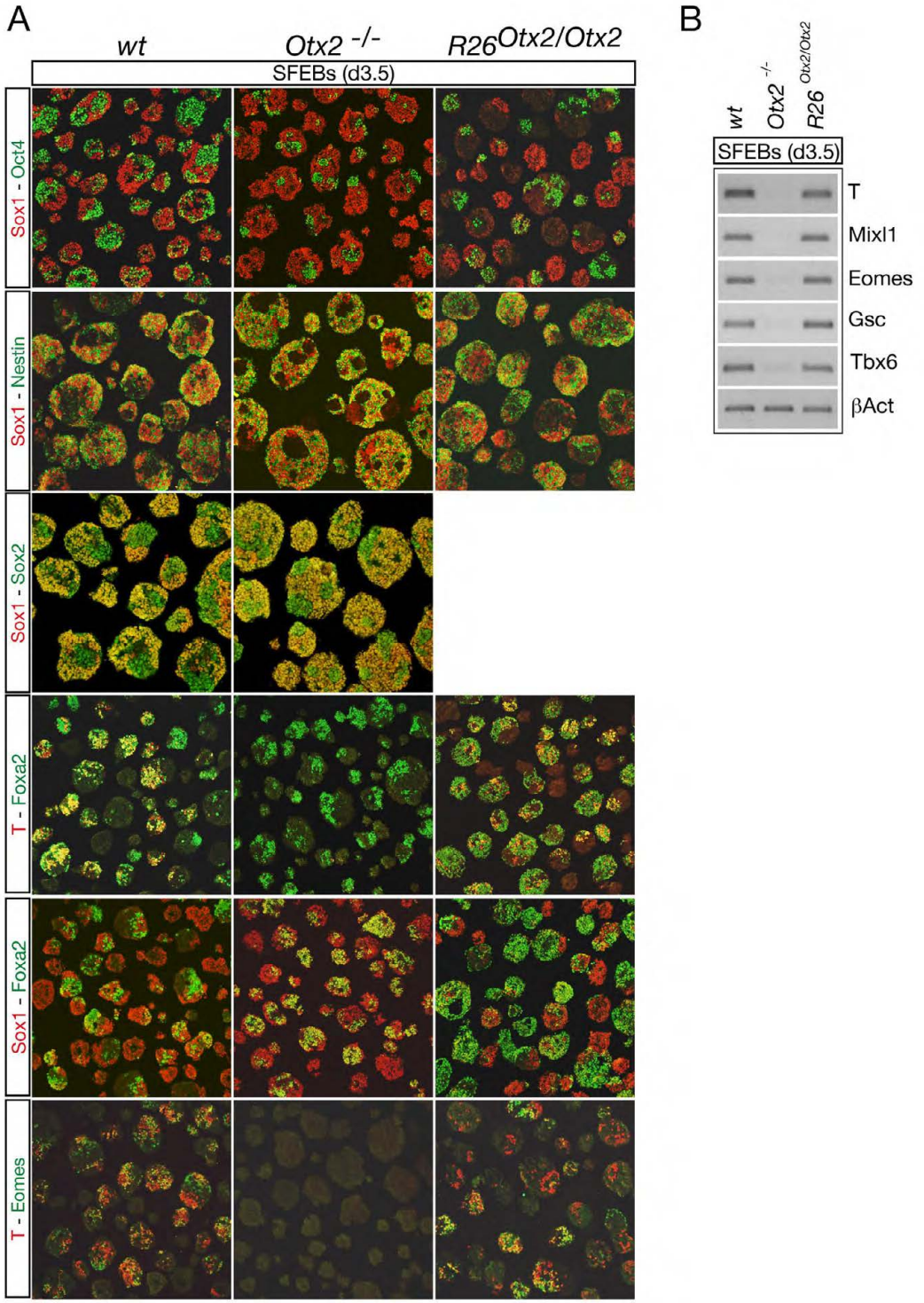

Fig. S5. Otx2 affects cell lineage decisions in differentiating SFEBs. (**A**) Immunohistochemistry experiments on d3.5 wt, *Otx2*$^{-/-}$ and *R26*$^{Otx2/Otx2}$ SFEB sections with Sox1 and Oct4, Sox1 and nestin, Sox1 and Sox2 (only for wt and *Otx2*$^{-/-}$ SFEBs), T and Foxa2, Sox1 and Foxa2, and T and Eomes show that, compared with wt, *Otx2*$^{-/-}$ SFEBs differentiate only in Sox1+ Nestin+ Sox2+ Oct4– neural cells, which are negative for the expression of T and Eomes and fully co-express Foxa2 with Sox1; by contrast, *R26*$^{Otx2/Otx2}$ ESCs generate fewer neural cells, exhibit at d3.5 a number of T^{+} cells similar to that of wt SFEBs, but show a substantial increase in Foxa2^{+} Sox1^{-} presumptive endodermal cells. (**B**) Expression analysis of mesendoderm markers shows that in *Otx2*$^{-/-}$ SFEBs, lack of *T* correlates with loss of *Mixl1*, *Eomes*, *Gsc* and *Tbx6*, whose expression is retained in *R26*$^{Otx2/Otx2}$ SFEBs.

