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Supplementary Materials for

Augmented BMP signaling commits cranial neural crest cells to a chondrogenic fate by suppressing autophagic β-catenin degradation

Jingwen Yang, Megumi Kitami, Haichun Pan, Masako Toda Nakamura, Honghao Zhang, Fei Liu, Lingxin Zhu, Yoshihiro Komatsu*, Yuji Mishina*

*Corresponding author. Email: yoshihiro.komatsu@uth.tmc.edu (Y.K.); mishina@umich.edu (Y.M.)

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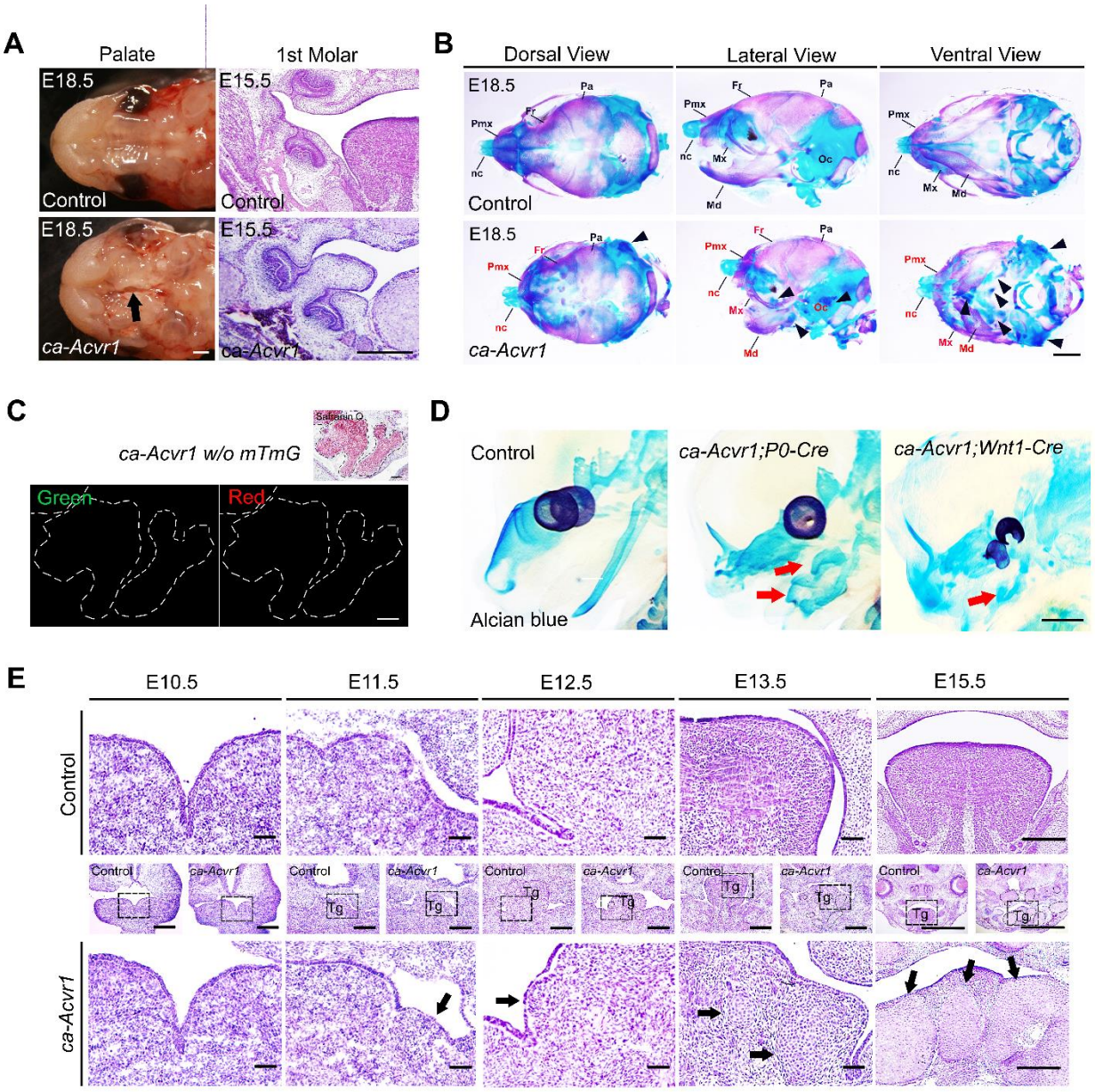
A
Palate
1st Molar
E18.5
E15.5
Control
Control
E18.5
E15.5
ca-Acvr1
ca-Acvr1
B
Dorsal View
Lateral View
Ventral View
E18.5
Pa
Fr
Pmx
nc
Mx
Oc
Md
Control
E18.5
ca-Acvr1
C
ca-Acvr1 w/o mTmG
Safranin O
Green
Red
D
Control
ca-Acvr1;P0-Cre
ca-Acvr1;Wnt1-Cre
Alcian blue
E
E10.5
E11.5
E12.5
E13.5
E15.5
Control
ca-Acvr1
Control
ca-Acvr1
Tg

Fig. S1. Phenotypes due to expression of ca-ACVR1 in CNCCs during craniofacial development. (**A**) Representative images showing the gross morphology of the palate and histological morphology of the first molar in control and ca-*Acvr1* mutant embryos. Scale bars, 500 μm. n=3 mice per group. Black arrow indicates the cleft palate in mutant. (**B**) Representative images showing the cartilage and bone structures of the heads from control and ca-*Acvr1* embryos. Scale bars, 1 mm. n=3 mice per group. Arrowheads indicate ectopic cartilages, and red labels indicate defective structures. Fr, frontal bone; Pa, parietal bone; Pmx, premaxilla bone; Mx, maxilla; Md, mandible; mc, Meckel's cartilage; nc, nasal capsule; Oc, otic capsule. (**C**) Representative images showing GFP and RFP signals in ectopic cartilages in a coronal section of a mutant embryo without the *R26R*^{mTmG} reporter. Pictures were taken using the same parameters as the images in Fig. 1F. Scale bar, 100 μm. n=3 mice per group. (**D**) Representative images showing the cartilage structures in heads of mutant embryos with the indicated genotypes. Red arrows indicate ectopic cartilages. Scale bar, 500 μm. n=3 mice per group. (**E**) Histological analysis of BA1 tissues during development. Representative coronal head sections (H&E staining) of control and mutant embryos at indicated time points are shown. The BA1 tissues (E10.5) and the tongue (E11.5-E15.5) that are shown in high magnification in the top and bottom rows are indicated in the lower magnification images in the middle row by black boxes. Scale bars, 300 μm (low magnification) and 50 μm (high magnification)). n=3 mice per group. Black arrows indicate abnormal structures.

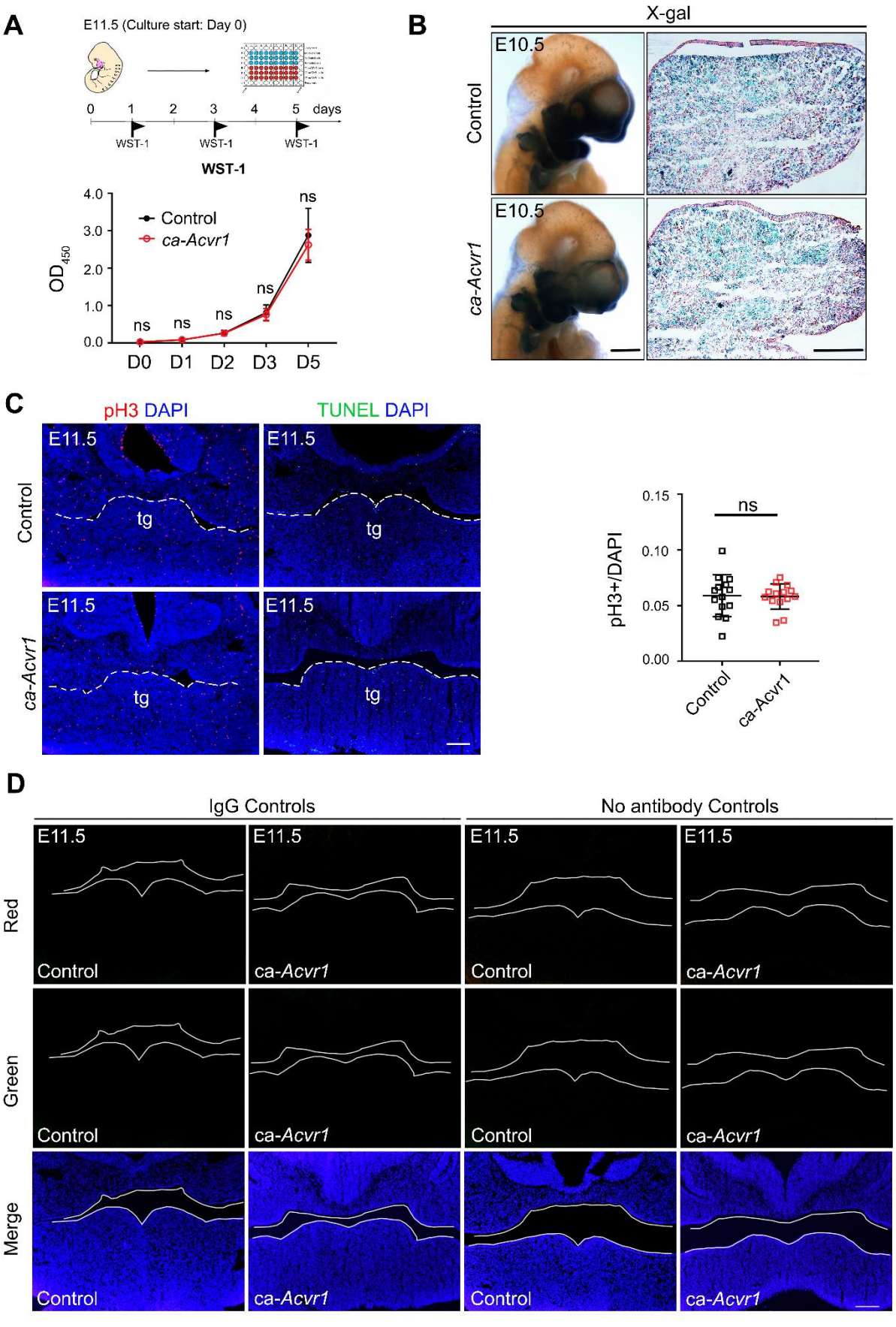
A
E11.5 (Culture start: Day 0)
0 1 2 3 4 5 days
WST-1
WST-1
WST-1
WST-1
4.0
3.0
2.0
1.0
0.0
OD450
Control
ca-Acvr1
ns
ns
ns
ns
ns
D0 D1 D2 D3 D5
B
X-gal
E10.5
E10.5
Control
ca-Acvr1
C
pH3 DAPI
TUNEL DAPI
E11.5
E11.5
E11.5
E11.5
Control
ca-Acvr1
tg
tg
tg
tg
0.15
0.10
0.05
0.00
pH3+/DAPI
ns
Control
ca-Acvr1
D
IgG Controls
No antibody Controls
E11.5
E11.5
E11.5
E11.5
Red
Green
Merge
Control
ca-Acvr1
Control
ca-Acvr1
Control
ca-Acvr1
Control
ca-Acvr1
Control
ca-Acvr1
Control
ca-Acvr1

