Hepatocyte-specific Prominin-1 protects against liver injury-induced fibrosis by stabilizing SMAD7

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Supplementary Figure Legends

Supplementary Table 1. Antibodies used in immunostaining and immunoblotting analysis.

Supplementary Table 2. Primers used in qRT-PCR and genotyping PCR.

Supplementary Figure 1. Global *Prom1* deficiency aggravates BDL-induced liver fibrosis. Eight-week-old male *Prom1*$^{+/+}$ (WT) and *Prom1*$^{-/-}$ (KO) mice were subjected to sham (n=3) or BDL (n=5-6) for one week. (a) Each liver specimen was analyzed by H&E, Masson's Trichrome, Sirius Red staining, and immunofluorescence for PROM1, αSMA and CK19. (b) Each liver specimen was analyzed by RT-qPCR for mRNA of *Prom1*, *Acta2*, *Col1a1*, *Krt19*, *Tgfbr1*, *Tfgb1*, *Ctgf*, *Pai1*, *Timp1*, *Il1b*, *Il6*, *and Tnfa*. The level of each mRNA was normalized to those of 18S rRNA. (c) The serum levels of aspartate transaminase (AST), alanine aminotransferase (ALT), and bilirubin were determined. Scale bar = 20 μm. *t*-test *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. All data are means ± S.E.M. *ACTA2*, Alpha-actin-2; *COL1A1*, Alpha-1 type 1 collagen; *Ctgf*, Connective tissue growth factor; *Il1b*, Interleukin 1b; *Il6*, Interleukin 6; *Pai1*, Plasminogen activator inhibitor 1; *Prom1*, Prominin-1; *Tgf1*, Transforming growth factor beta 1; *Tgfbr1*, Transforming growth factor beta receptor 1; *Timp1*, Tissue inhibitor of metalloprotease; *Tnf1*, Tumor necrosis factor 1.

Supplementary Figure 2. Preparation of liver-specific *Prom1* deficient mice (a) A scheme for the generation of liver specific *Prom1* deficient mice. Mice were created by crossing *Prom1*$^{f/f}$ mice (*f/f*), containing two loxP sequences flanking exon 2 of *Prom1*, with *Alb-Cre*

transgenic mice (*Alb-Cre*). Arrows indicate primers used for genotyping (Supplementary Table 2 shows the sequences of the primers used for genotyping PCR.). (b) Representative PCR products from tail-tip genomic DNA of WT, *f/f* and *f/f; Alb-Cre* mice. 1st, 2nd, and 4th lanes are for the PCR products by primers of F1 and R1; 3rd and 5th lanes for those by primers of F2, R2 and internal control (*Il-6*). (c) The expression levels of PROM1 in primary hepatocytes (PHs), liver and kidney of *f/f* and *f/f; Alb-Cre* were determined by immunoblotting.

Supplementary Figure 3. Preparation of cholangiocyte-specific *Prom1* deficient mice (a) scheme for the generation of cholangiocyte-specific *Prom1* deficient mice. Mice were created by crossing *Prom1*$^{f/f}$ mice (*f/f*) with *Krt19-Cre* transgenic mice (*Krt19-Cre*). Arrows indicates primers used for genotyping. (b) Representative PCR products from tail-tip genomic DNA of WT, *f/f* and *Prom1*$^{f/f}$*; Krt19-Cre* mice. Left panel shows the PCR product by primers of F1 and R2 whereas right panel shows those with primer of F2, R2 and R3. (c) *Prom1* deficiency in cholangiocyte was determined by PROM1 and CK19 immunofluorescence. Arrows indicate CK19-staining cholangiocyte.

Supplementary Figure 4. Global *Prom1* deficiency enhances TGFβ signaling by reducing the SMAD7 level in fibrotic liver. Eight-week-old *Prom1*$^{+/+}$ (WT) and *Prom1*$^{-/-}$ (KO) were subjected to sham (n=3) and BDL (n=7) for 7 days. (a) Band intensity of p-SMAD2/3, αSMA, SMAD7 and Cleaved Caspase-3 in BDL liver of WT and KO mice (Fig. 4a) was statistically analyzed after normalized by the band intensity of GAPDH. (b) Each liver specimen was analyzed by immunofluorescence for p-SMAD2/3 and SMAD7 and TUNEL assay. (c) The mRNA levels of *Smad7* from liver specimen of WT and KO mice were determined by RT-qPCR and normalized to those of 18S rRNA. *$p < 0.05$, **$p < 0.01$, n.s; non-significant. All

data are means ± S.E.M. Scale bar = 20 μm. TUNEL, Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labeling.

