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Supplementary Materials for

RhoA inhibits neural differentiation in murine stem cells through multiple mechanisms

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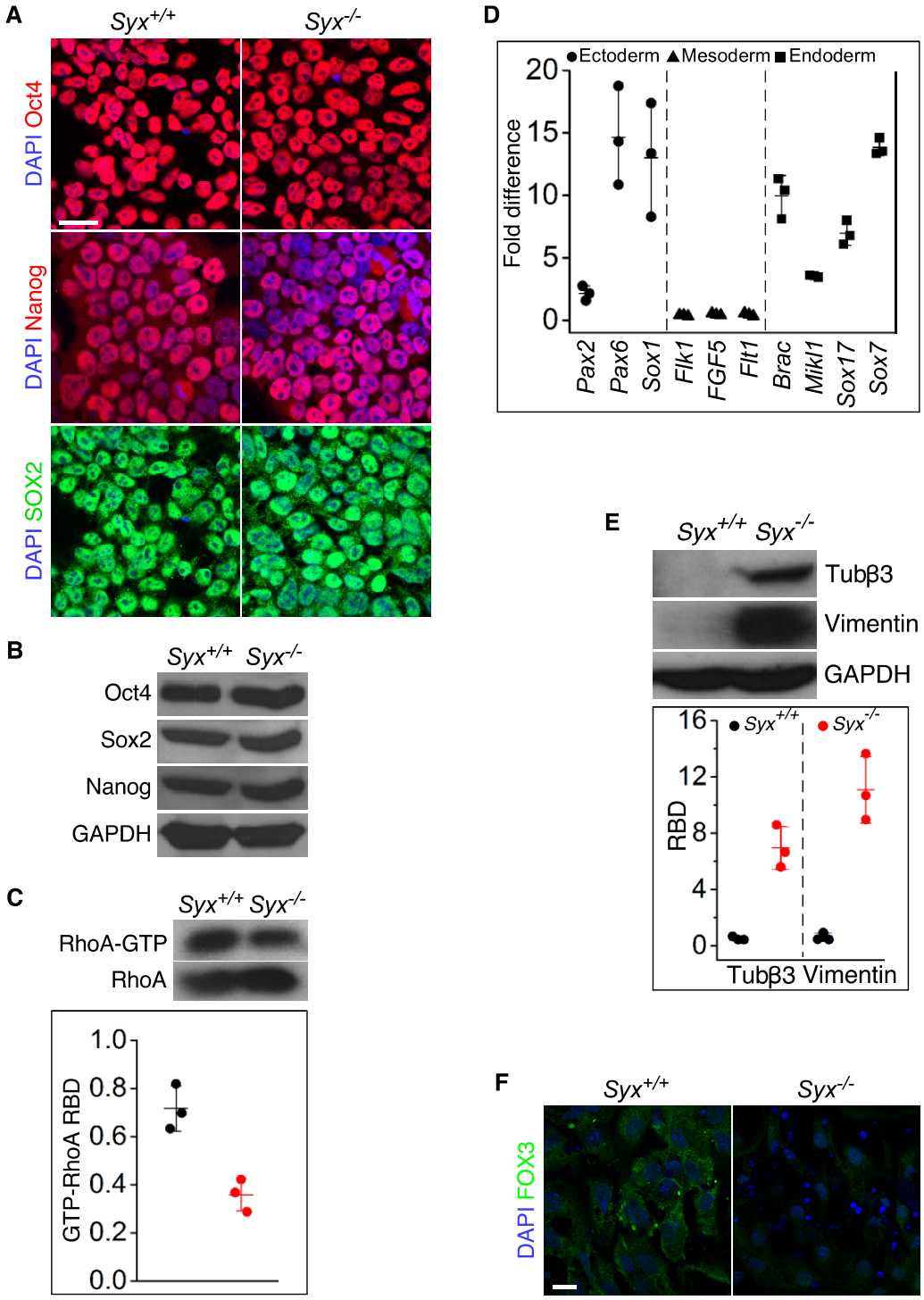

Fig. S1. Comparisons of gene expression patterns and GTP-RhoA abundance in *Syx*$^{-/-}$ EBs or mESCs, respectively, versus their *Syx*$^{+/+}$ counterparts. (A) Immunofluorescence images showing the core pluripotency transcription factors Oct4, Nanog, and Sox2 in *Syx*$^{+/+}$ or *Syx*$^{-/-}$ mESCs (one of two independent experiments). (B) Immunoblots showing the same transcription factors as in A (one of two independent experiments). (C) Immunoblots of RhoA-GTP and of total RhoA in *Syx*$^{+/+}$ and *Syx*$^{-/-}$ mESCs (two sample *t*-test, equal variance, mean ± SD, n = 3 independent experiments, $P = 0.006$). (D) qRT-PCR measurements of the mRNA abundances of the indicated markers of ectodermal, mesodermal, and endodermal

lineages, presented as fold difference of *Syx*$^{-/-}$ relative to *Syx*$^{+/+}$ cells from 13-day old RA-naïve EBs (n=3 replicates). (E) Immunoblots of EBs for the neuronal differentiation markers Tubβ3 and Vimentin (two sample *t* -test, unequal variance, mean ± SD, n = 3 independent experiments, $P = 0.017$ for Tubβ3, $P = 0.015$ for Vimentin). (F) Immunofluorescence images of the neuronal cell marker FOX3 in RA-treated *Syx*$^{+/+}$ or *Syx*$^{-/-}$ mESCs (one of two independent experiments).