Fig. S2. Cell migration, proliferation, and survival are unaltered in ca-*Acvr1* mutants. (**A**) The proliferation rate of CNCCs isolated from BA1 tissues (BA1 cells). n=3 independent experiments. (**B**) In vivo fate mapping of CNCCs expressing β-galactosidase in control and mutant embryos at E10.5. Scale bars, 1 mm (whole-mount), 100 μm (tissue sections). n=3 mice per group. (**C**) Representative images of pH3 (red) immunofluorescence and TUNEL staining (green) in coronal head sections from control or mutant embryos at E11.5. Nuclei are labelled with DAPI (blue). The percentage of pH3-positive cells in the CNCCs of BA1 tissues were quantified. Scale bar, 100 μm. n=13 sections from 4 embryos per group. (**D**) Representative images showing GFP (green) or RFP (red) signal in coronal sections of control and mutant embryos without primary antibody (negative controls) or with IgG as primary antibody (IgG controls). Pictures were taken using the same parameters as images in Figs. 2A, 3A, and 3B. Scale bar, 100 μm. n=3 mice per group. Error bars are mean ± s.d. ns $P > 0.05$; t-test.

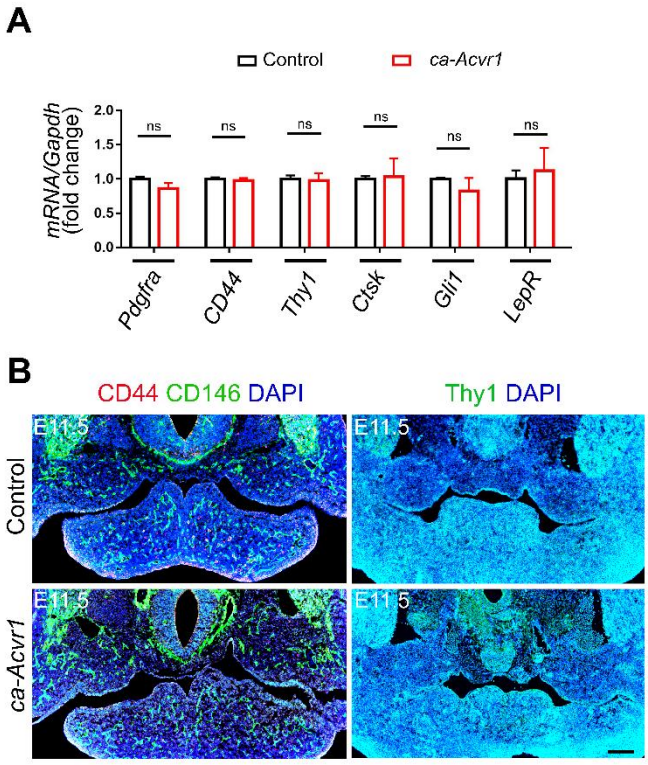

Fig. S3. Skeletal stem cell populations are unaltered in ca-*Acvr1* mutants. (**A**) Quantification of the expression of the skeletal stem cells markers *Pdgfra*, *CD44*, *Thy1*, *Ctsk*, *Gli1*, and *LepR* in BA1 cells from control and mutant embryos. n=3 independent experiments. (**B**) Representative images of CD146 (Green), CD44 (red), and Thy1 (green) immunofluorescence in coronal head sections from control and mutant embryos at E11.5. Nuclei were stained with DAPI (blue). Scale bar, 100 μm. n=3 mice per group. Error bars are mean ± s.d. ns $P > 0.05$; t-test.

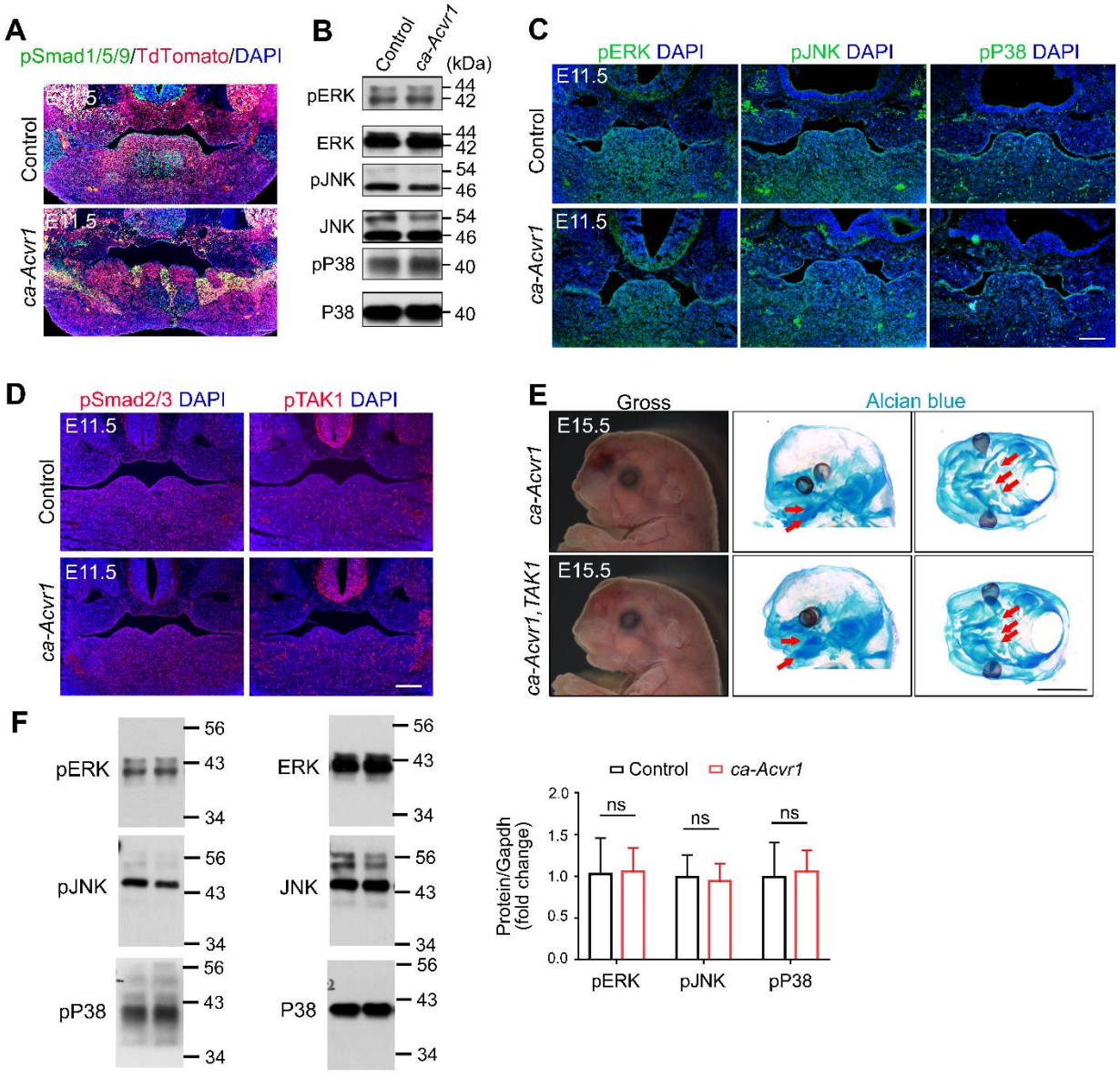
A
pSmad1/5/9/TdTomato/DAPI
Control
ca-Acvr1
E11.5
B
Control
ca-Acvr1
(kDa)
pERK
ERK
pJNK
JNK
pP38
P38
44
42
54
46
40
C
pERK DAPI
pJNK DAPI
pP38 DAPI
Control
ca-Acvr1
E11.5
D
pSmad2/3 DAPI
pTAK1 DAPI
Control
ca-Acvr1
E11.5
E
Gross
Alcian blue
ca-Acvr1
ca-Acvr1; TAK1
E15.5
F
pERK
pJNK
pP38
ERK
JNK
P38
56
43
34
Control
ca-Acvr1
Protein/Gapdh (fold change)
2.0
1.5
1.0
0.5
0.0
ns