Supplementary Figure 5. Truncation mutants of PROM1 (a) and SMAD7 (b) for domain analysis. EX, extracellular domain; TM, transmembrane domain; IC, intracellular domain; N domain, N-terminal domain; MH2 domain, Mad homology 2 domain.

Supplementary Figure 6. Adenoviral expression of overexpression of PROM1 in global *Prom1* deficient mice ameliorates BDL-induced liver fibrosis. Eight-week-old global *Prom1* deficient mice were infected by adenovirus with *LacZ* or *PROM1*. The mice were operated by sham (n=3) or BDL (n=5-9). (a) PROM1 expression in the liver was determined by immunoblotting 3 days after BDL. (b) Seven days after BDL, the liver was analyzed by H&E, and Sirius Red staining and immunofluorescence for αSMA and CK19, p-SMAD2/3 and SMAD7. (c) Quantification of liver fibrosis was determined by measuring areas stained by Sirius Red. 2-3 images for Sirius Red staining were obtained from each liver. (d) Each specimen was analyzed by immunoblotting for p-SMAD2/3, SMAD2/3, SMAD4, SMAD7, αSMA, Cleaved Caspase-3 and GAPDH. (e) Band intensity of p-SMAD2/3, αSMA, SMAD7 and Cleaved Caspase-3 in d was statistically analyzed after normalized by the band intensity of GAPDH. (f) Each liver specimen was analyzed by RT-qPCR for mRNAs of *PROM1, Acta2, Col1a1* and *Krt19*. The mRNA levels were normalized by those of 18S rRNA. Scale bar = 20 μm. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. All data are means ± S.E.M.

Supplementary Figure 7. Proposed model for hepatocytic Prominin-1 protects against

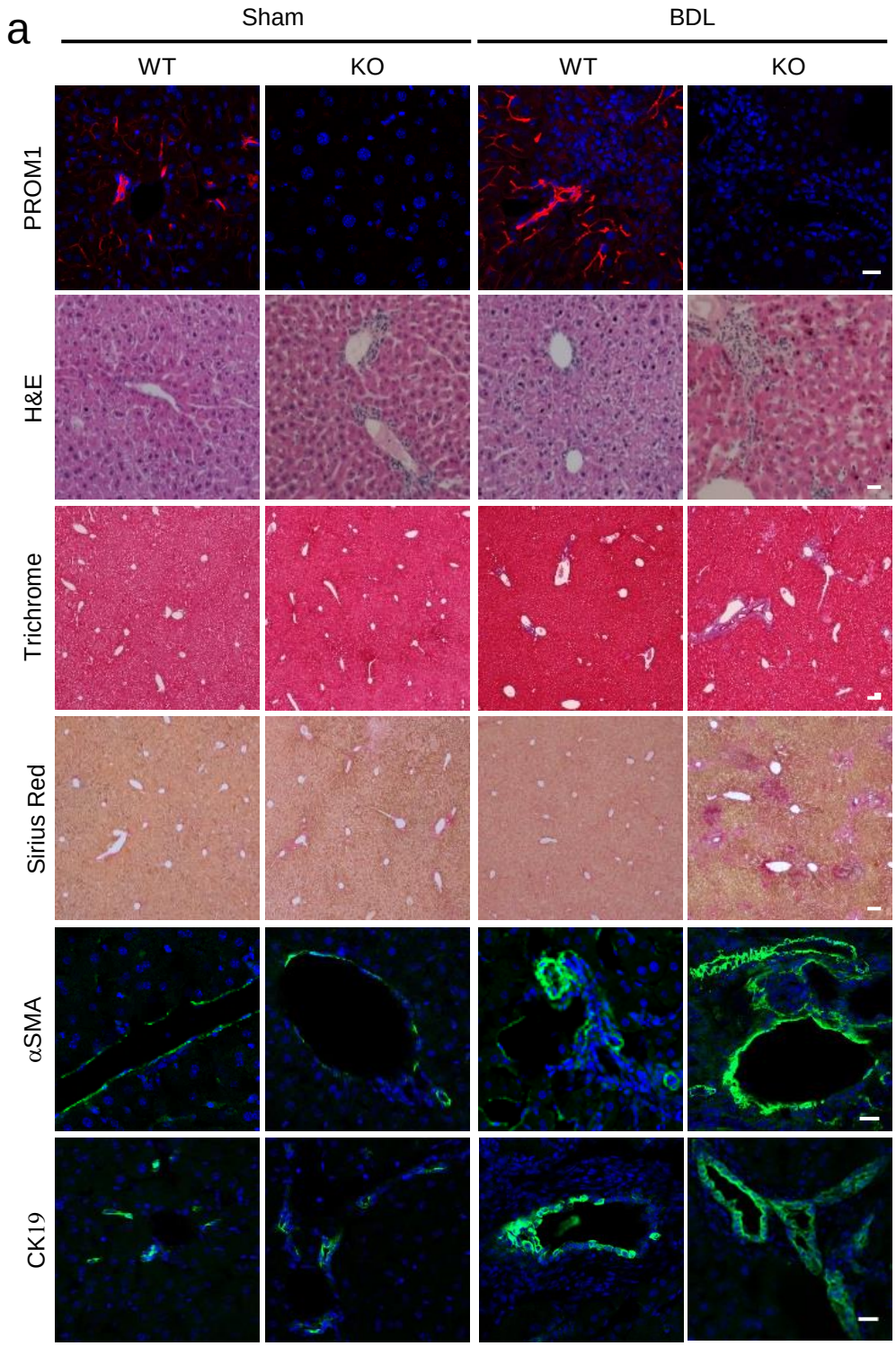
liver injury-induced fibrosis by stabilizing SMAD7. Prominin-1 in the hepatocytes decrease TGFβ signaling by regulating SMAD7 protein expression. PROM1 prevented SMURF2-induced SMAD7 ubiquitination and degradation by interfering with the molecular association of SMAD7 with SMURF2.

