

Molecular hydrogen suppresses activated Wnt/ β -catenin signaling

Yingni Lin¹, Bisei Ohkawara¹, Mikako Ito¹, Nobuaki Misawa², Kentaro Miyamoto¹, Yasuhiko Takegami¹, Akio Masuda¹, Shinya Toyokuni², Kinji Ohno^{1,*}

¹Division of Neurogenetics, Center for Neurological Diseases and Cancer, Nagoya University Graduate School of Medicine, Nagoya, Japan

²Department of Pathology and Biological Responses, Graduate school of Medicine, Nagoya University Graduate School of Medicine, Nagoya, Japan

*Address correspondence to Dr. Kinji Ohno

Division of Neurogenetics, Center for Neurological Diseases and Cancer, Nagoya University Graduate School of Medicine, 65 Tsurumai, Showa-ku, Nagoya 466-8550, Japan.

e-mail: ohnok@med.nagoya-u.ac.jp

Supplementary information includes:

Supplementary materials and methods.

Supplementary Tables S1-S2.

Supplementary Figures S1-S4.

Supplementary material and methods

Preparation of nuclear fraction

Cells were trypsinized, washed with PBS and resuspended in buffer A containing 10 mM HEPES-KOH (pH 7.8), 10 mM KCl, 0.1 mM EDTA and 0.1% NP-40, 10 mM sodium pyrophosphate, 1 µg/µl aprotinin, 1 µg/µl leupeptin, 1 µg/µl pepstatin A, 1 mM PMSF, 1 mM sodium orthovanadate, and the Phosphatase Inhibitor Cocktail. The cells were then vortexed and centrifuged at 5000 x g for 2 min. Supernatant was removed, and the pellet was washed with buffer A and resuspended in buffer C containing 50 mM HEPES-KOH (pH 7.8), 420 mM KCl, 0.1 mM EDTA, 5 mM MgCl₂, 2% glycerol, 10 mM sodium pyrophosphate, 1 µg/µl aprotinin, 1 µg/µl leupeptin, 1 µg/µl pepstatin A, 1 mM PMSF, 1 mM sodium orthovanadate, and the Phosphatase Inhibitor Cocktail. The pellet was vortexed and incubated at 4 °C on a rotating shaker for 30 min. The pellet was then centrifuged at 20,600 x g at 4 °C for 15 min. The supernatant was collected as nuclear fraction.

***In vivo* experiments with fed and starved mice**

Eight-week-old C57BL/6J mice were purchased from Japan SLC. Mice were randomly divided into 4 groups: free access to degassed water and food before euthanasia (fed control group), free access to hydrogen water and food before euthanasia (fed+H₂ group), free access to degassed water and starved before euthanasia (starved control group), and free access to hydrogen water and starved before euthanasia (starved+H₂ group). Mice had free access to either degassed water or H₂ water for 10 d. Mice had free access to food up to euthanasia or were starved for 18 h before euthanasia. The liver was isolated, flash-frozen in liquid nitrogen, and stored at -80 °C until further applications. For protein extraction, liver tissues were homogenized and lysed in RIPA buffer (Thermo Scientific Pierce, 89900) followed by centrifugation for 15 min at 20,600 x g at 4 °C.

***In vivo* ubiquitination assay**

Cells were harvested by a lysis buffer containing 2% SDS, 150 mM NaCl, 10 mM Tris-HCl (pH 8.0) with 2 mM sodium orthovanadate, 1 mM PMSF, 50 mM NaF, 1 µg/µl pepstatin A, 1mM DTT and the Protease Inhibitor Cocktail, and boiled for 10 min. Samples were then diluted in a dilution buffer (1: 10) containing 10 mM Tris-HCl (pH 8.0), 150 mM NaCl, 2 mM EDTA, 1% TritonX-100, incubated at 4 °C for 30 min on a rotating shaker, and centrifuged at 20,000 x g for 30 min. The total protein concentrations of the supernatants were measured by Pierce

660 nm Protein Assay Reagent, and 500 µg protein were incubated with anti-β-catenin antibody at 4 °C on a rotating shaker overnight, followed by addition of Dynabeads Protein G (Invitrogen) for another 1 h. Protein-antibody-bound beads were washed with a washing buffer [10 mM Tris-HCl (pH 8.0), 150 M NaCl, 1 mM EDTA, 1% NP-40] and boiled for 5 min with standard 2× Laemmli buffer. The immunoprecipitates were separated by SDS-PAGE and immunoblotted with anti-ubiquitin and anti-β-catenin antibody, as stated in Supplementary Table S1.