Fig. S4. Noncanonical BMP signaling and TGF-β signaling are unaffected in ca-*Acvr1* mutants. (**A**) Representative images showing tdTomato (red) and pSmad1/5/9 (green) immunofluorescence in coronal head sections from control and mutant embryos carrying the *R26R*^{tdTomato} reporter at E11.5. Nuclei are labelled with DAPI (blue). Scale bar, 100 μm. n=3 mice per group. (**B**) Western blot analysis of pERK, ERK, pJNK, JNK, pP38, and P38 in control and mutant BA1 tissues at E11.5. n=3 independent experiments. Uncropped western blot images and quantification are shown in (F). (**C**) Representative images showing pERK (green), pJNK (green), or pP38 (green) immunofluorescence in coronal head sections from control and mutant embryos at E11.5. Scale bars, 200 μm. n=3 mice per group. (**D**) Representative images showing pTak1 (red) or pSmad2 (red) immunofluorescence in coronal head sections from control and mutant embryos at E11.5. Scale bar, 200 μm. n=3 mice per group. (**E**) Representative images showing gross morphology and whole mount Alcian blue staining of the heads of E15.5 embryos from *Tak1*$^{+/+}$;ca-*Acvr1*$^{flox/+}$;*P0-Cre* (ca-*Acvr1*) and *Tak1*$^{flox/flox}$;ca-*Acvr1*$^{flox/+}$;*P0-Cre* (ca-*Acvr1*, *TAK1*) embryos. Scale bar, 1 mm. n= 4 mice per group. Red arrows indicate ectopic cartilages. (**F**) Uncropped western blot images for (B) and quantification results. Error bars are mean ± s.d. ns $P > 0.05$; t-test.

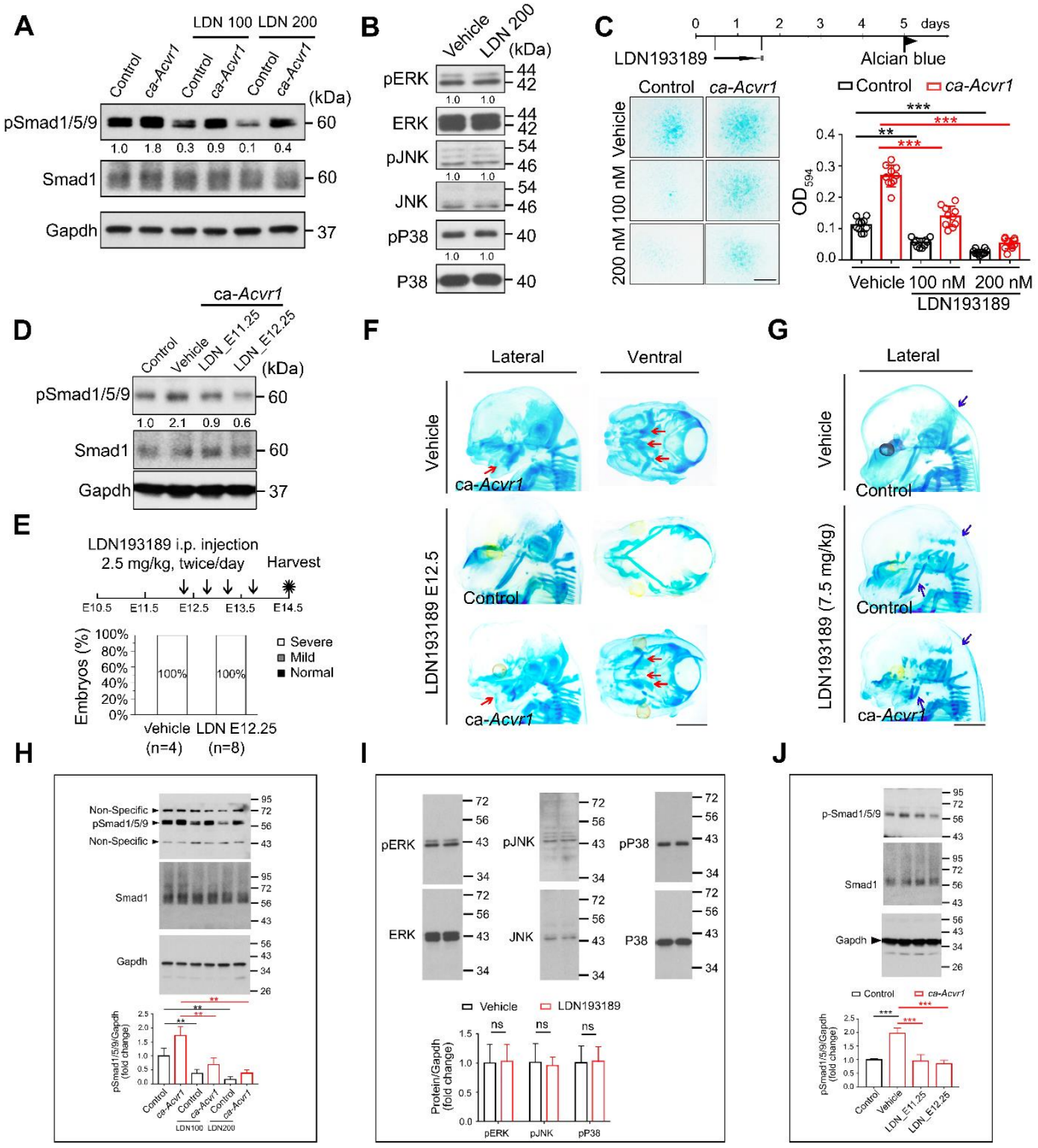
A
LDN 100
LDN 200
Control
ca-Acvr1
Control
ca-Acvr1
Control
ca-Acvr1
(kDa)
pSmad1/5/9
60
1.0 1.8 0.3 0.9 0.1 0.4
Smad1
60
Gapdh
37
B
Vehicle
LDN 200
(kDa)
pERK
44
42
1.0 1.0
ERK
44
42
pJNK
54
46
1.0 1.0
JNK
54
46
pP38
40
1.0 1.0
P38
40
C
0 1 2 3 4 5 days
LDN193189
Alcian blue
Control
ca-Acvr1
Vehicle
100 nM
200 nM
Control
ca-Acvr1
***
**
***
***
OD594
0.4
0.3
0.2
0.1
0.0
Vehicle
100 nM
200 nM
LDN193189
D
ca-Acvr1
Control
Vehicle
LDN_E11.25
LDN_E12.25
(kDa)
pSmad1/5/9
60
1.0 2.1 0.9 0.6
Smad1
60
Gapdh
37
E
LDN193189 i.p. injection
2.5 mg/kg, twice/day
Harvest
E10.5
E11.5
E12.5
E13.5
E14.5
Embryos (%)
100%
80%
60%
40%
20%
0%
100%
100%
Severe
Mild
Normal
Vehicle
(n=4)
LDN E12.25
(n=8)
F
Lateral
Ventral
Vehicle
ca-Acvr1
LDN193189 E12.5
Control
ca-Acvr1
G
Lateral
Vehicle
Control
LDN193189 (7.5 mg/kg)
Control
ca-Acvr1
H
Non-Specific
pSmad1/5/9
Non-Specific
95
72
56
43
Smad1
95
72
56
43
Gapdh
56
43
34
26
pSmad1/5/9/Gapdh (fold change)
Control
ca-Acvr1
Control
ca-Acvr1
Control
ca-Acvr1
LDN100
LDN200
I
pERK
ERK
pJNK
JNK
pP38
P38
72
56
43
34
Vehicle
LDN193189
ns
Protein/Gapdh (fold change)
pERK
pJNK
pP38
J
p-Smad1/5/9
Smad1
Gapdh
95
72
56
43
34
26
Control
ca-Acvr1
pSmad1/5/9/Gapdh (fold change)
Control
Vehicle
LDN_E11.25
LDN_E12.25

Fig. S5. LDN193189 suppresses Smad1/5/9 phosphorylation and chondrogenesis of BA1 cells in a concentration- and time-dependent manner. (**A**) Western blot analysis of pSmad1/5/9, total Smad1, and Gapdh in control and mutant BA1 cells stimulated with LDN193189 (100 nmol or 200 nmol) for 24 hr in the absence of ligands. n=3 independent experiments. Uncropped images and quantification are shown in (H). (**B**) Western blot analysis of pERK, ERK, pJNK, JNK, pP38, and P38 in control BA1 cells stimulated with LDN193189 (200 nmol) for 24 hr. n=3 independent experiments. Uncropped images and quantification are shown in (I). (**C**) Scheme, representative images of Alcian blue staining, and optical density quantification assessing chondrogenesis of BA1 cells treated as indicated. Scale bar, 1 mm. n=10 independent experiments. **(D)** Western blot analysis of pSmad1/5/9, total Smad1, and Gapdh in control and mutant BA1 tissues treated with LDN193189. n=3 mice per group. Uncropped images and quantification are shown in (J). (**E and F**) The ratios of each cartilage phenotype (E), and representative Alcian blue stains showing the cranial cartilage phenotypes (F) of embryos treated with LDN193189 in utero from E12.25 to E13.5. Scale bar, 1 mm. The numbers of mice examined are shown in parentheses. Red arrows indicate ectopic cartilages. (**G**) Representative Alcian blue stains showing cartilage phenotypes in embryos treated with LDN193189 in utero from E11.5 to E13.5. n=3 mice per group. Blue arrows indicate abnormal cartilages. (**H to J**) Uncropped western blot images for (A), (B), and (D), respectively, and quantification results. Error bars are mean ± s.d. ns $P > 0.05$; ** $P < 0.01$; and *** $P < 0.001$; t-test (I); ANOVA (C, H, J).