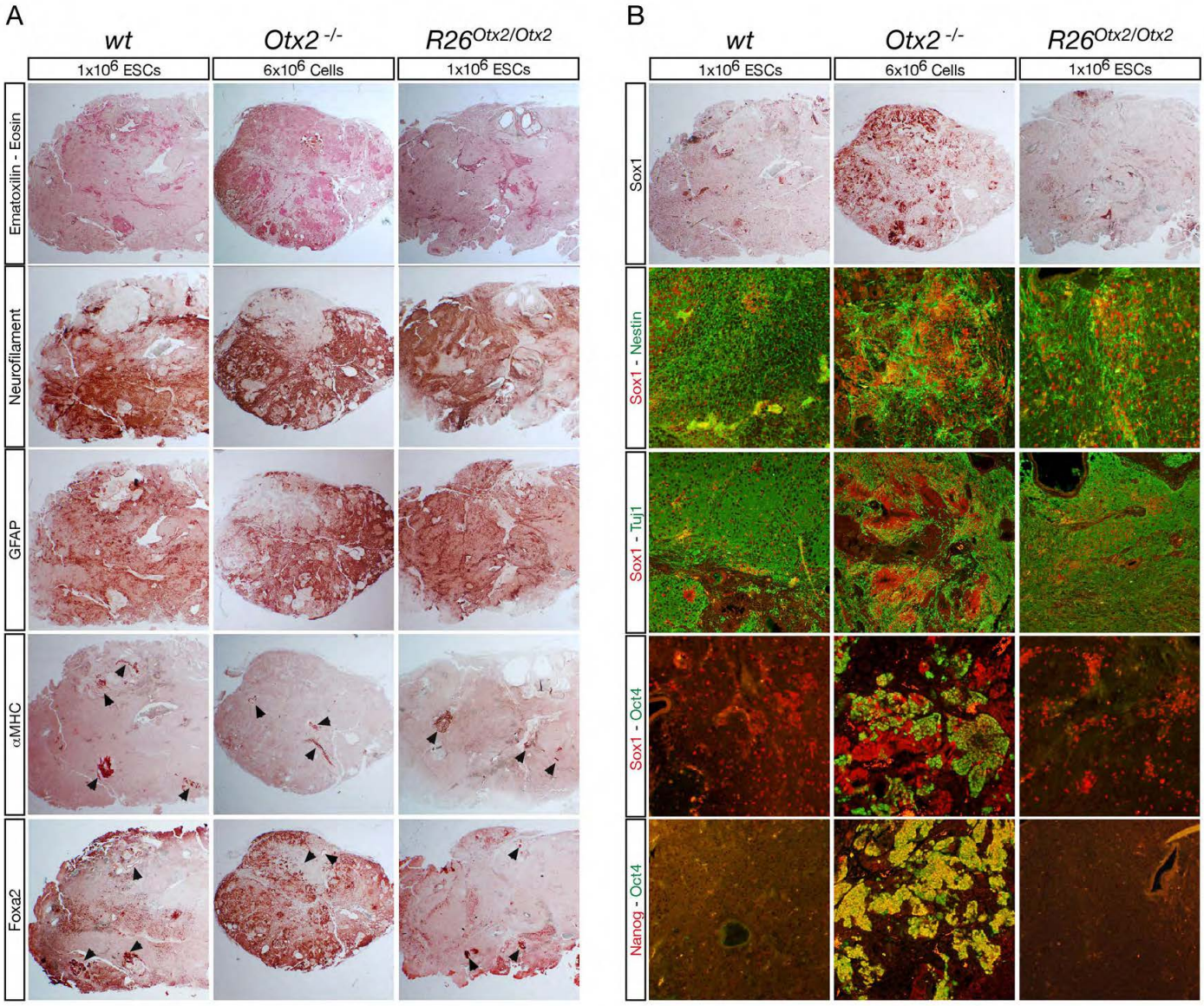

Fig. S6. Differentiation of teratomas generated by *Otx2* mutant ESC lines. (**A**) Wt, *Otx2*$^{-/-}$ and *R26*$^{Otx2/Otx2}$ ESC-derived teratomas generate neuronal and glial cells as revealed by neurofilament and Gfap staining, as well as muscle-like and endodermal-like structures as revealed by αMHC and Foxa2 staining (arrowheads). (**B**) However, *Otx2*$^{-/-}$ ESC-derived teratomas retain unusual enrichment of Sox1^{+} nestin^{+} rosette-like neural progenitors, which differentiate into Tuj1^{+} neurons, and widespread distribution of Oct4^{+} Nanog^{+} pluripotent cell clusters. Note that Oct4^{+} cells frequently co-expressed Sox1.