Co-immunoprecipitation

Cells were lysed in a buffer containing 20 mM Tris pH 8.0, 140 mM NaCl, 1 mM EDTA, 10% glycerol, 1% Triton X-100, 1mM DTT, 50 mM NaF, 1 mM PMSF, 1 mM sodium orthovanadate, 1 µg/µl pepstatin A, and the Phosphatase Inhibitor Cocktail. After centrifugation for 15 min at 20,600 x g at 4 °C, supernatants were collected and protein concentrations were measured by Pierce 660 nm Protein Assay Reagent. Lysates with the same amounts of proteins (750-1000 µg) were incubated with 1.0-1.5 µg of an antibody against Axin1 (05-1579, Millipore) or control mouse IgG (sc-2025, Santa Cruz Biotechnology) overnight at 4 °C. Dynabeads protein G (Invitrogen) were blocked with 1 µg/ml BSA for 1 h at 4 °C, added to the lysates with indicated antibody, and incubated for 1 h. Protein-antibody-bound beads were washed with PBS for 4 times and resuspended in standard 2× Laemmli buffer. Immunoprecipitated proteins were analyzed by Western blotting using specific antibodies against phospho-β-catenin (Ser45), phospho-β-catenin (Ser33/Ser37/Thr41), β-catenin, APC, GSK-3α/β, and Axin1, as stated in Supplementary Table S1.

Alcian blue staining

ATDC5 cells were seeded in a 12-well plate, and were differentiated into chondrocytes with insulin-transferrin-selenite (ITS, Invitrogen) for 21 days. Cells were treated with either 50% control CM, 50% Wnt3a CM, or 2 µM BIO with 10% H₂ or 10% N₂ gas for 48 h. Then, cells were fixed with methanol for 30 min at -20 °C, and stained overnight with 0.5% Alcian Blue 8 GX (Sigma) in 1N HCl. The stained cells were lysed in 200 µl or 400 µl of 6 M guanidine HCl for 6 h at room temperature. The optical density of the extracted Alcian blue was measured at 630 nm using PowerScan 4 (DS Pharma Biomedical).

Cell proliferation assay

Proliferation of ATDC5 cells was estimated by the BrdU cell proliferation ELISA kit (Roche) according to the manufacturer's recommendations. Briefly, ATDC5 cells were seeded at 5×10^3 /well in a 96-well plate and were incubated with 10% H₂ or 10% N₂ gas for 48 h. Then, cells were labeled with 10 μ M BrdU for 2 h at 37 °C, fixed and incubated with anti-BrdU antibody for 90 min at room temperature. After incubating the lysate with the substrate solution for 10 min, the reaction was stopped by H₂SO₄. Absorbance was measured at 450 nm using an absorbance microplate reader (Sunrise Remote, Tecan).