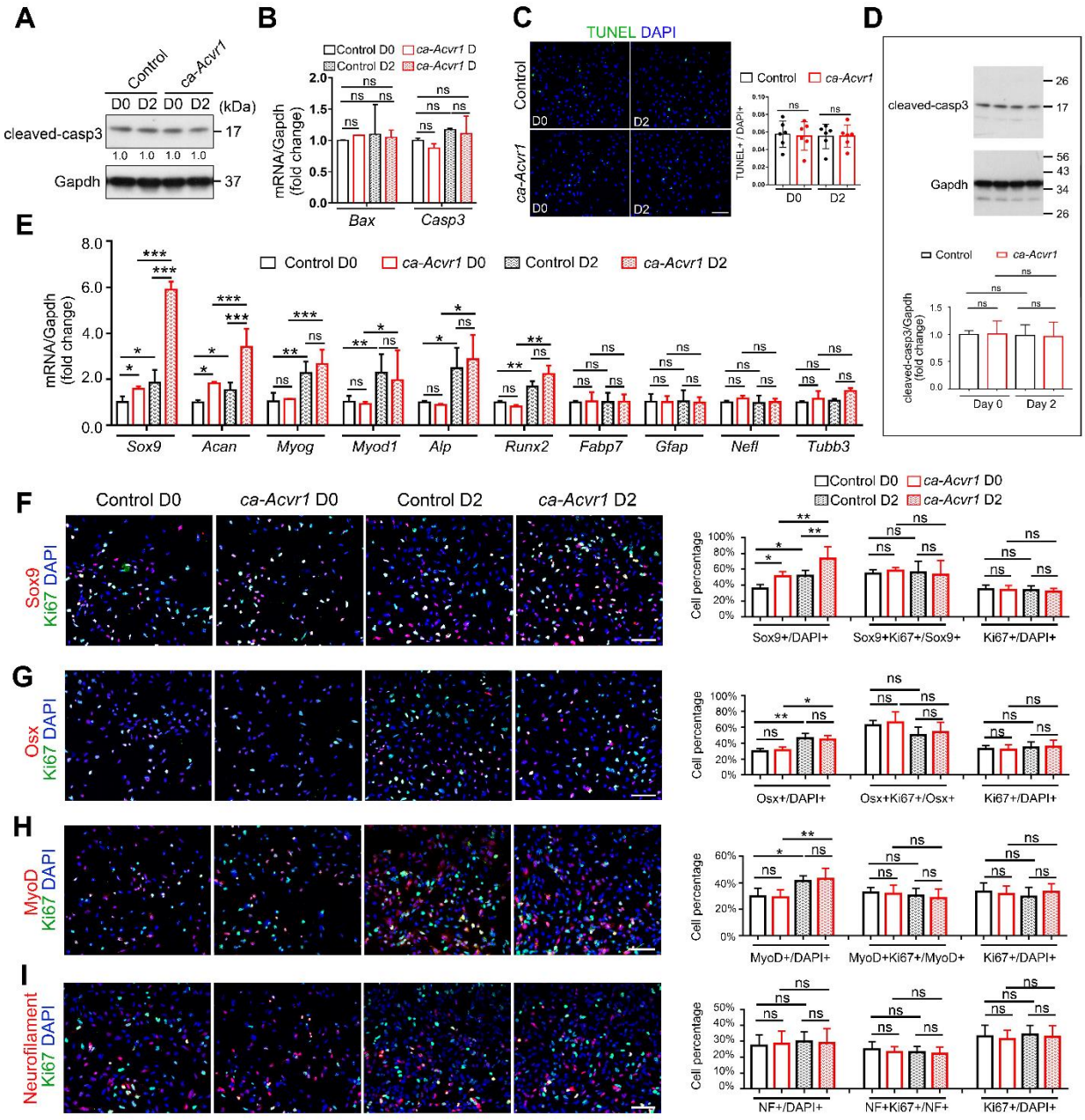
A
Control
ca-Acvr1
D0 D2 D0 D2 (kDa)
cleaved-casp3
17
1.0 1.0 1.0 1.0
Gapdh
37
B
Control D0
ca-Acvr1 D0
Control D2
ca-Acvr1 D2
mRNA/Gapdh (fold change)
Bax
Casp3
C
TUNEL DAPI
Control
ca-Acvr1
D0
D2
TUNEL+ / DAPI+
D
cleaved-casp3
Gapdh
26
17
56
43
34
26
cleaved-casp3/Gapdh (fold change)
Day 0
Day 2
E
mRNA/Gapdh (fold change)
Sox9
Acan
Myog
Myod1
Alp
Runx2
Fabp7
Gfap
Nefl
Tubb3
F
Control D0
ca-Acvr1 D0
Control D2
ca-Acvr1 D2
Sox9
Ki67
DAPI
Cell percentage
Sox9+/DAPI+
Sox9+Ki67+/Sox9+
Ki67+/DAPI+
G
Osx
Ki67
DAPI
Osx+/DAPI+
Osx+Ki67+/Osx+
Ki67+/DAPI+
H
MyoD
Ki67
DAPI
MyoD+/DAPI+
MyoD+Ki67+/MyoD+
Ki67+/DAPI+
I
Neurofilament
Ki67
DAPI
NF+/DAPI+
NF+Ki67+/NF+
Ki67+/DAPI+

Fig. S6. Increased commitment of BA1 cells to the chondrocyte fate in ca-*Acvr1* mutants. (**A**) Western blot analysis of cleaved-caspase 3 in control and mutant BA1 cells on day 0 and day 2 of culture. n=3 independent experiments. Uncropped images and quantification are shown in (D). (**B**) Relative expression of *Bax* and *Casp3* in BA1 cells on day 0 and day 2 in micromass culture. n=3 independent experiments. (**C**) Representative images and quantification of TUNEL staining in coronal head sections from control and mutant embryos at E11.5. Nuclei were stained with DAPI. Scale bar, 100 μm. n=3 mice per group. n=9 sections from 4 embryos per group. (**D**) Uncropped western blot images for (A) and quantification results. (**E**) Relative expression of chondrogenic markers (*Sox9, Acan*), myogenic markers (*Myog, MyoD1*), osteogenic markers (*Runx2, Alp*), and neurogenic markers (*Nefl, Tubb3, Fabp7,* and *Gfap*) in BA1 cells on day 0 and day 2 in micromass culture. n=3 independent experiments. (**F to I**) Representative double immunostaining images and quantification of the lineage markers Sox9 (F), Osx (G), MyoD (H) and Neurofilament (I) and the cell proliferation marker Ki67 in control and mutant BA1 cells at day 0 and day 2 of micromass culture. Scale bars, 100 μm. The percentages of single- and double-positive cells were quantified. n = 6 independent experiments. Error bars are mean ± s.d. ns $P > 0.05$; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; t-test (C); ANOVA (B, D-I).