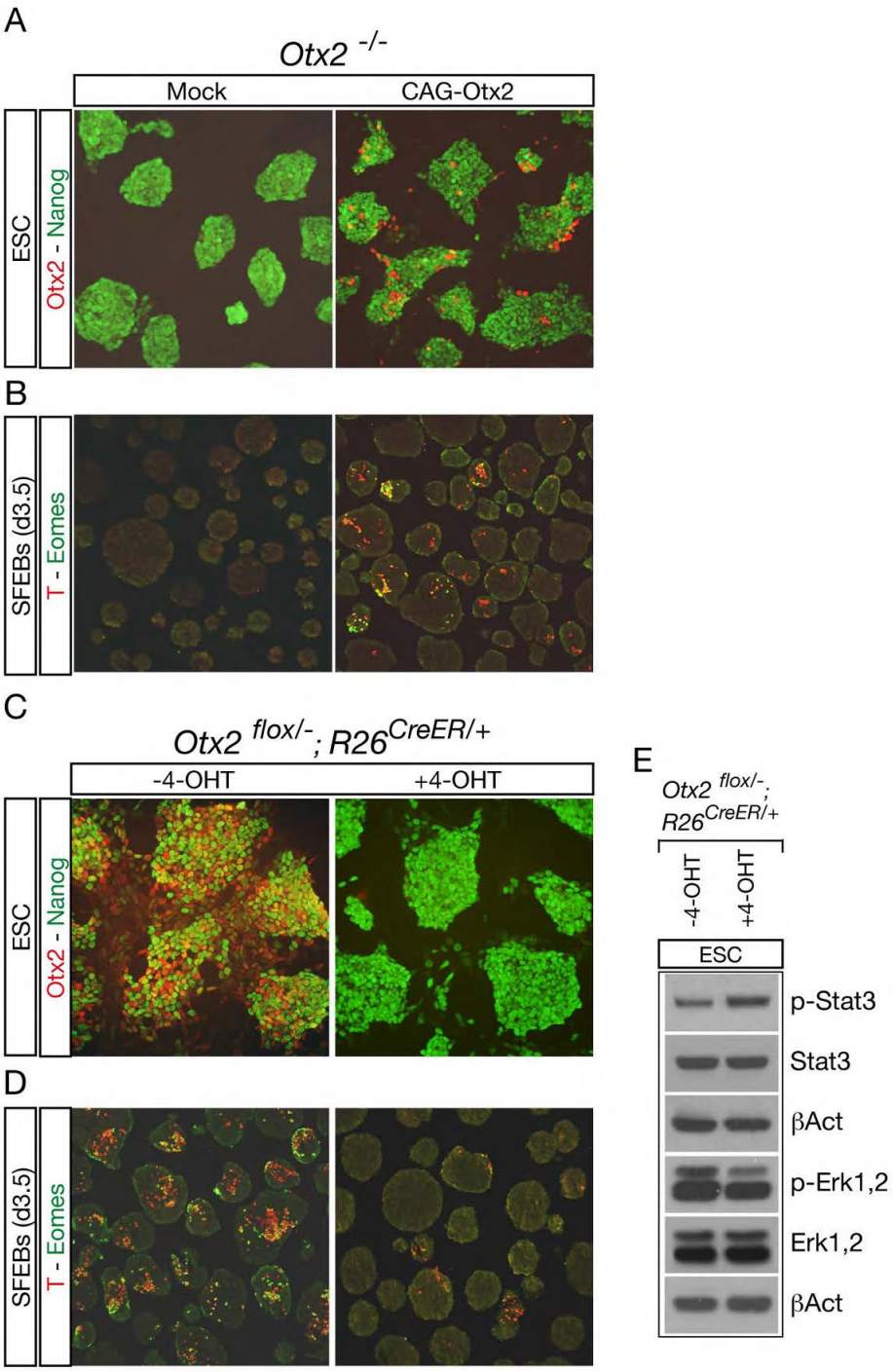

Fig. S7. Phenotypic features of *Otx2*$^{-/-}$ ESCs are not due to adaptation and can be rescued by Otx2 reintroduction. (**A**,**B**) Immunohistochemistry experiments with Otx2 and Nanog on *Otx2*$^{-/-}$ ESCs transfected (48 hours before) or not with the pCAG-Otx2 plasmid (A), and with T and Eomes on *Otx2*$^{-/-}$ d3.5 SFEBs generated from *Otx2*$^{-/-}$ ESCs transfected or not with the pCAG-Otx2 plasmid (B). (**C**,**D**) Immunohistochemistry with Otx2 and Nanog and with T and Eomes, respectively, on *Otx2*$^{flox/-}$;*R26*$^{CreER/+}$ ESCs previously treated or not with 4-OHT (C), and on *Otx2*$^{flox/-}$;*R26*$^{CreER/+}$ SFEBs generated from ESCs previously treated or not with 4-OHT for 3 days (D). (**E**) Western blots to detect the endogenous level of p-Stat3, total Stat3, p-Erk1,2 and total Erk1,2 in *Otx2*$^{flox/-}$;*R26*$^{CreER/+}$ ESCs treated or not for 3 days with 4-OHT. Western blots are normalized by β-actin.

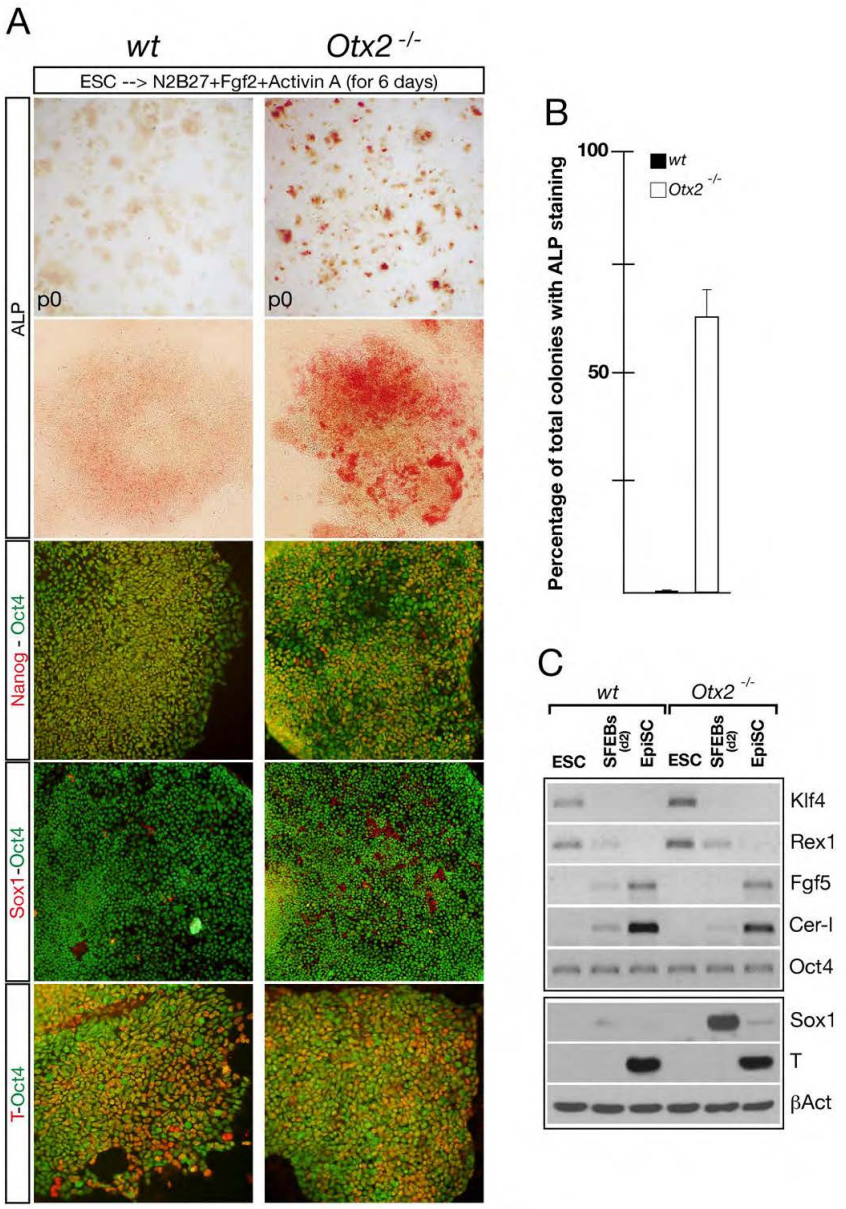

Fig. S8. Fgf2 and activin A induce a fairly normal initial specification of EpiSCs in the absence of Otx2. (**A-C**) Compared with wt, *Otx2*$^{-/-}$ EpiSCs induced for 6 days with Fgf2 and activin A exhibit similar expression for *Fgf5*, *Cer-l*, Sox1 and T (A,C), but show abnormal ALP staining in about 60% of the EpiSC colonies (A,B). RT-PCRs are normalized by *Oct4* and western blots by β-actin.