Name	Company	Host	Experiments
PROM1	eBioscience	Rat polyclonal	IB(1:2000), IHC(1:100)
PROM1	MACS	Mouse Monoclonal	IB(1:200), IFA(1:25)
PROM1	Merck Millipore	Mouse monoclonal	IB(1:1000), IFA(1:100)
PROM1	R&D systems	Mouse monoclonal	IB(1:1000), IFA(1:100)
a-SMA	abcam	Rabbit polyclonal	IB(1:1000),IHC(1:200)
CK19	abcam	Mouse Monoclonal	IB(1:1000),IHC(1:200)
SMAD2/3	Cell Signaling Technology	Rabbit polyclonal	IB(1:1000)
pSMAD2/3	Cell Signaling Technology	Rabbit polyclonal	IB(1:500), IFA(1:100)
SMAD4	Cell Signaling Technology	Rabbit polyclonal	IB(1:1000)
SMAD7	R&D systems	Mouse monoclonal	IB(1:1000), IFA(1:100)
FLAG	Sigma-Aldrich	Rabbit monoclonal	IB(1:2000)
HA	Merck Millipore	Mouse monoclonal	IB(1:1000)
Myc	Santa Cruz Biotechnology	Mouse monoclonal	IB(1:1000)
GAPDH	Santa Cruz Biotechnology	mouse monoclonal	IB(1:2000)
COL1A1	Cell Signaling Technology	Rabbit monoclonal	IFA(1:200)
Laminin	abcam	Rabbit polyclonal	IFA(1:200)