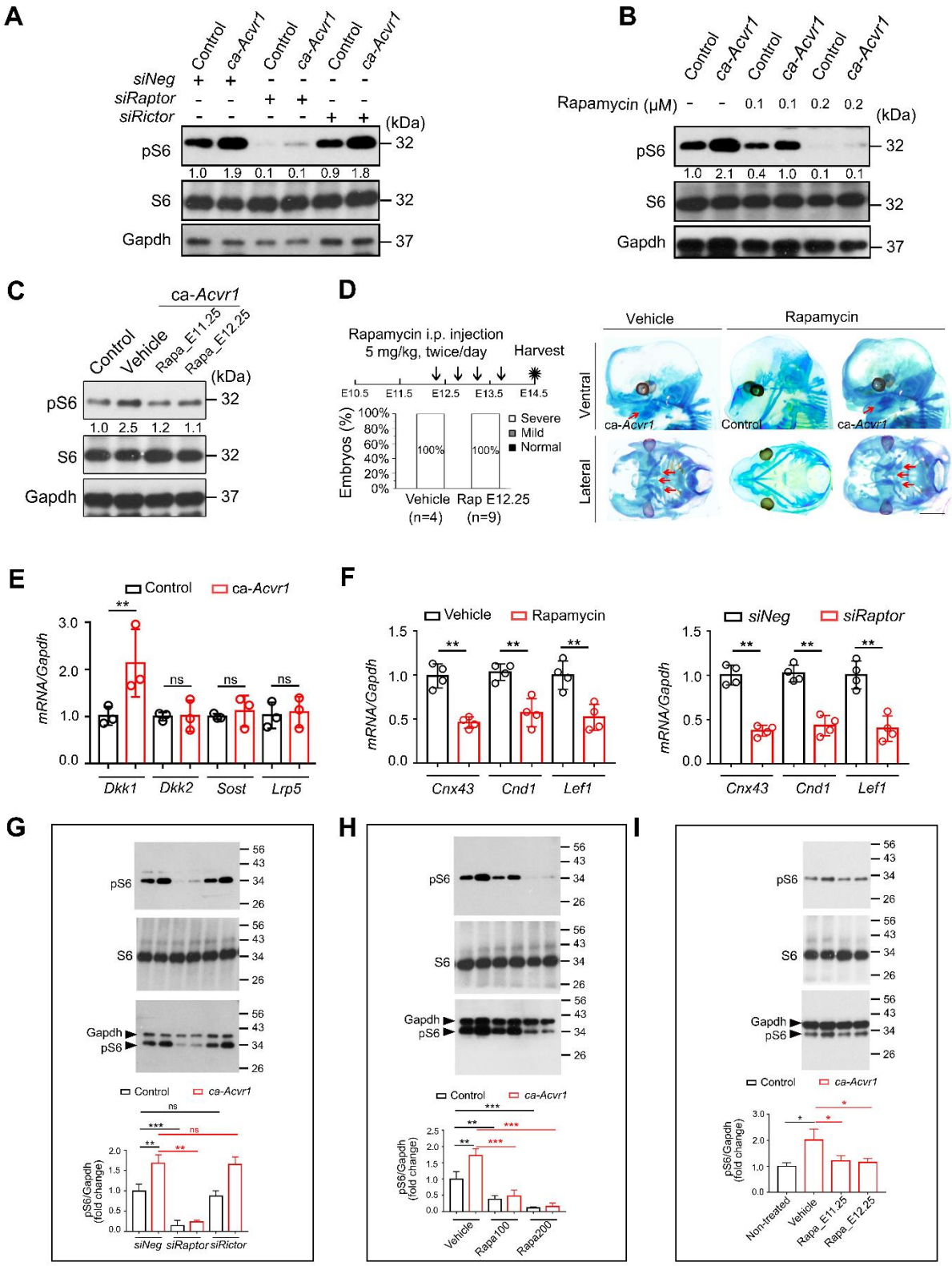
A
Control
ca-Acvr1
siNeg
siRaptor
siRictor
(kDa)
pS6
S6
Gapdh
32
37
1.0 1.9 0.1 0.1 0.9 1.8
B
Rapamycin (μM)
1.0 2.1 0.4 1.0 0.1 0.1
C
ca-Acvr1
Control
Vehicle
Rapa_E11.25
Rapa_E12.25
1.0 2.5 1.2 1.1
D
Rapamycin i.p. injection
5 mg/kg, twice/day
Harvest
E10.5 E11.5 E12.5 E13.5 E14.5
Embryos (%)
Severe
Mild
Normal
100%
Vehicle (n=4)
Rap E12.25 (n=9)
Vehicle
Rapamycin
Ventral
Lateral
E
mRNA/Gapdh
Dkk1
Dkk2
Sost
Lrp5
ns
F
Vehicle
Rapamycin
siNeg
siRaptor
Cnx43
Cnd1
Lef1
G
pS6/Gapdh (fold change)
siNeg
siRaptor
siRictor
H
Vehicle
Rapa100
Rapa200
I
Non-treated
Vehicle
Rapa_E11.25
Rapa_E12.25

Fig. S7. *siRaptor* or rapamycin suppresses mTORC1 signaling and Wnt–β-catenin signaling. (**A, B**) Western blot analysis of pS6, total S6, and Gapdh in control and mutant BA1 cells transfected with *siRaptor* or *siRictor* (A) or stimulated with the indicated concentrations rapamycin for 24 hr (B). n=3 independent experiments. Uncropped images and quantification are shown in (G) and (H), respectively. (**C**) Western blot analysis of pS6, total S6, and Gapdh in control and mutant BA1 tissues of embryos treated in utero with rapamycin. n=3 mice per group. Uncropped images and quantification are shown in (I). (**D**) Scheme, the ratios of each cartilage phenotype (severe, mild, and normal), and representative showing the head cartilage phenotypes of the embryos treated with rapamycin in utero from E12.25 to E13.5. Scale bar, 1 mm. The numbers of mice examined are shown in parentheses. Red arrows indicate ectopic cartilages. (**E**) Relative expression of *Dkk1*, *Dkk2*, *Sost,* and *Lrp5* in BA1 tissues of control and mutant embryos. n=4 mice per group. (**F**) Relative expression of *Cnx43*, *Ccnd1,* and *Lef1* in BA1 cells transfected with *siRaptor* or stimulated with rapamycin for 24 hr. n=4 independent experiments. (**G to I**) Uncropped western blot images for (A to C), respectively, and quantification results. Error bars are mean ± s.d. ns $P > 0.05$; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; t-test (E, F); ANOVA (G-I).

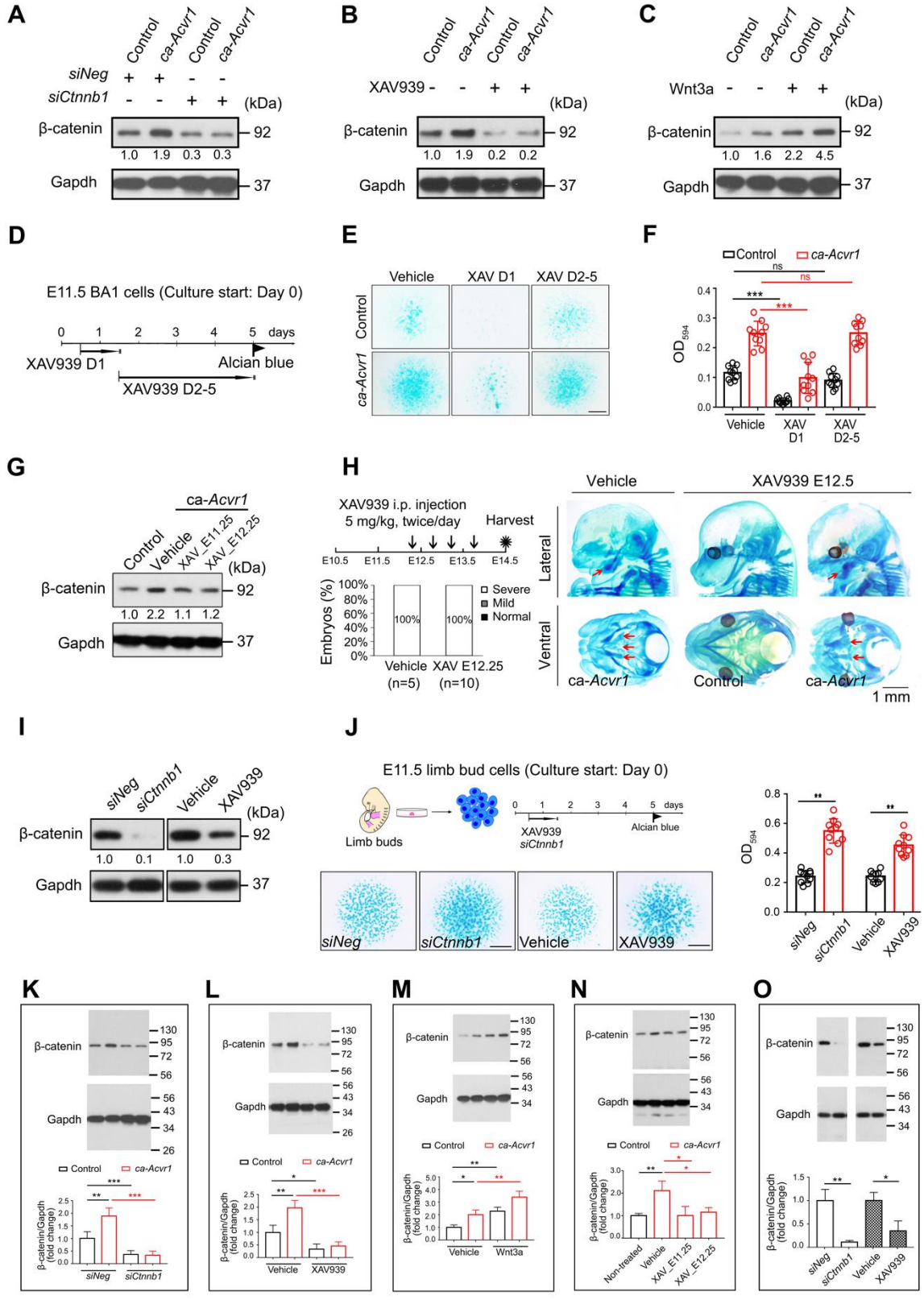
A
Control
ca-Acvr1
Control
ca-Acvr1
siNeg + + - -
siCtnnb1 - - + +
(kDa)
β-catenin
92
1.0 1.9 0.3 0.3
Gapdh
37
B
Control
ca-Acvr1
Control
ca-Acvr1
XAV939 - - + +
(kDa)
β-catenin
92
1.0 1.9 0.2 0.2
Gapdh
37
C
Control
ca-Acvr1
Control
ca-Acvr1
Wnt3a - - + +
(kDa)
β-catenin
92
1.0 1.6 2.2 4.5
Gapdh
37
D
E11.5 BA1 cells (Culture start: Day 0)
0 1 2 3 4 5 days
XAV939 D1
Alcian blue
XAV939 D2-5
E
Vehicle
XAV D1
XAV D2-5
Control
ca-Acvr1
F
Control
ca-Acvr1
ns
ns
***
***
OD594
0.4
0.3
0.2
0.1
0.0
Vehicle
XAV D1
XAV D2-5
G
ca-Acvr1
Control
Vehicle
XAV_E11.25
XAV_E12.25
(kDa)
β-catenin
92
1.0 2.2 1.1 1.2
Gapdh
37
H
XAV939 i.p. injection
5 mg/kg, twice/day
Harvest
E10.5 E11.5 E12.5 E13.5 E14.5
Embryos (%)
100%
80%
60%
40%
20%
0%
100%
100%
Severe
Mild
Normal
Vehicle (n=5)
XAV E12.25 (n=10)
Vehicle
XAV939 E12.5
Lateral
Ventral
ca-Acvr1
Control
ca-Acvr1
1 mm
I
siNeg
siCtnnb1
Vehicle
XAV939
(kDa)
β-catenin
92
1.0 0.1 1.0 0.3
Gapdh
37
J
E11.5 limb bud cells (Culture start: Day 0)
Limb buds
0 1 2 3 4 5 days
XAV939
siCtnnb1
Alcian blue
siNeg
siCtnnb1
Vehicle
XAV939
OD594
0.8
0.6
0.4
0.2
0.0
**
**
siNeg
siCtnnb1
Vehicle
XAV939
K
β-catenin
130
95
72
56
Gapdh
56
43
34
26
Control
ca-Acvr1
***
**
***
β-catenin/Gapdh (fold change)
2.5
2.0
1.5
1.0
0.5
0.0
siNeg
siCtnnb1
L
β-catenin
130
95
72
56
Gapdh
56
43
34
26
Control
ca-Acvr1
*
**
***
β-catenin/Gapdh (fold change)
2.5
2.0
1.5
1.0
0.5
0.0
Vehicle
XAV939
M
β-catenin
130
95
72
56
Gapdh
56
43
34
Control
ca-Acvr1
**
*
**
β-catenin/Gapdh (fold change)
5.0
4.0
3.0
2.0
1.0
0.0
Vehicle
Wnt3a
N
β-catenin
130
95
72
56
Gapdh
56
43
34
Control
ca-Acvr1
**
*
*
β-catenin/Gapdh (fold change)
3.0
2.0
1.0
0.0
Non-treated
Vehicle
XAV_E11.25
XAV_E12.25
O
β-catenin
130
95
72
56
Gapdh
56
43
34
β-catenin/Gapdh (fold change)
1.5
1.0
0.5
0.0
**
*
siNeg
siCtnnb1
Vehicle
XAV939