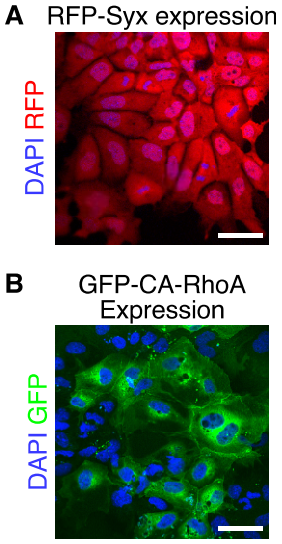

Fig. S2. Transfection of RFP-Syx or GFP–CA-RhoA into *Syx*$^{-/-}$ cells. Immunofluorescence images showing the presence of either (A) RFP-Syx or (B) GFP-CA-RhoA (scale bars, 50 μm) transfected into cells dissociated from RA-treated *Syx*$^{-/-}$ EBs 8 days post aggregation (one of two independent experiments is shown).

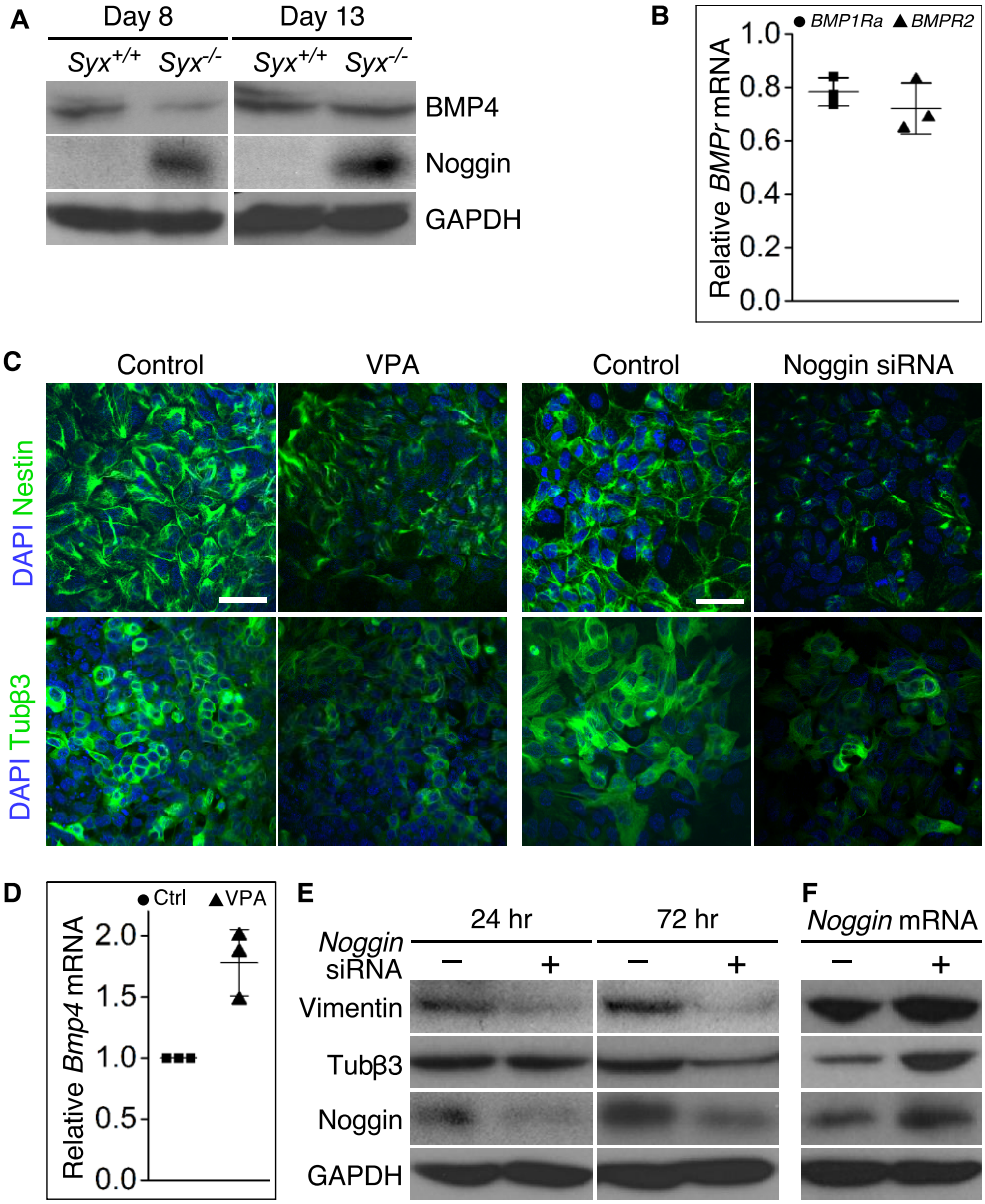

Fig. S3. Noggin production increased during neural differentiation, and VPA inhibited neural differentiation. (A) BMP4 and noggin abundances at the indicated time points after induction of differentiation in 8-day EBs treated with RA (one of two independent experiments). **(**B) qRT-PCR results showing that the expression of Bmp receptors BMPR1a and BMPR2 mRNA in 13-day *Syx*$^{-/-}$ EBs was not substantially different relative to *Syx*$^{+/+}$ EBs (n=3 replicates). (C) Immunofluorescence images showing the effect of 6-day VPA treatment or *Nog* silencing on neural marker expression in differentiating mESCs (scale bars, 50 μm) (one of two independent experiments). (D) qRT-PCR results showing the effect of 6-day 400 μM VPA treatment