Immunofluorescence staining

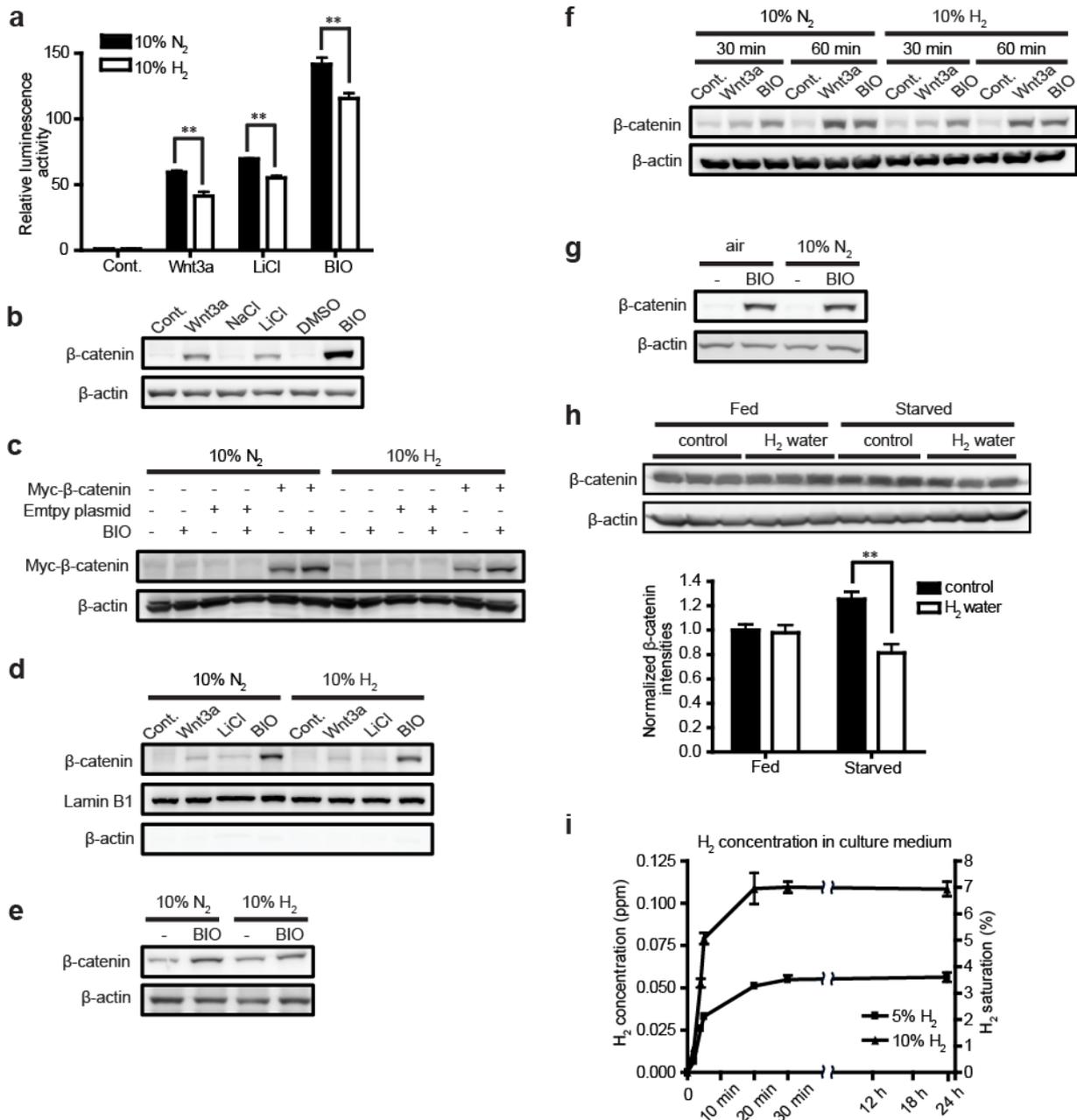
For immunofluorescence staining, the paraffin-embedded sections were first deparaffinized and rehydrated. Then, the sections were unmasked by 10 mM sodium citrate buffer (pH 6.0) at 95 °C for 15 min and were incubated with 3% H₂O₂ for 15 min to inactivate endogenous peroxidases. Sections were blocked with 5% goat serum in TBS-T for 1 h at room temperature followed by overnight incubation with antibodies against β -catenin (1: 100 in 5% goat serum with TBS-T, #9587, Cell Signaling Technology) or Sox9 (1: 400 in 5% goat serum with TBS-T, AB5535, Chemicon International) at 4 °C. After washing with TBS-T, sections were incubated with Alexa Fluor 488 donkey anti-rabbit IgG (1: 500, Invitrogen) for 1 h at room temperature. Finally, the specimens were mounted in VectaShield containing 2 μ g/ml diamidino-2-phenylindole (DAPI) (Vector Laboratories). The percentage of β -catenin-positive cells and Sox9-positive cells to DAPI-positive cells, and average signal intensities of β -catenin and Sox9 were blindly estimated using MetaMorph (Molecular Device). Three fields of the tibial cartilage area per section and 3 different sections per group were analyzed.