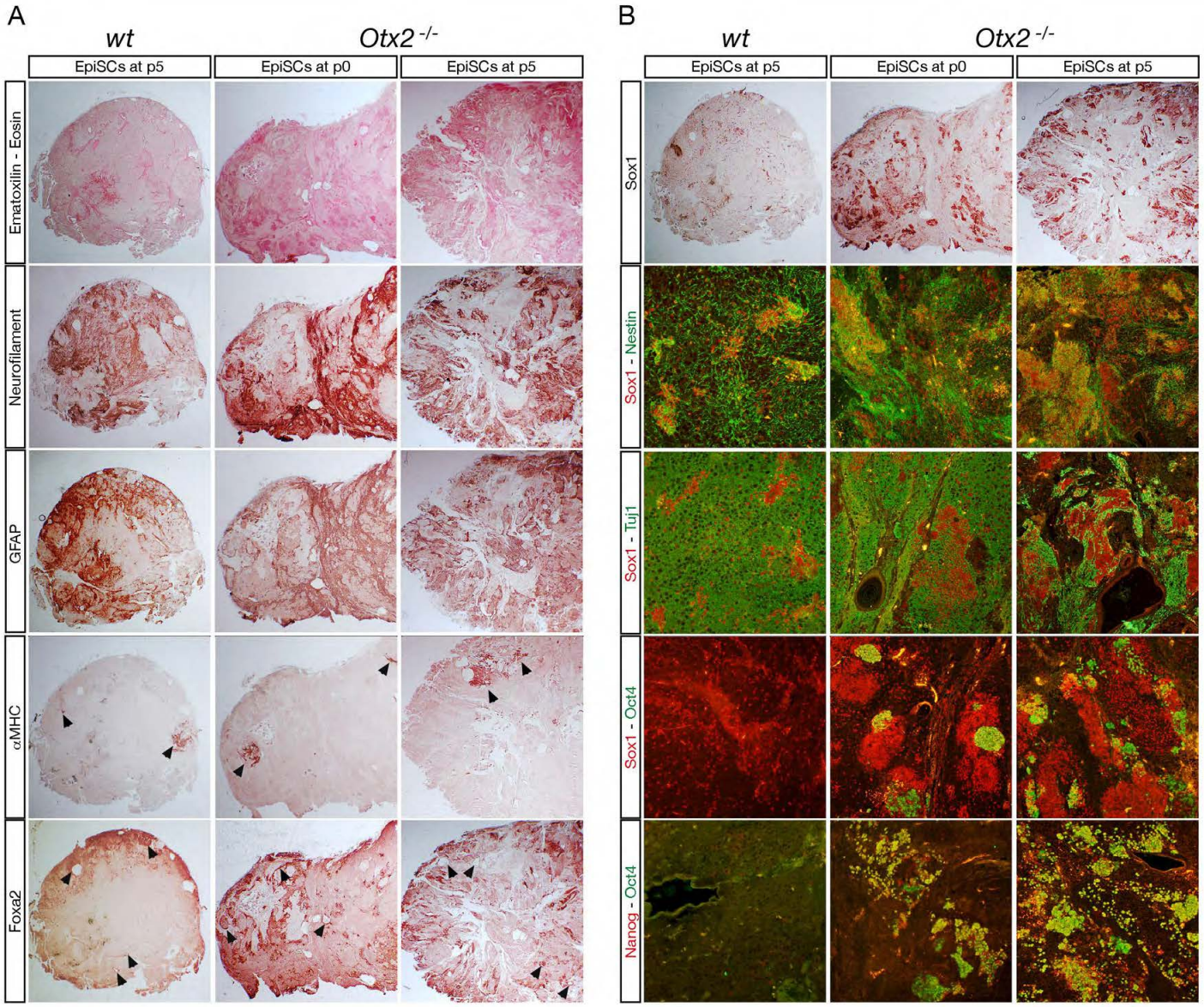

Fig. S9. Differentiation of *Otx2*$^{-/-}$ EpiSC-derived teratomas. (**A**) Compared with p5 wt EpiSC-derived teratomas, those derived from p0 and p5 *Otx2*$^{-/-}$ EpiSCs exhibit neuronal, glial, muscle-like and endodermal-like structures as revealed by neurofilament, Gfap, αMHC (arrowheads) and Foxa2 (arrowheads) staining. (**B**) However, *Otx2*$^{-/-}$ teratomas show numerous Sox1^{+} nestin^{+} rosette-like structures and Oct4^{+} Nanog^{+} cell clusters. Note that the number of Sox1^{+} nestin^{+} and Oct4^{+} Nanog^{+} cell clusters appears increased in teratomas generated by *Otx2*$^{-/-}$ EpiSCs passaged several times.