on *Bmp4* mRNA expression in 13-day *Syx*$^{-/-}$ EBs (two sample *t* -test, unequal variance, mean ± SD, n = 3 independent experiments, $P = 0.008$). (E) *Nog* silencing in *Syx*$^{-/-}$ cells dissociated from differentiating EBs 8 days post aggregation reduced the abundance of the neural differentiation markers vimentin and Tubβ3 (one of two independent experiments). (F) *Nog* transfection into *Syx*$^{+/+}$ cells dissociated from EBs 8 days post aggregation increased the abundance of the neural differentiation markers vimentin and Tubβ3 (one of two independent experiments).

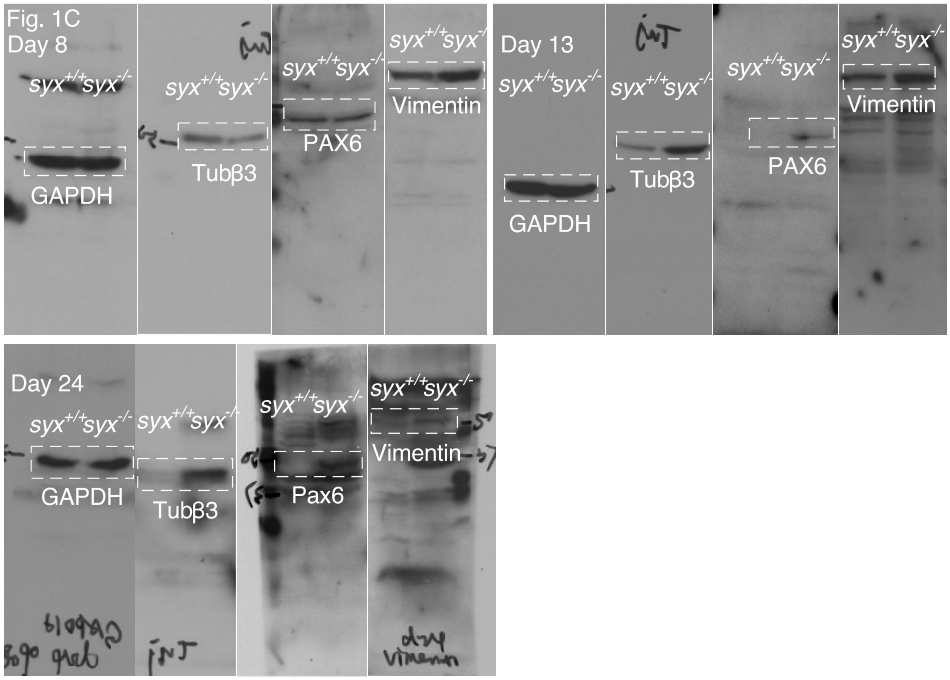
Fig. 1C
Day 8
syx+/+syx-/-
GAPDH
syx+/+syx-/-
Tubβ3
syx+/+syx-/-
PAX6
syx+/+syx-/-
Vimentin
Day 13
syx+/+syx-/-
GAPDH
syx+/+syx-/-
Tubβ3
syx+/+syx-/-
PAX6
syx+/+syx-/-
Vimentin
Day 24
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Tubβ3
syx+/+syx-/-
Pax6
syx+/+syx-/-
Vimentin

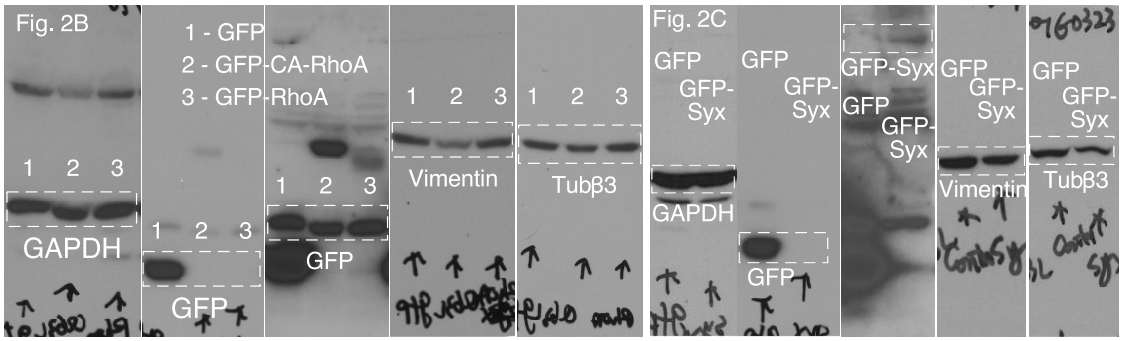
Fig. 2B
1 2 3
GAPDH
1 - GFP
2 - GFP-CA-RhoA
3 - GFP-RhoA
1 2 3
GFP
1 2 3
GFP
1 2 3
Vimentin
1 2 3
Tubβ3
Fig. 2C
GFP
GFP-Syx
GAPDH
GFP
GFP-Syx
GFP
GFP-Syx
GFP
GFP-Syx
Vimentin
GFP
GFP-Syx
Tubβ3