Table S1. Antibodies and concentrations for Western blotting

Name	Manufacturer	Clone	Cat. No.	Dilution
β -catenin	BD Transduction Laboratories	Mouse monoclonal Clone 14	610153	1: 2000
c-myc	Santa Cruz Biotechnology	Mouse monoclonal Clone 9E10	sc-40	1: 500
ubiquitin	Santa Cruz Biotechnology	Mouse monoclonal Clone P4D1	sc-8017	1: 400
β -actin	Santa Cruz Biotechnology	Mouse monoclonal Clone C-4	sc-47778	1: 400
GSK-3 α/β	Santa Cruz Biotechnology	Mouse monoclonal Clone 1H8	sc-7291	1:200
Axin1	Millipore	Mouse monoclonal Clone A5	05-1579	1: 1000
phospho-c-Jun (Ser73)	Cell Signaling Technology	Rabbit monoclonal Clone D47G9	#3270	1: 1000
c-Jun	Santa Cruz Biotechnology	Rabbit polyclonal Clone H-79	sc-1694	1:200
phospho-SAPK/JNK (p-JNK) (Thr183/Tyr185)	Cell Signaling Technology	Rabbit monoclonal Clone 81E11	#4668	1: 1000
SAPK/JNK (JNK)	Cell Signaling Technology	Rabbit polyclonal	#9252	1: 1000
phospho- β -catenin (Ser45)	Cell Signaling Technology	Rabbit polyclonal	#9564	1: 1000
phospho- β -catenin (Ser33/Ser37/Thr41)	Cell Signaling Technology	Rabbit polyclonal	#9561	1: 1000
glycogen synthase (GS)	Cell Signaling Technology	Rabbit polyclonal	#3893	1: 1000
phospho-GS (Ser641)	Cell Signaling Technology	Rabbit polyclonal	#3891	1: 1000
lamin B1	Abcam	Rabbit polyclonal	ab16048	1: 1000
APC	Santa Cruz Biotechnology	Rabbit polyclonal Clone C-20	sc-896	1: 200

Table S2. Primer sequences for qRT-PCR

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
Mouse <i>Axin2</i>	GAAACTGGCAAGTGTCCACG	CGCAGGCAAATTCGTCCTC
Human <i>AXIN2</i>	CTCCCCACCTTGAATGAAGA	GTTTCCGTGGACCTCACACT
Mouse <i>Axin1</i>	GGAGCTATTCCGAGAACGCA	ACTGGGTACCTCAGCATTGG
Mouse <i>Apc</i>	TCCCTTCGCTCCTACGGAA	TGAGCATAATACCAGTCCTTTTCCT
Mouse <i>Ctnnb1</i>	TTAAACTCCTGCACCCACCAT	AGGGCAAGGTTTCGAATCAA
Human <i>CTNNB1</i>	GCTTTCAGTTGAGCTGACCA	CAAGTCCAAGATCAGCAGTCTC
Human <i>MMP3</i>	GCAGTTTGCTCAGCCTATCC	GAGTGTCGGAGTCCAGCTTC
Human <i>SOX9</i>	CTGGGCAAGCTCTGGAGA	ATGTGCGTCTGCTCCGTG
Human <i>ACAN</i>	CCAGGAGGTATGTGAGGA	CGATCCACTGGTAGTCTTG
Human <i>COL2A1</i>	AGAGGGGATCGTGGTGACAA	GGCAGCAAAGTTTCCACCAA
Mouse <i>B2m</i>	ATTCACCCCCACTGAGACTG	TGCTATTTCTTTCTGCGTGC
Human <i>B2M</i>	TGGCACCTGCTGAGATACTG	AGGATGCTAGGACAGCAGGA