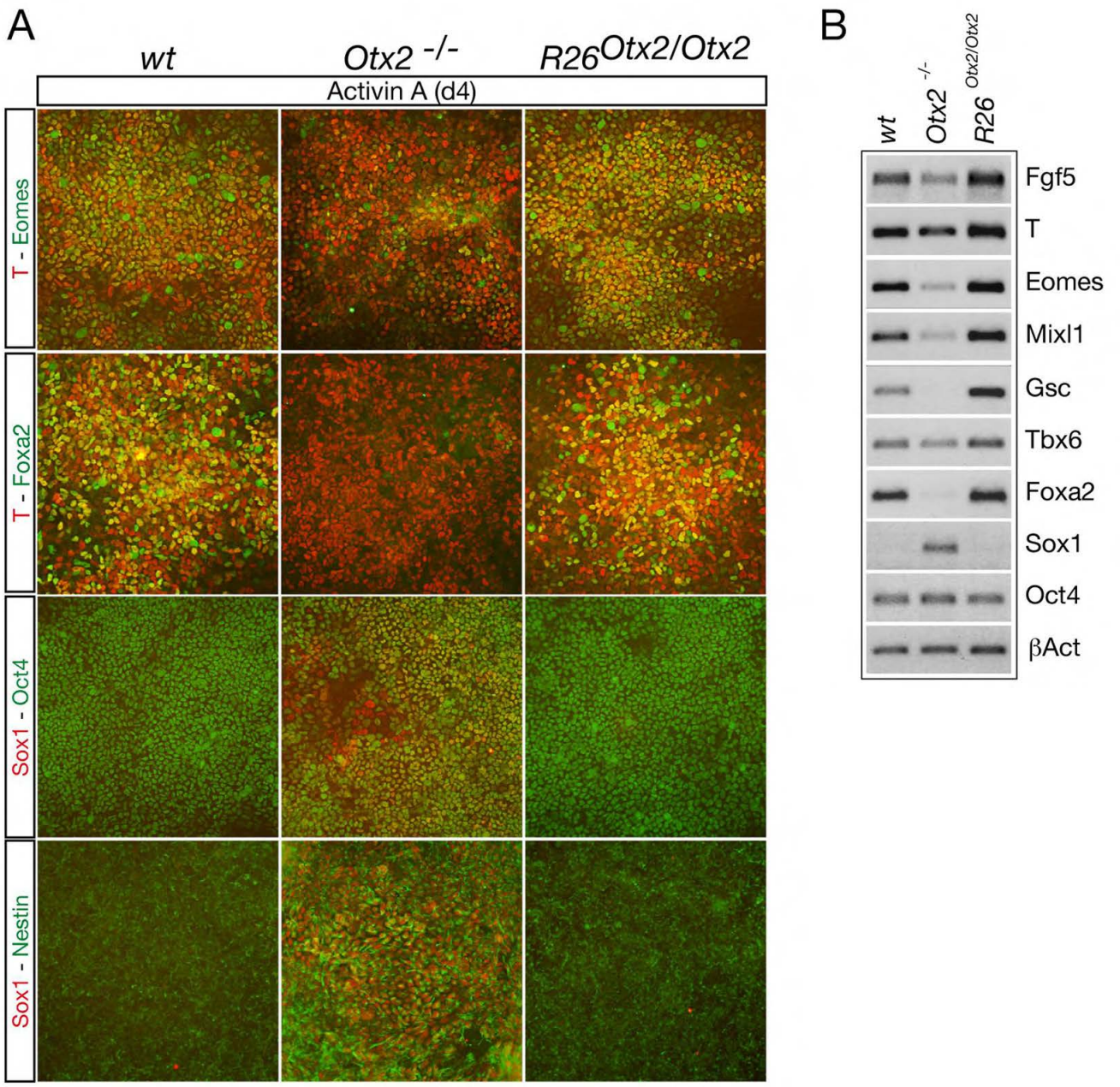

Fig. S10. Mesendoderm induction by activin A is affected in *Otx2*$^{-/-}$ and enhanced in *R26*$^{Otx2/Otx2}$ ESCs. (**A**,**B**) Mesendoderm induction shows that in the absence of Otx2 the expression level of *Eomes*, *Mixl1*, *Gsc* and *Foxa2* is strongly reduced (A,B), *Fgf5* expression is moderately diminished (B), that of *Tbx6* and *T* shows only a mild decrease (A,B), and, importantly, Sox1^{+} nestin^{+} neural progenitors are detected in numerous Oct4^{+} patches (A,B); conversely, the expression of mesendoderm markers is enhanced in *R26*$^{Otx2/Otx2}$ mutant cells (A,B).

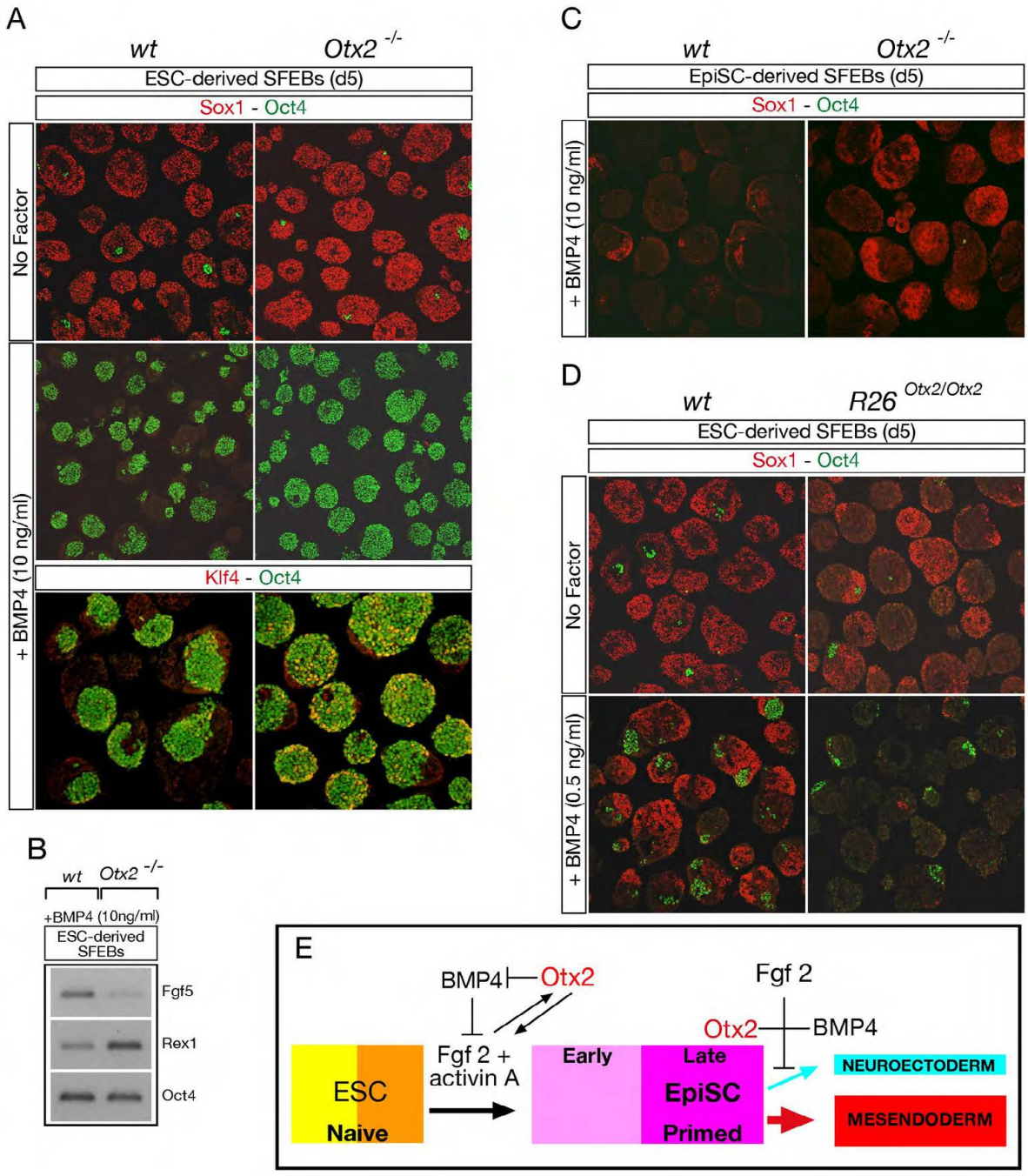

Fig. S11. Otx2 cooperates with BMP4 to suppress neural fate and promote differentiation of non-neural cells. (**A**) Immunohistochemistry assays with Sox1 and Oct4 and Klf4 and Oct4 on d5 wt and *Otx2*$^{-/-}$ ESC-derived SFEBs cultured without (no factor) or with BMP4 show that, compared with wt, *Otx2*$^{-/-}$ SFEBs cultured without BMP4 generate only neural cells, whereas when administered with BMP4 at high dosage (10 ng/ml) *Otx2*$^{-/-}$ SFEBs contain almost exclusively Oct4^{+} pluripotent cells and exhibit a significant increase in Oct4^{+} cells co-expressing the ESC marker Klf4. (**B**) RT-PCR assays showing that in *Otx2*$^{-/-}$ SFEBs the expression of *Rex1* and *Fgf5* is respectively higher and lower than that exhibited by wt SFEBs. RT-PCRs are normalized by *Oct4*. (**C**) Immunohistochemistry assays with Sox1 and Oct4 on wt and *Otx2*$^{-/-}$ EpiSC-derived SFEBs show that, in contrast to wt, high dosage of BMP4 (10 ng/ml) is not sufficient to efficiently suppress neural fate in *Otx2*$^{-/-}$ SFEBs. (**D**) Immunohistochemistry assays with Sox1 and Oct4 on wt and *R26*$^{Otx2/Otx2}$ ESC-derived SFEBs untreated or treated with BMP4 show that, compared with wt, untreated *R26*$^{Otx2/Otx2}$ SFEBs generate fewer Sox1^{+} neural cells and, when administered with a very low dosage of BMP4 (0.5 ng/ml), *R26*$^{Otx2/Otx2}$ SFEBs exhibit a substantial enhancement of the BMP4 anti-neuralizing activity. (**E**) Schematic representation of putative Otx2 actions in ESC transition to EpiSCs and maintenance of the EpiSC condition shows that Otx2 might be involved in the initial priming of ESC transition into EpiSCs by establishing a mutual positive loop with Fgf2 signaling and an antagonism on BMP4 signaling, which, in turn, antagonizes Fgf2-mediated priming. Later, in mature EpiSCs, Otx2 cooperates with Fgf2 and BMP4 to prevent EpiSC instability and the mesendoderm-to-neural fate switch.