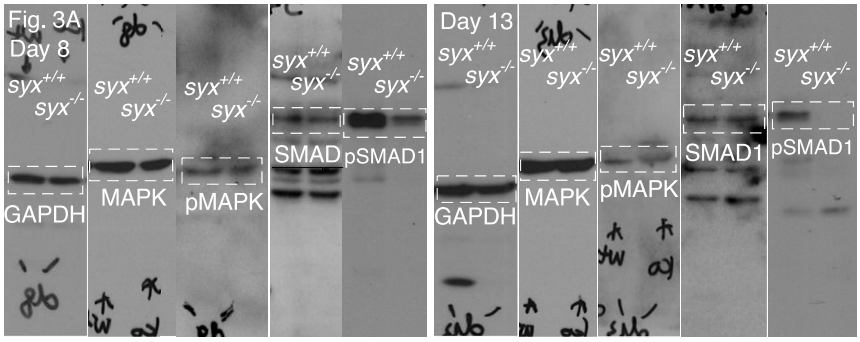
Fig. 3A
Day 8
syx+/+ syx-/-
GAPDH
syx+/+ syx-/-
MAPK
syx+/+ syx-/-
pMAPK
syx+/+ syx-/-
SMAD
syx+/+ syx-/-
pSMAD1
Day 13
syx+/+ syx-/-
GAPDH
syx+/+ syx-/-
MAPK
syx+/+ syx-/-
pMAPK
syx+/+ syx-/-
SMAD1
syx+/+ syx-/-
pSMAD1

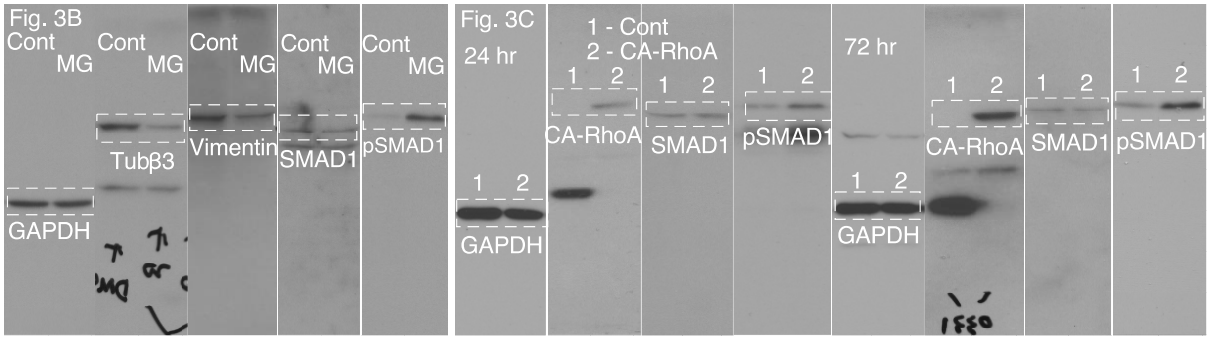
Fig. 3B
Cont MG
GAPDH
Cont MG
Tubβ3
Cont MG
Vimentin
Cont MG
SMAD1
Cont MG
pSMAD1
Fig. 3C
24 hr
1 - Cont
2 - CA-RhoA
1 2
GAPDH
1 2
CA-RhoA
1 2
SMAD1
1 2
pSMAD1
72 hr
1 2
GAPDH
1 2
CA-RhoA
1 2
SMAD1
1 2
pSMAD1

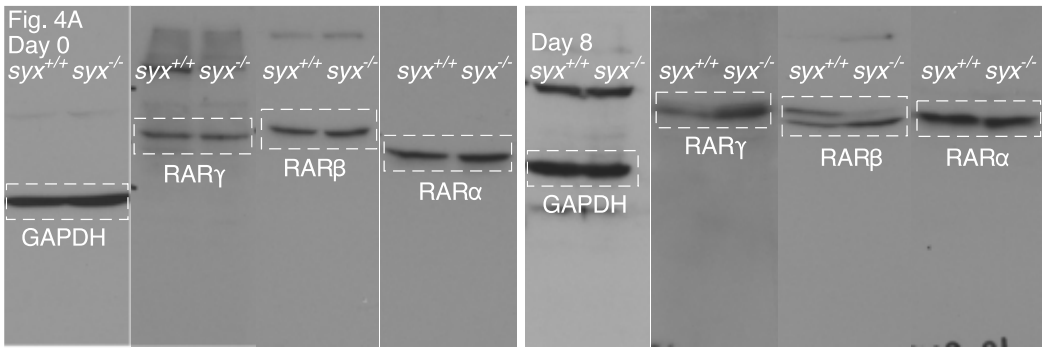
Fig. 4A
Day 0
syx+/+ syx-/-
syx+/+ syx-/-
syx+/+ syx-/-
syx+/+ syx-/-
RARγ
RARβ
RARα
GAPDH
Day 8
syx+/+ syx-/-
syx+/+ syx-/-
syx+/+ syx-/-
syx+/+ syx-/-
RARγ
RARβ
RARα
GAPDH