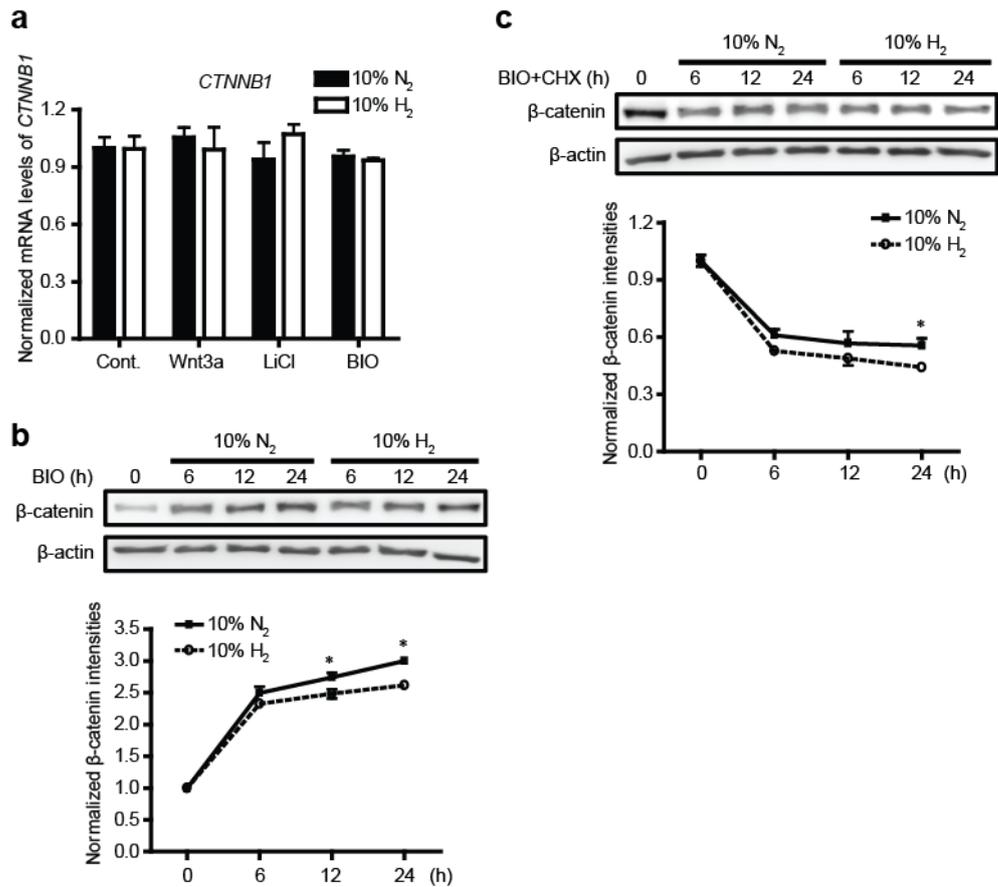


Supplemental Figure 1. H₂ suppresses Wnt/β-catenin signaling.

- (a) HeLa cells were treated with control CM (Cont.), Wnt3a CM, 30 mM LiCl, or 4 μM BIO with 10% H₂ or 10% N₂ gas for 24 h. The Wnt/β-catenin signaling activity was measured by Topflash luciferase reporter assay ($n = 3$). Mean and SEM are plotted. Values are normalized to the mean of Cont. and 10% N₂. $**P < 0.01$ by Student's t test.
- (b) L cells were treated with pairs of control CM (Cont.) and Wnt3a CM; 30 mM NaCl and 30 mM LiCl; and 0.02% DMSO and 2 μM BIO/0.02% DMSO for 24 h. Representative Western blots are shown.
- (c) L cells were transfected with an empty or myc-β-catenin plasmid. Cells were treated with 2 μM BIO with