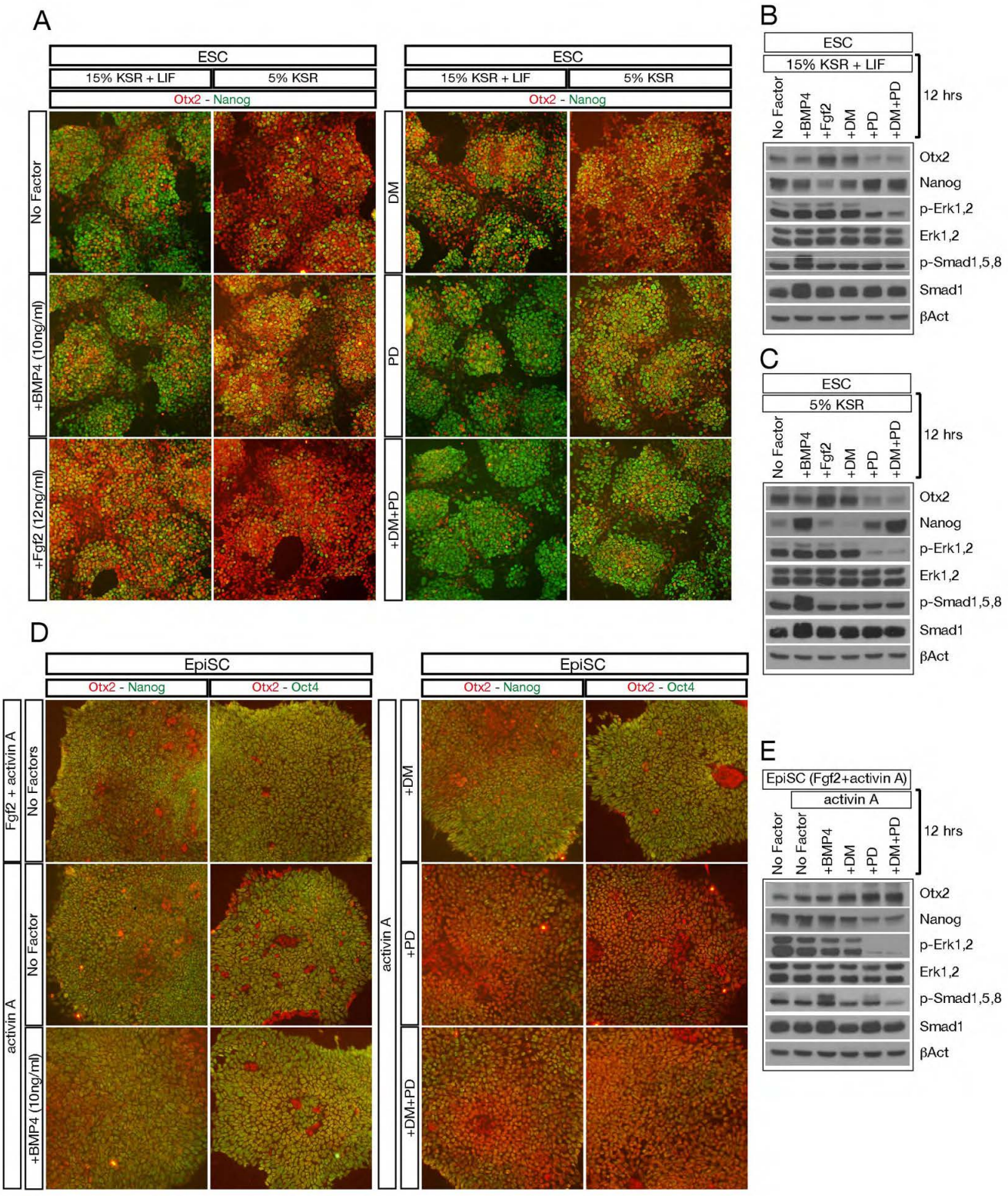

Fig. S12. Otx2 response to Fgf2 and BMP4 factors and their inhibitors. (**A-C**) Otx2 and Nanog expression analyzed by immunohistochemistry (A) and western blotting (B,C) in ESCs cultured in 15% KSR plus LIF without the addition of any factor or inhibitor (no factors) or 12 hours in the presence of BMP4 or Fgf2, or DM or PD, or both DM and PD (A,B); Otx2 and Nanog expression is also analyzed in ESCs cultured for 12 hours in 5% KSR only or with the same factors or inhibitors as for ESCs cultured in 15% KSR+LIF (A,C). (**D**,**E**) Immunohistochemistry with Otx2 and Nanog and Otx2 and Oct4 (D) and western blot analysis with Otx2 and Nanog in EpiSCs induced with Fgf2 and activin A, or Fgf2-deprived and cultured for 12 hours in activin A only or supplemented with BMP4 or DM or PD or both DM and PD (E). The expression level of p-Smad1,5,8, p-Erk1,2, total Erk1,2 and Smad1 is monitored in all experiments (B,C,E) to control the activity of BMP4 and Fgf2 and their inhibitors. β-actin is employed to normalize western blots