Fig. S8. Blocking Wnt–β-catenin signaling suppresses chondrogenesis of BA1 cells but enhances chondrogenic differentiation of limb bud cells. (**A to C**) Western blot analysis of β-catenin and Gapdh in control and mutant BA1 cells transfected with *siCtnnb1* (A), treated with XAV939 (B), or stimulated with Wnt3a (C) for 24 hr. n=3 independent experiments. Uncropped images and quantification are shown in (K to M), respectively. (**D to F**) Scheme (D), representative images of Alcian blue staining (E), and the optical density quantification (F) to assess chondrogenesis of BA1 cells treated with XAV939 during the indicated time period. Scale bar, 1 mm. n=10 independent experiments. (**G**) Western blot analysis of β-catenin and Gapdh in control and mutant BA1 tissues of embryos treated with XAV939. n=3 mice per group. Uncropped images and quantification are shown in (N). (**H**) Scheme, the ratios of each cartilage phenotype (severe, mild, and normal), and representative images showing the head cartilage phenotypes of embryos treated in utero with XAV939 from E12.25 to E13.5. Scale bar, 1 mm. The numbers of mice examined are shown in parentheses. Red arrows indicate ectopic cartilages. (**I**) Western blot analysis of β-catenin and Gapdh in limb bud cells transfected with *siCtnnb1* or treated with XAV939 for 24 hr. n=3 independent experiments. Uncropped images and quantification are shown in (O). (**J**) Scheme showing the isolation and micromass culture of cells from limb buds from E11.5 control embryos, representative images of Alcian blue staining, and the optical density quantification to show the chondrogenic ability of limb bud cells transfected with *siCtnnb1* or treated with XAV939 at day 1. Scale bar, 1 mm. n=10 independent experiments. (**K** to **O**) Uncropped western blot images and quantification results for (A), (B), (C), (G), and (I), respectively. Error bars are mean ± s.d. ns $P > 0.05$; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; t-test (J, O); ANOVA (F, K-N).

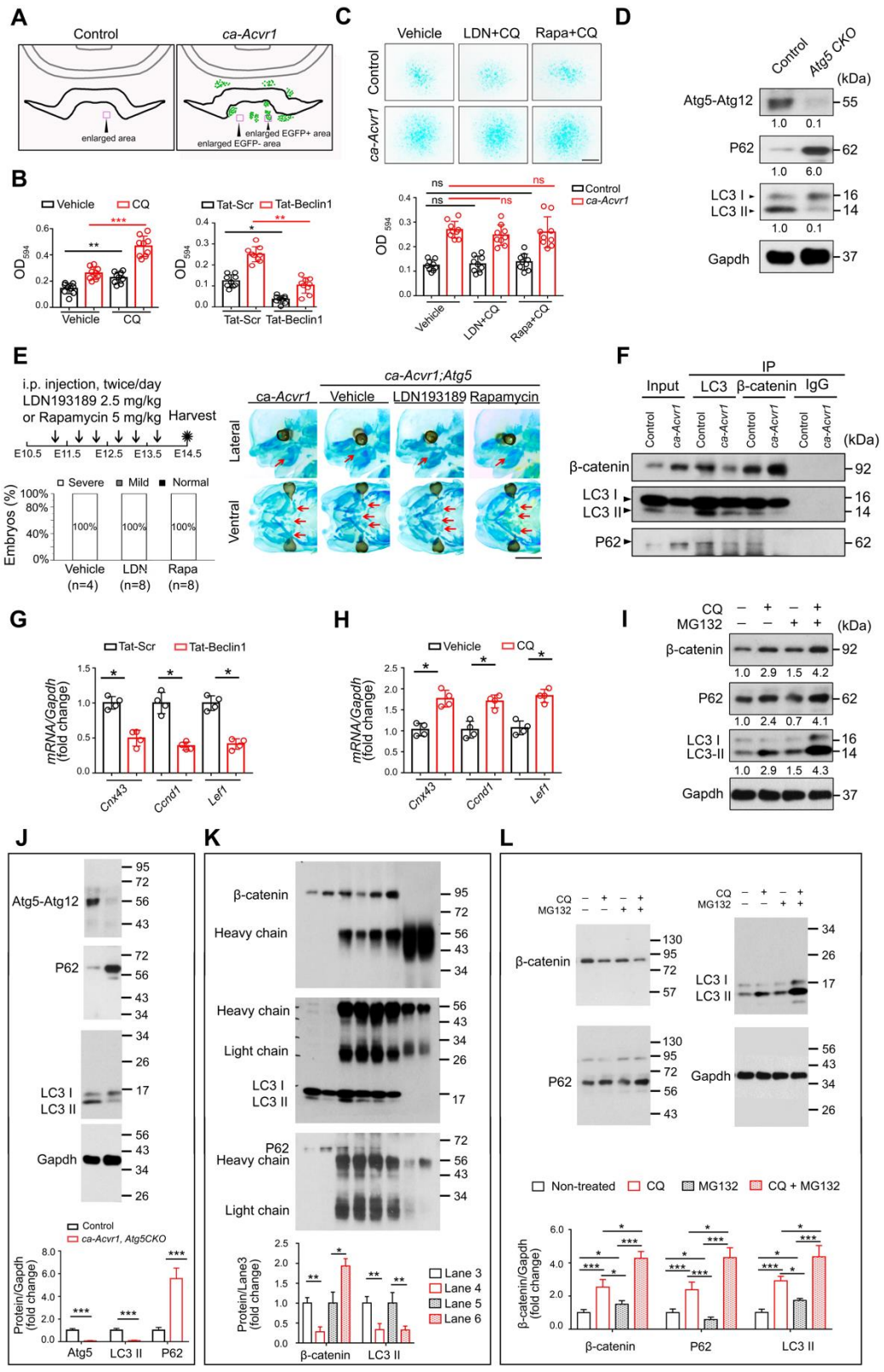
A
Control
ca-Acvr1
enlarged area
enlarged EGFP+ area
enlarged EGFP- area
B
Vehicle
CQ
OD594
Tat-Scr
Tat-Beclin1
C
Vehicle
LDN+CQ
Rapa+CQ
Control
ca-Acvr1
ns
D
Control
Atg5 CKO
(kDa)
Atg5-Atg12
P62
LC3 I
LC3 II
Gapdh
E
i.p. injection, twice/day
LDN193189 2.5 mg/kg
or Rapamycin 5 mg/kg
Harvest
E10.5
E11.5
E12.5
E13.5
E14.5
Severe
Mild
Normal
Embryos (%)
Vehicle (n=4)
LDN (n=8)
Rapa (n=8)
ca-Acvr1;Atg5
ca-Acvr1
Vehicle
LDN193189
Rapamycin
Lateral
Ventral
F
IP
Input
LC3
β-catenin
IgG
β-catenin
LC3 I
LC3 II
P62
G
Tat-Scr
Tat-Beclin1
mRNA/Gapdh (fold change)
Cnx43
Ccnd1
Lef1
H
Vehicle
CQ
I
CQ
MG132
β-catenin
P62
LC3 I
LC3-II
Gapdh
J
Atg5-Atg12
P62
LC3 I
LC3 II
Gapdh
Control
ca-Acvr1, Atg5CKO
Protein/Gapdh (fold change)
Atg5
LC3 II
P62
K
β-catenin
Heavy chain
Light chain
LC3 I
LC3 II
P62
Protein/Lane3 (fold change)
Lane 3
Lane 4
Lane 5
Lane 6
L
Non-treated
CQ
MG132
CQ + MG132
β-catenin/Gapdh (fold change)
β-catenin
P62
LC3 II