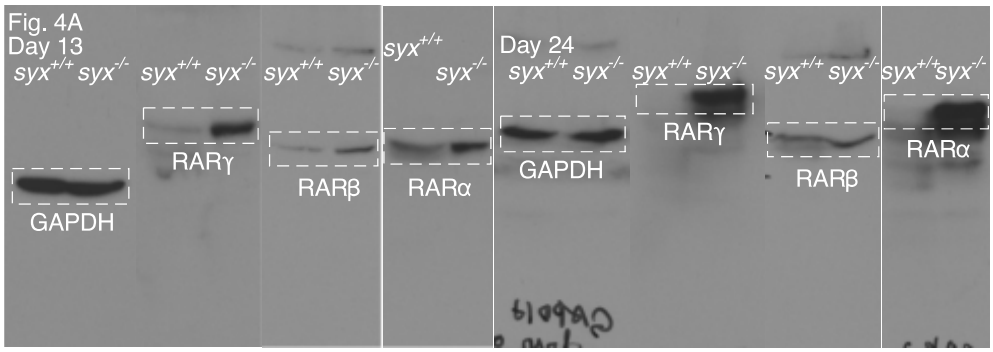
Fig. 4A
Day 13
syx+/+ syx-/-
syx+/+ syx-/-
syx+/+ syx-/-
syx+/+
syx-/-
RARγ
RARβ
RARα
GAPDH
Day 24
syx+/+ syx-/-
syx+/+ syx-/-
syx+/+ syx-/-
syx+/+syx-/-
RARγ
RARβ
RARα
GAPDH

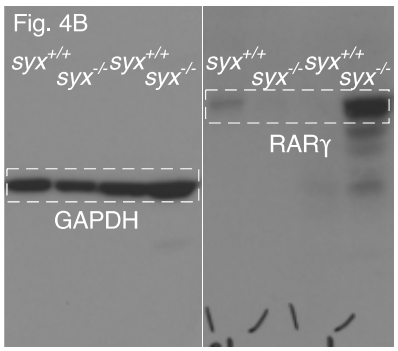
Fig. 4B
syx+/+
syx-/-syx+/+
syx-/-
syx+/+
syx-/-syx+/+
syx-/-
RARγ
GAPDH

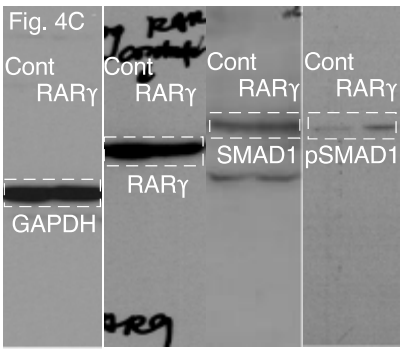
Fig. 4C
Cont
RARγ
Cont
RARγ
Cont
RARγ
Cont
RARγ
RARγ
SMAD1
pSMAD1
GAPDH

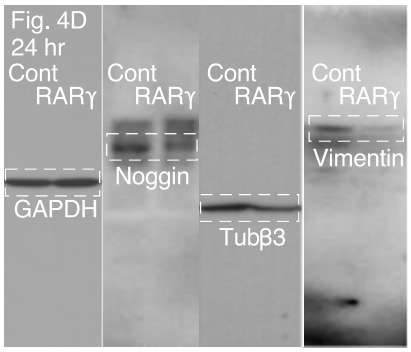
Fig. 4D
24 hr
Cont
RARγ
Cont
RARγ
Cont
RARγ
Cont
RARγ
Noggin
Tubβ3
Vimentin
GAPDH

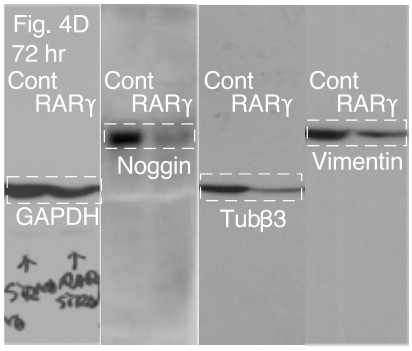
Fig. 4D
72 hr
Cont
RARγ
Cont
RARγ
Cont
RARγ
Cont
RARγ
Noggin
Tubβ3
Vimentin
GAPDH

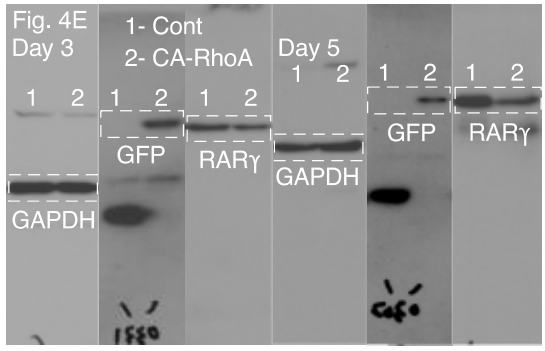
Fig. 4E
Day 3
1- Cont
2- CA-RhoA
Day 5
1 2
1 2
1 2
1 2
1 2
1 2
GFP
RARγ
GAPDH
GFP
RARγ
GAPDH