Table S1. RT-PCR primers

mRNA	Forward primer	Reverse primer	Size (bp)	N° cycles
β-actin	GGTTCCGATGCCCTGAGGCTC	ACTTGCGGTGCACGATGGAGG	360	18
Klf4	TGCTGAACAGCAGGGACTGTCAC	AGGTGTGCCTTGAGATGAGAACTC	280	24
Rex1	ACTGTGCTGCCTCCAAGTGTTGTC	AGGGAAGCCATCTTCCTCAGTCTC	330	20
Fgf5	TCGGTTTCCATCTGCAGATCTACC	TTCTGTGGATCGCGGACGCATAG	252	22
Cer-l	GTGGAAAGCGATCATGTCTCATCG	GCAAAGGTTGTTCTGGACAACGAC	261	28
Oct4	GCCGACAACAATGAGAACCTTCAG	CGCCGGTTACAGAACCATACTCG	215	22
Fgf4	GCAACGTGGGCATCGGATTC	GTTACCTTCATGGTAGGCGACA	316	25
Fgfr1	GTCACAGCCACTCTCTGCACTG	GACGGAGAAGTAGGTGGTATCGCT	310	26
Fgfr2	GCTCCAATGCAGAAGTGCTGGCTC	GGCAGAACTGTCAACCATGCAGAG	276	28
Fgfr3	GGAGGAGCTGATGGAAACTGATG	GAACAGGACCTTCTCCTGAGGACAG	270	28
Fgfr4	GCTTTGTCCCTTGAGGCCTCTGAG	GTATCGGCCAGCATCCTCAGGAAG	243	28
Mta1	CAAGTCGGAATCTCCTGCTCAATG	GGCGCAGGGCAATGGGTTTGTAGG	225	25
Mta2	GAGAACTCCTCCAGCAATCCTTAC	GTGGCTGGTAATGATTCAAACTGC	255	25
Hdac1	CCTCACAAAGCCAATGCTGAGGAG	GTTCACAGCGATGTCCGTCTGCTG	237	27
Rbap46	GAAGATACTGTGGAGGAGCGTGTC	CATCAAACTGTGCATCATCATTGG	260	25
Mbd3	TTCCAGGTCTCAGTGCAGGGA	TGACTTCCTGGTGGGCTGCT	334	25
T	CACCAGCATGCTGCCTGTGAGTCA	CTGGCTGTCAGAAATGTCTGTGAC	264	27
Goosecoid	TCTTCACCGATGAGCAGCTCGAAG	CAGCTGTCCGAGTCCAAATCGCT	276	29
Eomes	GCTTCAACATAAACGGACTCAACC	GTTCATTCAAGTCCTCCACACCGT	344	27
Mixl1	AGTTGCTGGAGCTCGTCTTCCGA	ATCCGGAACGTGGTTCACATCTG	266	27
Tbx6	GCTTCCTCTCTGGGATCGAGGCAG	CCTCTGGGTCCAGGCCAGTGACTG	264	28
Foxg1	ACTTTGAGTTACAACGGGACCACG	AAAGTAACTGGTCTGGCCCGC	282	27
Emx2	ACGACACAAGTCCCGAGAGTTTCC	TGCTTGGTAGCAATTCTCCACCG	310	28
Dmbx1	CCATCAGTGCATGCGCTTACGTT	GGCAAACCAGGAGGCTGTTCTG	399	29
En1	CAACCCTGCGATCCTACTCATGG	GATATAGCGGTTTGCCTGGAACTC	247	29
Gbx2	ATGGCGCTCACCTCCACGCTCAT	CATCTGAGCTGTAATCCACATCG	340	28
Sox1	GCACCAAGGCCAACCAGGATCGG	TTCTTGAGCAGCGTCTTGGTCTTG	268	27
Hnf3b	TCCGACTGGAGCAGCTACTACG	TCAGACTCGGACTCAGGTGAGGTC	300	27
Emx1	CAGGACGGGCTGCTTTTGCACG	GTGACATCAATGTCCTCCCCGTTG	326	30
Tbr1	GGAGACTCAGTTCATCGCTGTCA	CTTGGCGTAGTTGCTCACGAACTG	241	30
En2	TCCGACTCGGACAGCTCTCAAG	TCTTGATCTAGACTCGTTCAGG	280	29
Hoxa2	CCTGCCTGCCTCGGCCACAAAG	CACTGGGTTTGCCTCTTATGCTTC	242	31
Hoxa11	CACACTGAGGACAAGGCCGGTG	CCCTCCCAATTCCAGTAGGCTGG	270	33
Hoxb1	CAACCTTTGCATCAGCCTACGAC	CACCTGCGTTTCATTGAGCTCCA	290	32
Hoxb5	GCCAATTTCACCGAAATAGACGAG	ATCTGACGCTCGGACAGGCAAAG	330	33

Table S2. ESC subsets co-expressing Otx2 and Nanog

Genotype	N° of Exp.	ESC subpopulations co-expressing different levels of Otx2 and Nanog								
		Total $Otx2^{+}$ cells	$Otx2^{+}$ $Nanog^{h+m}$	$Otx2^{+}$ $Nanog^{l+a}$	Total $Otx2^{h+m}$	$Otx2^{h+m}$ $Nanog^{h+m}$	$Otx2^{h+m}$ $Nanog^{l+a}$	Total $Otx2^{l+a}$	$Otx2^{l+a}$ $Nanog^{h+m}$	$Otx2^{l+a}$ $Nanog^{l+a}$
wt	4	1436±201	672±55	736±149	625±63	234±23	398±54	399±50	261±23	138±28

h, m, l or a indicate high, moderate, low or absent expression, respectively; $Otx2^{+}$ or $Nanog^{+}$ indicate ESCs expressing the indicated factor regardless of the expression level.

Table S3. Total Oct4^{+} cells and Oct4^{+} cells co-expressing Nanog or Otx2 in ESCs and SFEBs

Genotype	No of Exp.	Time course (hours)	Total Oct4^{+} cells and Oct4^{+} subtypes expressing Nanog or Otx2 (mean ± s.d.)					
			Total cells	Oct4^{+}	Total Oct4^{+} cells	Oct4^{+} Nanog^{+}	Total Oct4^{+} cells	Oct4^{+} Otx2^{+}
wt	4	0 (ESC)	*	*	2813±125	1991±138	2770±286	1281±191
wt	4	6	*	*	2613±232	1472±154	2338±285	1496±128
wt	4	12	*	*	2930±142	824±64	3261±182	2901±206
wt	4	24	*	*	2566±243	622±80	2742±214	2555±192
wt	4	48	2643±108	2459±145	2995±301	1828±137	3255±165	2728±243
wt	4	72	2709±136	1935±104	2067±167	1503±141	2127±149	1856±112
wt	4	90	2423±94	1197±109	**	**	**	**
Otx2$^{-/-}$	4	0 (ESC)	*	*	**	**	n/a	n/a
Otx2$^{-/-}$	4	12	*	*	**	**	n/a	n/a
Otx2$^{-/-}$	4	24	*	*	**	**	n/a	n/a
Otx2$^{-/-}$	4	48	3207±39	1922±198	**	**	n/a	n/a
Otx2$^{-/-}$	4	72	3212±202	1223±188	**	**	n/a	n/a
Otx2$^{-/-}$	4	90	3904±431	664±138	**	**	n/a	n/a
R26$^{Otx2/Otx2}$	4	0 (ESC)	3024±169	2393±190	**	**	n/a	n/a
R26$^{Otx2/Otx2}$	4	12	3125±131	2566±154	**	**	n/a	n/a
R26$^{Otx2/Otx2}$	4	24	3131±149	2337±166	**	**	n/a	n/a
R26$^{Otx2/Otx2}$	4	48	2965±162	1986±126	**	**	n/a	n/a
R26$^{Otx2/Otx2}$	4	72	2859±95	1459±154	**	**	n/a	n/a
R26$^{Otx2/Otx2}$	4	90	4980±332	1407±150	**	**	n/a	n/a

*At 6, 12 and 24 hours all cells were Oct4^{+} in wt and *Otx2*$^{-/-}$ ESC lines and differentiating SFEBs.
**Not determined.