Fig. S9. Autophagy inhibits β-catenin signaling and is suppressed by BMP-mTORC1 signaling in BA1 cells (**A**) Schematic drawing to indicate the position of magnified regions in Fig. 6B. (**B**) Quantification of Alcian blue staining of BA1 cells stimulated with CQ or Tat-Beclin1. n=10 independent experiments. (**C**) Representative images and quantification of Alcian blue staining to assess chondrogenesis by BA1 cells treated with LDN193189 (LDN) and plus CQ or with rapamycin (rapa) plus CQ at day 1 of culture. Scale bar, 1 mm. n=10 independent experiments. (**D**) Western blot for the Atg5-Atg12 conjugate, P62, LC3, and Gapdh in control and *Atg5 CKO* (*Atg5*$^{flox/flox}$*;P0-Cre*) BA1 tissues at E11.5. n=3 independent experiments. Uncropped images and quantification are shown in (J). (**E**) Schematic representations, the ratios of each cartilage phenotype, and representative images showing cartilage structures in the heads of ca-*Acvr1,Atg5* compound mutant embryos (*Atg5*$^{flox/flox}$*;*ca-*Acvr1*$^{flox/+}$*;P0-Cre*) treated in utero with LDN193189 or rapamycin from E11.25 to E13.5. Red arrows indicate ectopic cartilages. Scale bar, 1 mm. The numbers of mice examined are shown in parentheses. (**F**) Immunoblotting for β-catenin, LC3, and P62 in LC3 and β-catenin immunoprecipitates (IP) from lysates of control and mutant BA1 tissues. n = 3 independent experiments. Uncropped images and quantification are shown in (K). (**G, H**) Relative expression of *Cnx43*, *Ccnd1*, and *Lef1* in BA1 cells treated with Tat-Beclin1 peptide (G) or CQ (H) for 24 hr. n=3 independent experiments. (**I**) Western blot for β-catenin, LC3, P62, and Gapdh in mutant BA1 cells after stimulation with MG-132 and/or CQ as indicated for 24 hr. n= 3 independent experiments. Uncropped images and quantification are shown in (L). (**J** to **L**) Uncropped western blot images and quantification results for (D), (F), and (I), respectively. Error bars are mean ± s.d. ns $P > 0.05$; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; t-test (G, H, J, K); ANOVA (B, C, L).

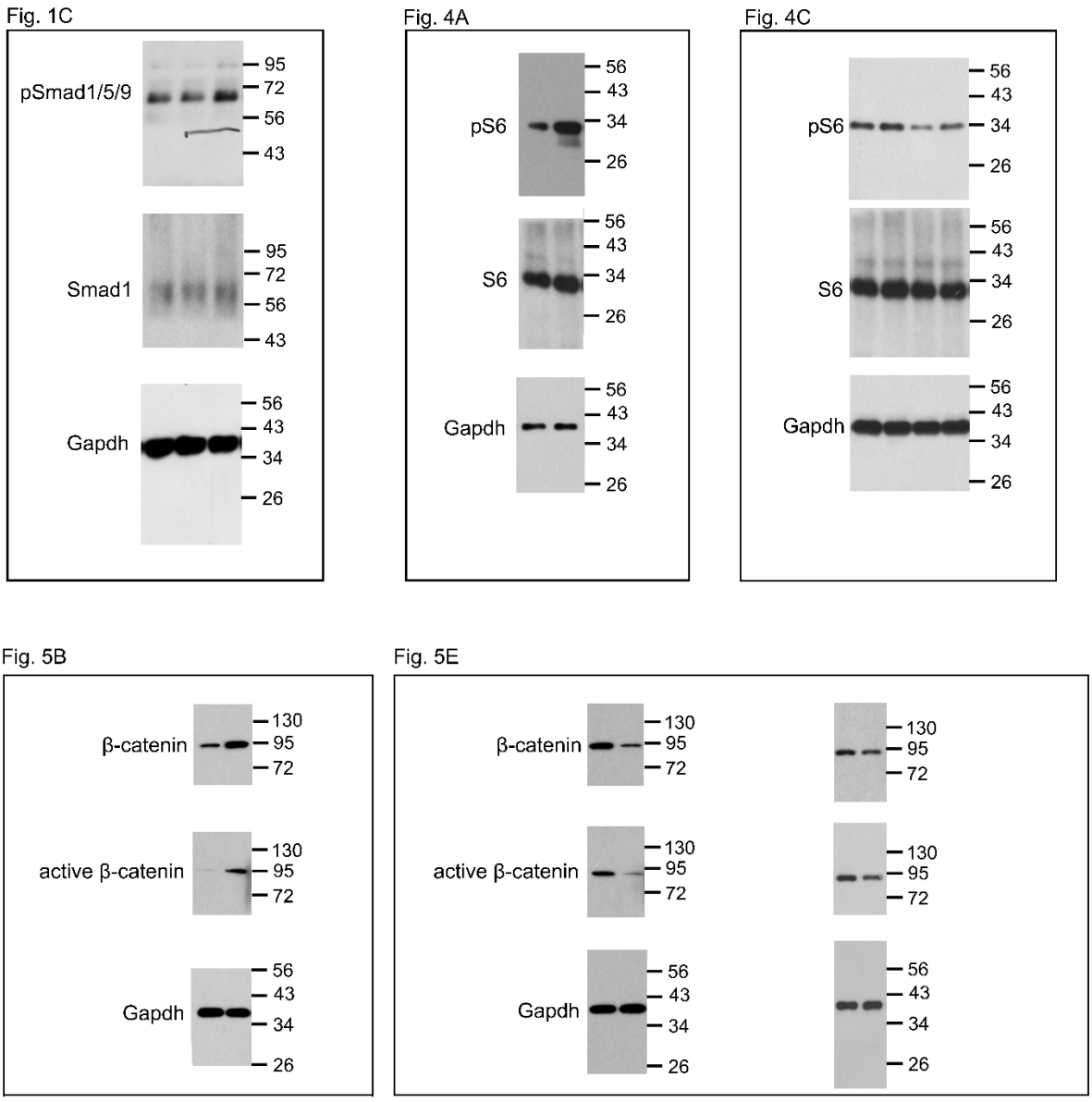

Fig. S10. Uncropped Western blots for Figs. 1, 4, and 5. Uncropped images of scanned western blots shown in Figs. 1, 4, and 5 are provided.

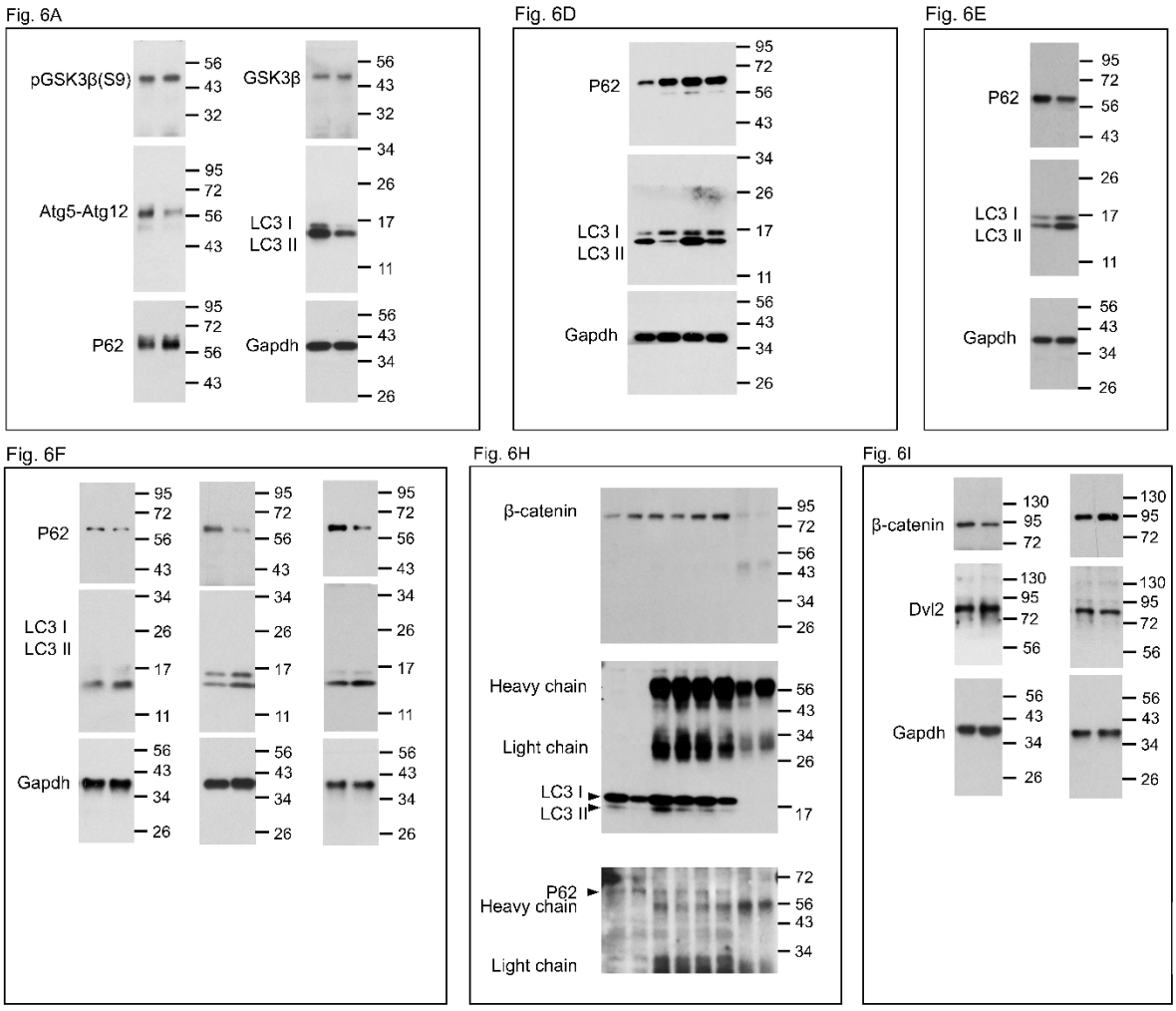

Fig. S11. Uncropped Western blots for Fig. 6. Uncropped images of scanned western blots shown in Fig.6 are provided.