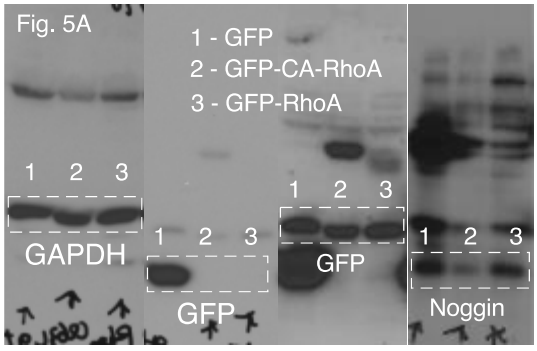
Fig. 5A
1 - GFP
2 - GFP-CA-RhoA
3 - GFP-RhoA
1 2 3
1 2 3
1 2 3
1 2 3
GAPDH
GFP
GFP
Noggin

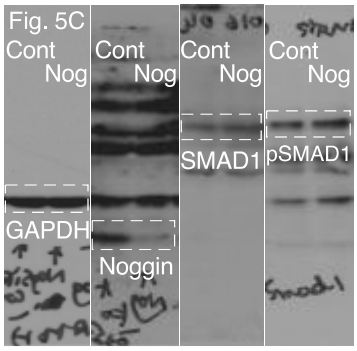
Fig. 5C
Cont
Nog
Cont
Nog
Cont
Nog
Cont
Nog
SMAD1
pSMAD1
GAPDH
Noggin

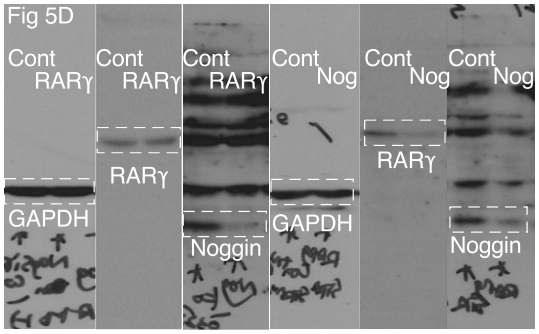
Fig 5D
Cont
RARγ
GAPDH
Noggin
Nog

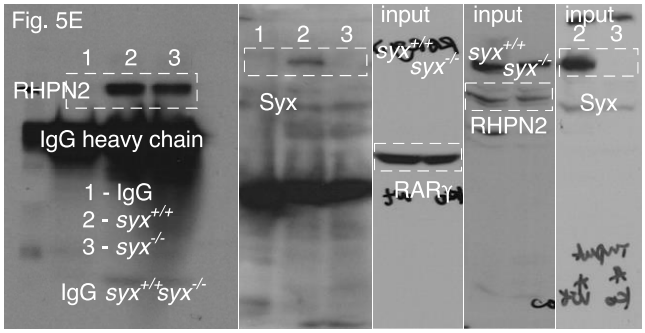
Fig. 5E
1 2 3
RHPN2
IgG heavy chain
1 - IgG
2 - syx+/+
3 - syx-/-
IgG syx+/+syx-/-
Syx
input
RHPN2
RARγ

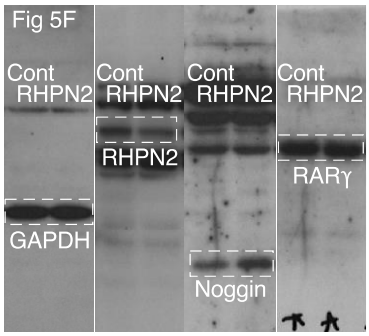
Fig 5F
Cont
RHPN2
RARγ
GAPDH
Noggin

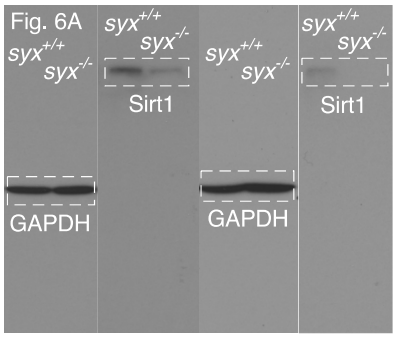
Fig. 6A
syx+/+
syx-/-
Sirt1
GAPDH

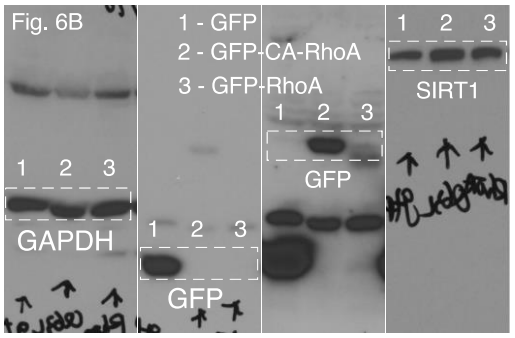
Fig. 6B
1 - GFP
2 - GFP-CA-RhoA
3 - GFP-RhoA
GAPDH
GFP
SIRT1