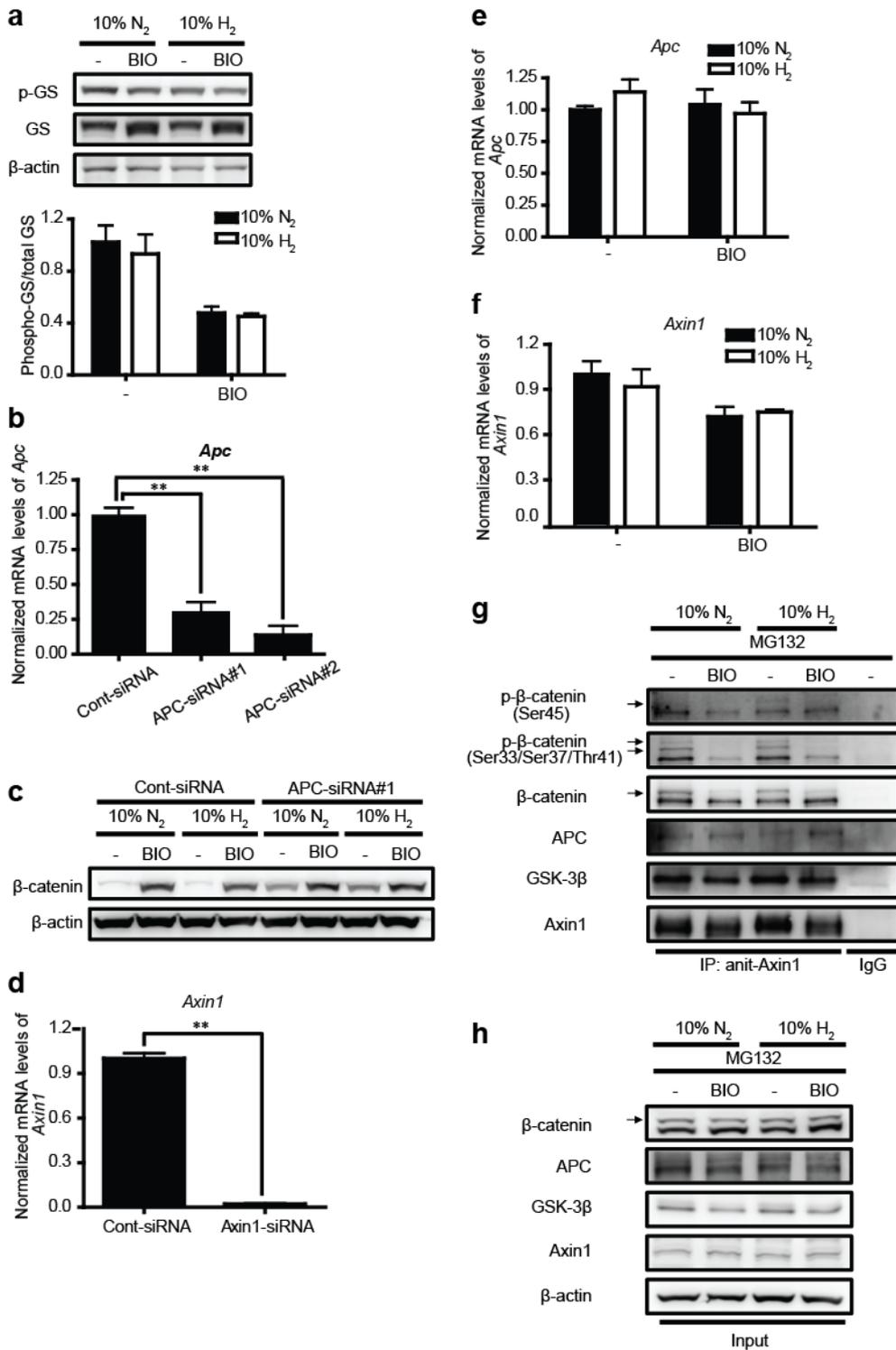
10% H₂ or 10% N₂ gas for 24 h. Representative Western blots with anti-c-myc antibody are shown.

- (d) L cells were treated with control CM (Cont.), Wnt3a CM, 30 mM LiCl, or 2 μM BIO with 10% H₂ or 10% N₂ gas for 24 h. Representative Western blots with nuclear fractions are shown.
- (e) HeLa cells were treated with 4 μM BIO with 10% H₂ or 10% N₂ gas for 24 h. Representative Western blots with anti-β-catenin antibody are shown.
- (f) L cells were treated with control CM (Cont.), Wnt3a CM, or 2 μM BIO with 10% H₂ or 10% N₂ gas for 30 min and 60 min. Representative Western blots with anti-β-catenin antibody are shown.
- (g) L cells were treated with 2 μM BIO with air or 10% N₂ gas for 24 h. Representative Western blots with anti-β-catenin antibody are shown.
- (h) C57BL/6J mice were given degassed water (control) or H₂ water *ad libitum* for 10 d and were fed or starved for 18 h before sacrifice. Representative Western blots of the liver of 3 mice in each group are shown with densitometry of β-catenin/β-actin ($n = 6$ in each group). Values are normalized to control fed group. $**P < 0.01$ by Student's t test.
- (i) Time course analysis of H₂ concentrations in culture medium treated with 5% or 10% H₂ gas for indicated time periods ($n = 3$). Mean and SEM are plotted. Note that the concentration of saturated H₂ under standard ambient temperature and pressure (SATP) is 1.6 ppm.