Table S1. Genotyping PCR primers.

Allele		Forward (5' to 3')	Reverse (5' to 3')
ca-Acvr1$^{flox/lofx}$	floxed (580 bp)	GTGCTGGTTATTGTGCTGTCTC	GACGACAGTATCGGCCTCAGGAA
	wild type (334 bp)	GAGGACGCAGTCCAGTACCT	TAGCCTCTGCCTCACGCCCTGC
Tak1$^{flox/lofx}$	floxed (300 bp)	GATACCTTACACTGGGGACCA	GGCATTCAGTTGTGGAGCATT
	wild type (200 bp)		
Atg5 $^{flox/flox}$	floxed (700 bp)	ACAACGTCGAGCACAGCTGCGCAAGG	GTACTGCATAATGGTTTAACTCTTGC
	wild type (350 bp)	GAATATGAAGGCACACCCCTGAAATG	GTACTGCATAATGGTTTAACTCTTGC
Cre	Cre (696 bp)	GAGTGATGAGGTTCGCAAGA	CTACACCAGAGACGGAAATC
	wild type (371 bp)	ATGCTAGACCTGGGCAGCCATA	CATGCTAGCAGCTCGGAGAAAC
R26R^{mTmG}	mTmG (195 bp)	CTCGTGATCTGCAACTCCAGTC	TCAATGGGCGGGGGTCGTT
	wild type (290 bp)	CTCGTGATCTGCAACTCCAGTC	CTCGATGGAAAATACTCCGAGG
R26R^{LacZ}	LacZ (550 bp)	CTCGTGATCTGCAACTCCAGTC	GAGACTAGTGAGACGTGCTACT
	wild type (290 bp)	CTCGTGATCTGCAACTCCAGTC	CTCGATGGAAAATACTCCGAGG
R26R^{tdTomato}	tdTomato (196 bp)	GGCATTAAAGCAGCGTATCC	CTGTTCCTGTACGGCATGG
	wild type (297 bp)	AAGGGAGCTGCAGTGGAGTA	CCGAAAATCTGTGGGAAGTC

Table S2. Quantitative real-time PCR primers.

Gene	Forward (5' to 3')	Reverse (5' to 3')
Mouse Sox9	GACTTCCGCGACGTGGAC	GTTGGGCGGCAGGTACTG
Mouse Col2a1	GGCAATAGCAGGTTCACGTACA	CGATAACAGTCTTGCCCCACTT
Mouse Acan	TCGAGGACAGCGAGGCC	TCGAGGGTGTAGCGTGTAGAGA
Mouse Cnx43	TGGGGGAAAGGCGTGAGGGA	ACCCATGTCTGGGCACCTCTCTT
Mouse Ccnd1	GCGTACCCTGACACCAATCTC	CTCCTCTTCGCACTTCTGCTC
Mouse Lef1	TCTCAAGGACAGCAAAGCTC	CACTTGAGGCTTCATGCACAT
Mouse Dkk1	CTCATCAATTCCAACGCGATCA	GCCCTCATAGAGAACTCCCG
Mouse Dkk2	CTGATGCGGGTCAAGGATTCA	CTCCCCTCCTAGAGAGGACTT
Mouse Sost	AGCCTTCAGGAATGATGCCAC	CTTTGGCGTCATAGGGATGGT
Mouse Lrp5	AAGGGTGCTGTGTACTGGAC	AGAAGAGAACCTTACGGGACG
Mouse Axin2	TGACTCTCCTTCCAGATCCCA	TGCCCACACTAGGCTGACA
Mouse Alp	GGACAGGACACACACACACA	CAAACAGGAGAGCCACTTCA
Mouse Runx2	GACTGTGGTTACCGTCATGGC	ACTTGGTTTTTCATAACAGCGGA
Mouse Myog	GAGACATCCCCCTATTTCTACCA	GCTCAGTCCGCTCATAGCC
Mouse MyoD1	CCACTCCGGGACATAGACTTG	AAAAGCGCAGGTCTGGTGAG
Mouse Nestin	CCCTGAAGTCGAGGAGCTG	CTGCTGCACCTCTAAGCGA
Mouse Nefl	CCGTACTTTTCGACCTCCTACA	CTTGTGTGCGGATAGACTTGAG
Mouse Tubb3	TAGACCCCAGCGGCAACTAT	GTTCCAGGTTCCAAGTCCACC
Mouse Fabp7	GGACACAATGCACATTCAAGAAC	CCGAACCACAGACTTACAGTTT
Mouse GFAP	CCCTGGCTCGTGTGGATTT	GACCGATACCACTCCTCTGTC
Mouse Bax	TGAAGACAGGGGCCTTTTTG	AATTCGCCGGAGACACTCG
Mouse Casp3	ATGGAGAACAACAAAACCTCAGT	TTGCTCCCATGTATGGTCTTTAC
Mouse Pdgfra	AGAGTTACACGTTTGAGCTGTC	GTCCCTCCACGGTACTCCT
Mouse Ctsk	GAAGAAGACTCACCAGAAGCAG	TCCAGGTTATGGGCAGAGATT
Mouse LepR	TGGTCCCAGCAGCTATGGT	ACCCAGAGAAGTTAGCACTGT
Mouse Gli1	CCAAGCCAACTTTATGTCAGGG	AGCCCGCTTCTTTGTTAATTTGA
Mouse Thy1	TGCTCTCAGTCTTGCAGGTG	TGGATGGAGTTATCCTTGGTGTT
Mouse CD44	TCGATTTGAATGTAACCTGCCG	CAGTCCGGGAGATACTGTAGC
Mouse Gapdh	AGGTCGGTGTGAACGGATTTG	AGGTCGGTGTGAACGGATTTG

Table S3. Antibodies used in this study.

Antibody	Clone NO.	Species	Supplier	Cat NO.	IF	IB	IP
Sox9	3C10	Mouse	Abcam	ab76997	1:400		
EGFP	polyclonal	Rabbit	Abcam	ab290	1:200		
GFP	B2	Mouse	Santa Cruz	sc-9996	1:100		
Phospho-Smad1/5/9	D5B10	Rabbit	Cell signaling	13820	1:100	1:1000	
Smad1	D59D7	Rabbit	Cell signaling	6944		1:1000	
Phospho-S6 ribosomal protein	D68F8	Rabbit	Cell signaling	5364	1:500	1:5000	
S6 ribosomal protein	5G10	Rabbit	Cell signaling	2217		1:5000	
LC3	polyclonal	Rabbit	Sigma-Aldrich	L7543	1:300	1:4000	1:400
Atg5	polyclonal	Rabbit	Cell signaling	2630		1:1000	
P62	polyclonal	Rabbit	Cell signaling	5114		1:1000	
β-catenin		Mouse	DB Bioscience	610154	1:200	1:1000	1:200
active β-catenin	D13A1	Rabbit	Cell signaling	8814		1:1000	
Phospho-Histone H3	polyclonal	Rabbit	Thermo Fisher	44-1190G	1:500	1:1000	
Ki67	D3B5	Rabbit	Cell signaling	9129	1:500		
Phospho- p44/42 MAPK (Erk1/2)	D13.14.4E	Rabbit	Cell signaling	4370	1:100	1:1000	
p44/42 MAPK (Erk1/2)	137F5	Rabbit	Cell signaling	4695		1:1000	
Phospho-SAPK/JNK	81E11	Rabbit	Cell signaling	4668	1:100	1:1000	
SAPK/JNK	polyclonal	Rabbit	Cell signaling	9252		1:1000	
Phospho-p38 MAPK	D3F9	Rabbit	Cell signaling	4511	1:100	1:1000	
p38 MAPK	D13E1	Rabbit	Cell signaling	8690		1:1000	
Phospho-TAK1	polyclonal	Rabbit	Cell signaling	9339	1:100		
Phospho-SMAD2	E8F3R	Rabbit	Cell signaling	18338	1:100		
CD146	EPR3208	Rabbit	Abcam	ab75769	1:200		
CD44	EPR18668	Rabbit	Abcam	ab189524	1:200		
CD90	IBL-6/23	Rabbit	Abcam	ab3105	1:200		
Osx	polyclonal	Rabbit	Abcam	ab22552	1:200		
MyoD	C-20	Rabbit	Santa Cruz	sc-304	1:100		
Neurofilament		Rabbit	DSHB	2H3	1:100		
GSK-3β	27C10	Rabbit	Cell Signaling	9315		1:1000	
Phospho-GSK-3β	D85E12	Rabbit	Cell Signaling	5558		1:1000	
cleaved caspase-3	Asp175	Rabbit	Cell Signaling	9661		1:1000	
Dvl2	polyclonal	Rabbit	Cell Signaling	3216		1:1000	
Gapdh	D16H11	Rabbit	Cell Signaling	5174		1:2000	