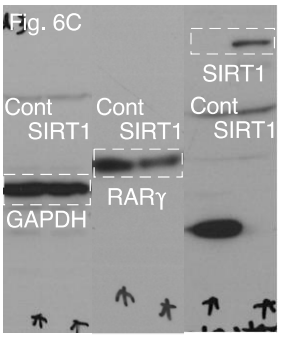
Fig. 6C
Cont
SIRT1
RARγ
GAPDH

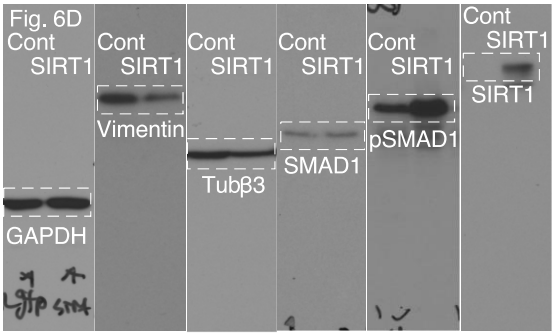
Fig. 6D
Cont
SIRT1
Vimentin
Tubβ3
GAPDH
SMAD1
pSMAD1

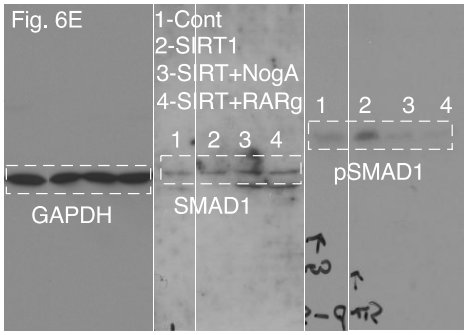
Fig. 6E
1-Cont
2-SIRT1
3-SIRT+NogA
4-SIRT+RARg
GAPDH
SMAD1
pSMAD1

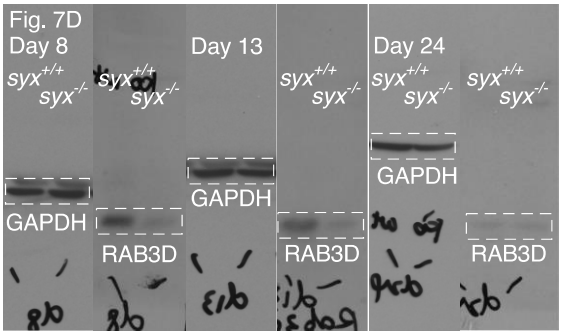
Fig. 7D
Day 8
Day 13
Day 24
syx+/+
syx-/-
GAPDH
RAB3D

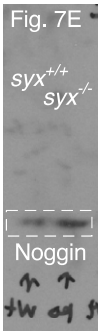
Fig. 7E
syx+/+
syx-/-
Noggin

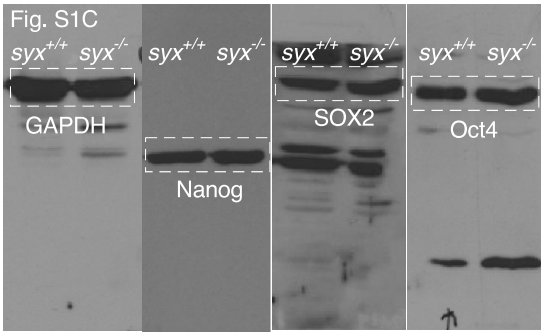
Fig. S1C
syx+/+
syx-/-
GAPDH
Nanog
SOX2
Oct4