Table S4. ALP in wt and *Otx2* mutant ESC colonies

		Undifferentiated ESC colonies ($\times 10^3$ plated ESCs) (mean ± s.d.)		
Genotype	**N° of Exp.**	**Uniform ALP$^+$ (+LIF)**	**Uniform ALP$^+$ (–LIF)**	**Uniform ALP$^+$ (–LIF+JAK inh.)**
wt	4	232±39	6±4	0
Otx2$^{-/-}$	4	823±66	311±61	124±30
R26$^{Otx2/Otx2}$	4	72±16	n/a	n/a

Table S5. Oct4, Nanog and Klf4 in wt, *Otx2*$^{-/-}$ and *R26*$^{Otx2/Otx2}$ ESCs

		Oct4, Nanog and Klf4 ESC subsets (mean ± s.d.)					
Genotype	**N° of Exp.**	**Total cells**	**Oct4^{+}**	**Total cells**	**Nanog^{+}**	**Total cells**	**Klf4^{+}**
wt	4	*	*	2630±127	1863±142	2328±71	1508±138
Otx2$^{-/-}$	4	*	*	2587±125	2444±131	2235±83	2036±142
R26$^{Otx2/Otx2}$	4	3024±169	2393±190	2606±133	1410±107	2446±237	437±87

*All cells were Oct4^{+} in wt and *Otx2*$^{-/-}$ ESCs.

Table S6. Chimeric embryos generated by control and *Otx2* mutant ESCs

Genotype	Injected embryos	Recovered embryos	Chimerism			
			High	Moderate	Low	Undetectable or very low
$R26^{GFP/+}$	76	65	39	16	6	4
$Otx2^{-/-};R26^{GFP/+}$	83	61	26	22	11	2
$R26^{Otx2/Otx2}$	105	78	0	0	13	65

Table S7. Teratoma occurrence in wt and *Otx2* mutant ESCs and EpiSCs

Genotype	Number of subcutaneous injections	Number of injected ESCs	Number of injected EpiSCs	Recovered teratomas	Size*		
					Large (>0.7 cm)	Small (<0.5 cm)	Undetectable
$R26^{GFP/+}$	12	1.5×10^6		12	10	2	0
$Otx2^{-/-};R26^{GFP/+}$	11	1.5×10^6		2	0	2	9
$Otx2^{-/-};R26^{GFP/+}$	10	6×10^6		6	1	5	4
$R26^{Otx2/Otx2}$	12**	1.5×10^6		10	8	2	1
$R26^{GFP/+}$	6		1.5×10^6	4	4	0	2
$Otx2^{-/-};R26^{GFP/+}$	8		1.5×10^6	7	4	3	1

*The size classification was based on the largest diameter after mid-sectioning of the teratomas.
**One of the injected mice died prematurely.

Table S8. Wt, *Otx2*$^{-/-}$ and *R26*$^{Otx2/Otx2}$ neural and mesendoderm cell lineages in SFEBs at d3.5

			Number of neural and mesendodermal cells (mean ± s.d.)					
Genotype	**N° of Exp.**	**Factor (ng/ml)**	**Total cells**	**Sox1^{+}**	**Total cells**	**T^{+}**	**Total cells**	**Sox1^{-} Foxa2^{+}**
wt	4	None	4218±575	2151±293	3857±383	679±184	3061±358	805±142
Otx2$^{-/-}$	4	None	3982±467	3464±273	n/a	n/a	n/a	n/a
R26$^{Otx2/Otx2}$	4	None	4780±576	1496±246	5342±349	1199±264	3951±149	2343±268

Table S9. Pluripotent, neural and non-neural cells in wt and *Otx2* mutant SFEBs derived from ESCs or EpiSCs

			Number of Oct4$^+$, Sox1$^+$ and Sox1$^-$ Oct4$^-$ cells in SFEBs at day 5 (mean ± s.d.)			
Genotype	**N° of Exp.**	**BMP4 (ng/ml)**	**Total cells**	**Oct4$^+$**	**Sox1$^+$**	**Sox1$^-$ Oct4$^-$**
wt ESC-derived SFEBs	4	—	4974±336	77±18	3730±314	1166±239
Otx2$^{-/-}$ ESC-derived SFEBs	4	—	4595±298	51±25	4357±231	202±108
R26$^{Otx2/Otx2}$ ESC-derived SFEBs	4	—	4240±303	44±10	1191±179	3005±183
wt ESC-derived SFEBs	4	10	5037±663	2450±266	9±3	2576±421
Otx2$^{-/-}$ ESC-derived SFEBs	4	10	4912±578	4213±333	12±7	686±265
wt ESC-derived SFEBs	4	0.5	3987±375	514±86	1943±236	1529±283
R26$^{Otx2/Otx2}$ ESC-derived SFEBs	4	0.5	4013±469	234±84	115±47	3688±281
wt EpiSC-derived SFEBs	4	10	6398±135	13±3	373±136	6021±252
Otx2$^{-/-}$ EpiSC-derived SFEBs	4	10	6478±181	17±4	1735±263	4736±331

Table S10. Pallial and sub-pallial differentiation in wt, *Otx2*$^{-/-}$ and *R26*$^{Otx2/Otx2}$ neural differentiation

			Number of Foxg1^{+} cells co-expressing pallial or sub-pallial markers (mean ± s.d.)			
Genotype	**N° of Exp.**	**Factor (ng/ml)**	**Total Foxg1^{+}**	**Pax6^{+} Foxg1^{+}**	**Total Foxg1^{+}**	**Nkx2.1^{+} Foxg1^{+}**
wt	4	None	1337±125	84±23	1407±146	930±55
Otx2$^{-/-}$	4	None	566±118	18±5	641±108	391±24
R26$^{Otx2/Otx2}$	4	None	1507±136	744±104	1491±125	18±8
wt	4	Dkk1 (500 ng/ml)	1531±145	1069±107	1418±109	131±30
Otx2$^{-/-}$	4	Dkk1 (500 ng/ml)	941±72	21±9	938±70	494±57
R26$^{Otx2/Otx2}$	4	Dkk1 (500 ng/ml)	1509±169	886±96	1493±161	15±5