Supplemental Figure 2. H₂ promotes degradation of endogenous β-catenin without decreasing its mRNA level in HeLa cells.

- (a) HeLa cells were treated with control CM (Cont.), Wnt3a CM, 30 mM LiCl, or 4 μM BIO with 10% H₂ or 10% N₂ gas for 24 h. Expression of *CTNNB1* encoding β-catenin was quantified by qRT-PCR ($n = 3$). No statistical difference by Student's *t*-test.
- (b, c) HeLa cells were added with 4 μM BIO (b) or a combination of 10 μg/ml CHX and 4 μM BIO (c) with 10% H₂ or 10% N₂ gas for indicated periods of time. Representative Western blots are shown with densitometry of β-catenin/β-actin ($n = 3$). Two groups in each panel were not statistically different by two-way repeated measures ANOVA [$P = 0.071$ for (b) and $P = 0.0531$ for (c)]. * $P < 0.05$ by Student's *t*-test with Bonferroni correction for each pair of H₂ and N₂. Values are normalized to the mean of 10% N₂ at 0 h.

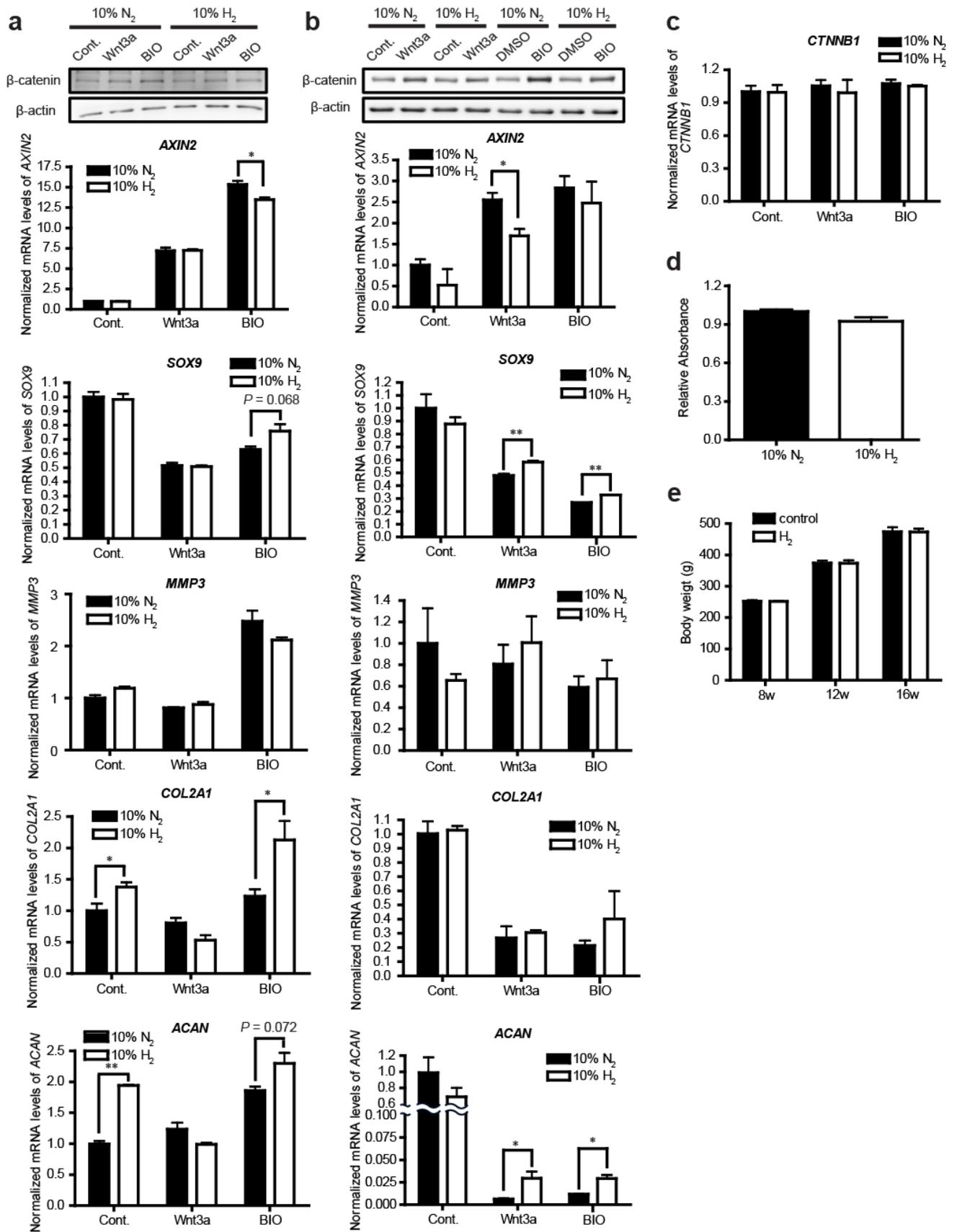


Supplemental Figure 3. H₂-mediated β-catenin degradation requires activities of APC and Axin1.

- (a) L cells were treated with 2 μM BIO with 10% H₂ or 10% N₂ gas for 24 h. Western blots are shown with densitometry of phospho-glycogen synthase (p-GS)/glycogen synthase (GS) ($n = 3$). No statistical difference by Student's *t*-test.
- (b) Knock-down efficiencies of endogenous APC by two siRNAs were assessed by qRT-PCR in L cells ($n = 3$).

**** $P < 0.01$ by Student's t -test.**

- (c)** L cells were transfected with Cont-siRNA or APC-siRNA#1, and were treated with 2 μ M BIO with 10% H₂ or 10% N₂ gas for 24 h. Representative Western blots are shown.
- (d)** Knock-down efficiency of endogenous Axin1 by siRNA was assessed by qRT-PCR in L cells ($n = 3$). **** $P < 0.01$ by Student's t -test.**
- (e, f)** L cells were treated with 2 μ M BIO with 10% H₂ or 10% N₂ gas for 24 h. Expressions of *Apc* ($n = 3$) **(e)** and *Axin1* ($n = 3$) **(f)** were quantified by qRT-PCR. No statistical difference by Student's t -test.