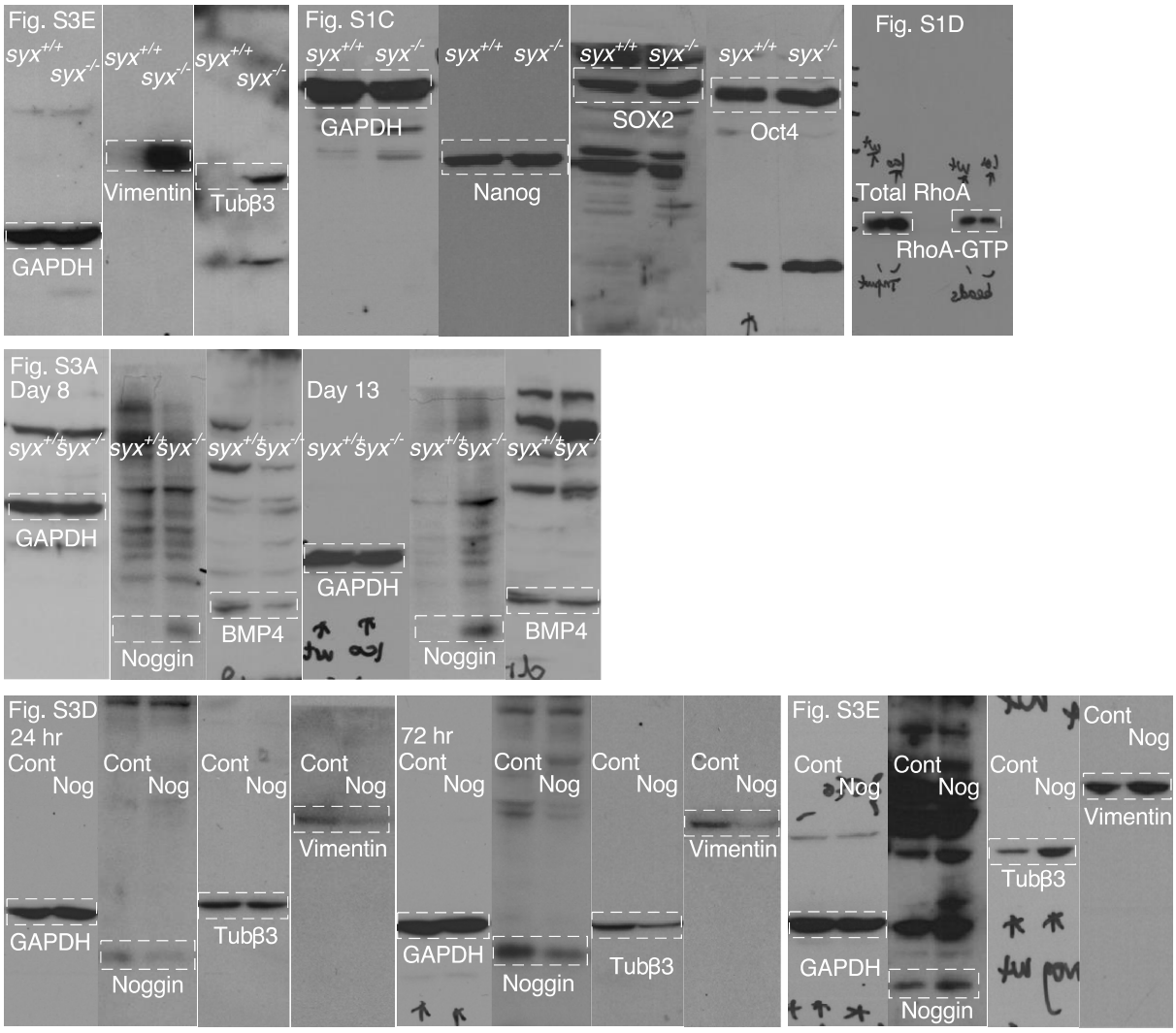

Fig. S4. Full-length images of the immunoblots shown in Figs. 1 to 7 and figs. S1 and S3. Lane identities are marked at the top; band identities appear under the frame surrounding the bands.

Gene	Primer sequence
Bmp4	F: 5’-TGCCATTGTGCAGACCCTAG-3’
	R: 5’-CACCACCTTGTCATACTCATCCAG-3’
Bmpr1a	F: 5'-GGGAGAAATCAAAAGGGGACA-3'
	R: 5'-AATTGAGGGTGGGGTGGTAGT-3'
Bmpr2	F: 5'-CCTATGAGGACATGCGTGAGGT-3'
	R: 5'-TGTGAGTCTGGAGGCTGGATTA-3'
Brac	F: 5’-GCGGACAATTCATCTGCTTG-3’
	R: 5’-AGTAGGTGGGCTGGCGTTAT-3’
Fgf5	F: 5’-CGTCTTCTGCCTCCTCACCA-3’
	R: 5’-AGTAGGTGGGCTGGCGTTAT-3’
Fgf8	F: 5’-ATGGCAGAAGACGGAGACCC-3’
	R: 5’-CTTGCCTTTGCCGTTGCTC-3’
Flk1	F: 5’-CCTGCCTACCTCACCTGTTT-3’
	R: 5’-GCTCTTTCGCTTACTGTTCTG-3’
Flt1	F: 5’-GGTCCTCGTTCCAGTCTTTC-3’
	R:5’-GTCTTCCTGCTGTGGTTTCC-3’
Foxa1	F: 5’-GTGGATCATGGACCTCTTCCC-3’
	R: 5’-CGTGCCACCTTGACGAAAC-3’
Gapdh	F: 5’-CCTTCCGTGTTCCTACCCCC-3’
	R: 5’-AGCCCAAGATGCCCTTCAGT-3’
Mixl1	F: 5’-TTCCGACAGACCATGTACCCA-3’
	R: 5’-GGCTGAAATGACTTCCCACTCT-3’
Ngfr	F: 5’-CCAGAGCGAGACCTCATAGCC-3’
	R: 5’-CACAACCACAGCAGCCAAGAT-3’
Noggin	F: 5’-CATGCCGAGCGAGATCAAA-3’
	R: 5’-CAGCCACATCTGTAACTTCCTCC-3’
Oct4	F: 5’-GATCACTCACATCGCCAATCA-3’
	R: 5’-CTGTAGCCTCATACTCTTCTCGTT-3’
Pax2	F: 5’-AACGGTGAGAAGAGGAAACG-3’
	R: 5’-CTGCTGCTGGGTGAAGGT-3’
Pax6	F: 5’-CACGTACAGTGCTTTGCCACC-3’
	R: 5’-TATCATAACTCCGCCCATTCA-3’
Pitx2	F: 5’-ACCTTACGGAAGCCCGAGTC-3’
	R: 5’-CAAAGCCATTCTTGCACAGC-3’
Sox1	F: 5’-GGCGGCATCCCTTACG-3’
	R: 5’-GGCTCCGACTTGACCAGA-3’
Sox7	F: 5’-ATTACTCCCATGCCACCTACC-3’
	R: 5’-TGTCTCCCAGAAGTTCCACC-3’
Sox17	F: 5’-ATACGCCAGTGACGACCAGAG-3’
	R: 5’-CCTCGCCTTTCACCTTTACATC-3'

Table S1. Primer sequences for qRT-PCRs.