- (g, h)** L cells were treated with 10 μ M MG132 in the presence or absence of 2 μ M BIO with 10% H₂ or 10% N₂ gas for 12 h. Cell lysates were then immunoprecipitated (IP) with antibodies against Axin1 or mouse control IgG (IgG). Representative Western blots of IP samples **(g)** and input samples **(h)** with indicated antibodies are shown. Arrows indicate mono- or di-ubiquitinated phospho- β -catenin.



Supplemental Figure 4. H₂ inhibits Wnt/β-catenin signaling in human OAC cells.

(a) Human OAC cells (clone 2) were treated with control CM (Cont.), Wnt3a CM, or 2 μM BIO with 10% H₂

or 10% N₂ gas for 24 h. β -catenin and β -actin were detected by Western blotting. Expression of *AXIN2* ($n = 3$), *SOX9* ($n = 3$), *MMP3* ($n = 3$), *COL2A1* ($n = 3$), and *ACAN* ($n = 3$) were quantified by qRT-PCR. * $P < 0.05$, ** $P < 0.01$ by Student's t -test. Non-significant P values less than 0.10 are indicated above each pair.

- (b) Human OAC cells (clone 3) were treated with control CM (Cont.), Wnt3a CM, 0.02% DMSO, or 2 μ M BIO with 10% H₂ or 10% N₂ gas for 24 h. Western blotting and qRT-PCR were performed as in (a). * $P < 0.05$, ** $P < 0.01$ by Student's t -test.
- (c) Human OAC cells (clone 1) were treated with control CM (Cont.), Wnt3a CM, or 2 μ M BIO with 10% H₂ or 10% N₂ gas for 24 h. Expression of *CTNNB1* was quantified by qRT-PCR ($n = 3$). No statistical difference by Student's t -test.
- (d) ATDC5 cells were treated with 10% H₂ or 10% N₂ gas for 48 h. Proliferation of ATDC5 cells was evaluated by the BrdU cell proliferation assay ($n = 4$). No statistical difference by Student's t -test.
- (e) Body weights of rats taking degassed water (control) or H₂ water were measured at indicated weeks of age ($n = 6$). No statistical difference by two-repeated measures ANOVA.