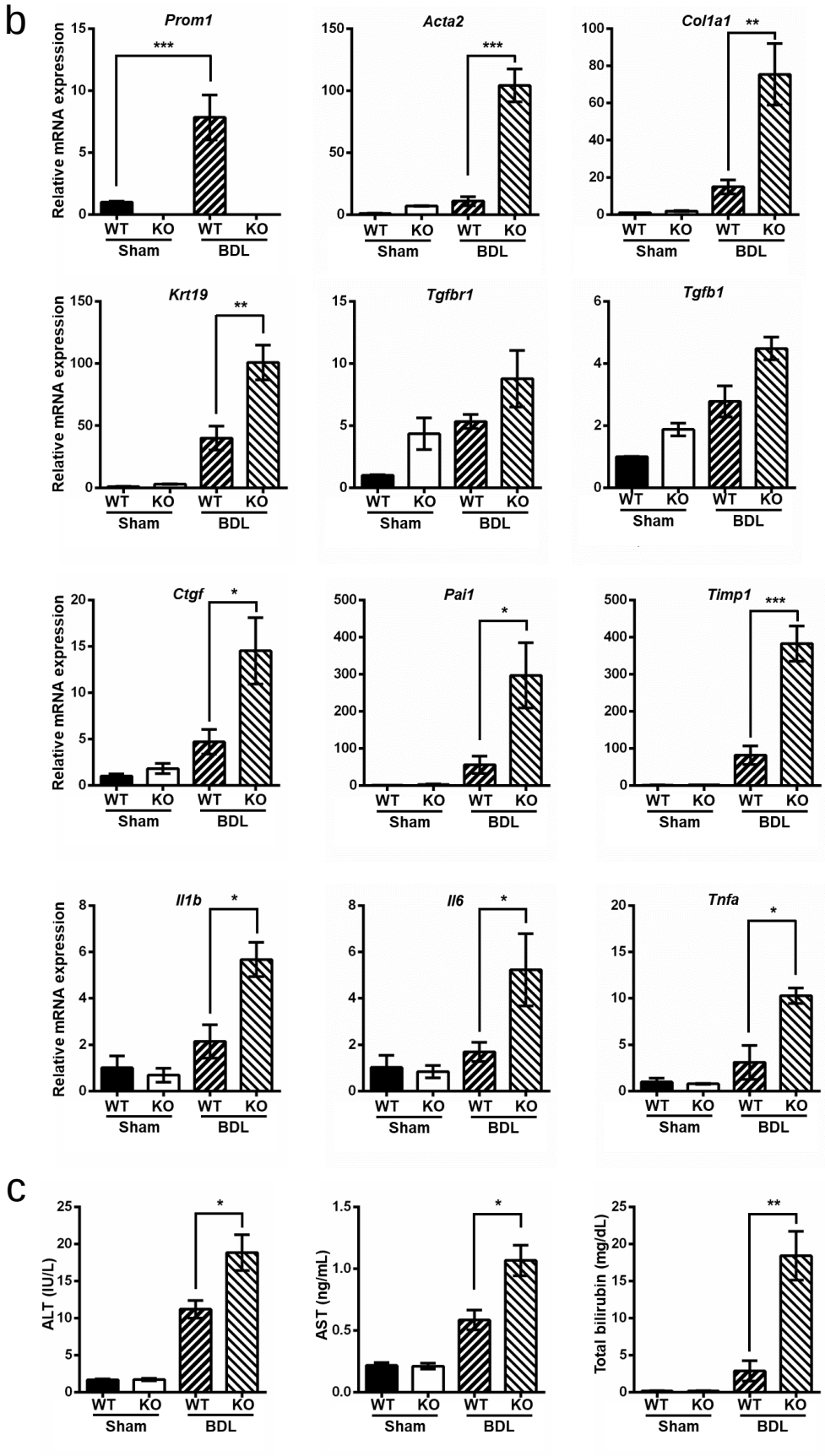
Supplementary Table 1

	Name	Forward	Reverse
qRT-PCR	***Prom1***	**CTCATGGCTGGGGTTGGATT**	**TGAGCAGATAGGGAGTGTCCA**
	Acta2	**AGCTACGAACTGCCTGACGG**	**CGTGGATGCCCGCTGAC**
	Col1a1	**AGCACGTCTGGTTTGGAGAG**	**GACATTAGGCGCAGGAAGGT**
	Tgfb1	**GTCACTGGAGTTGTACGGCA**	**GGGGCTGATCCCGTTGATTT**
	Tgfbr1	**GCATTGGCAAAGGTCGGTTT**	**TGCCTCTCGGAACCATGAAC**
	Krt19	**GTGCTGGATGAGCTGACTCTG**	**GATCTTGGCTAGGTCGACACC**
	Ctgf	**AGGGCCTCTTCTGCGATTTC**	**CTTTGGAAGGACTCACCGCT**
	Pai1	**CACAGGCACTGCAAAAGGTC**	**TGTGCCGAACCACAAAGAGA**
	Timp1	**GGCATCTGGCATCCTCTTGT**	**TGGTCTCGTTGATTTCTGGGG**
	Il1b	**GCCCATCCTCTGTGACTCAT**	**AGGCCACAGGTATTTTGTCG**
	Il6	**TGATGGATGCTACCAAACTGGA**	**ACTCTGGCTTTGTCTTTCTTGT**
	Tnfa	**TGGGACAGTGACCTGGACTGT**	**TTCGGAAAGCCCATTTGAGT**
	Smad7	**GGGGGCTTTCAGATTCCCAA**	**GACACAGTAGAGCCTCCCCA**
Genotype PCR	**Cre transgene**	**CCAGGCTAAGTGCCTTCTCTACA**	**AATGCTTCTGTCCGTTTGCCGGT**
	Internal control	**CTAGGCCAGAGAATTGAAAGATCT**	**GTAGGTGGAAATTCTAGCATCATCC**
	loxP	**ATGGGGAGCATTGATTTGCC**	**TCTTTCTGCCTGGTTGCTTT**

Supplementary Table 2



Supplementary Fig. 1



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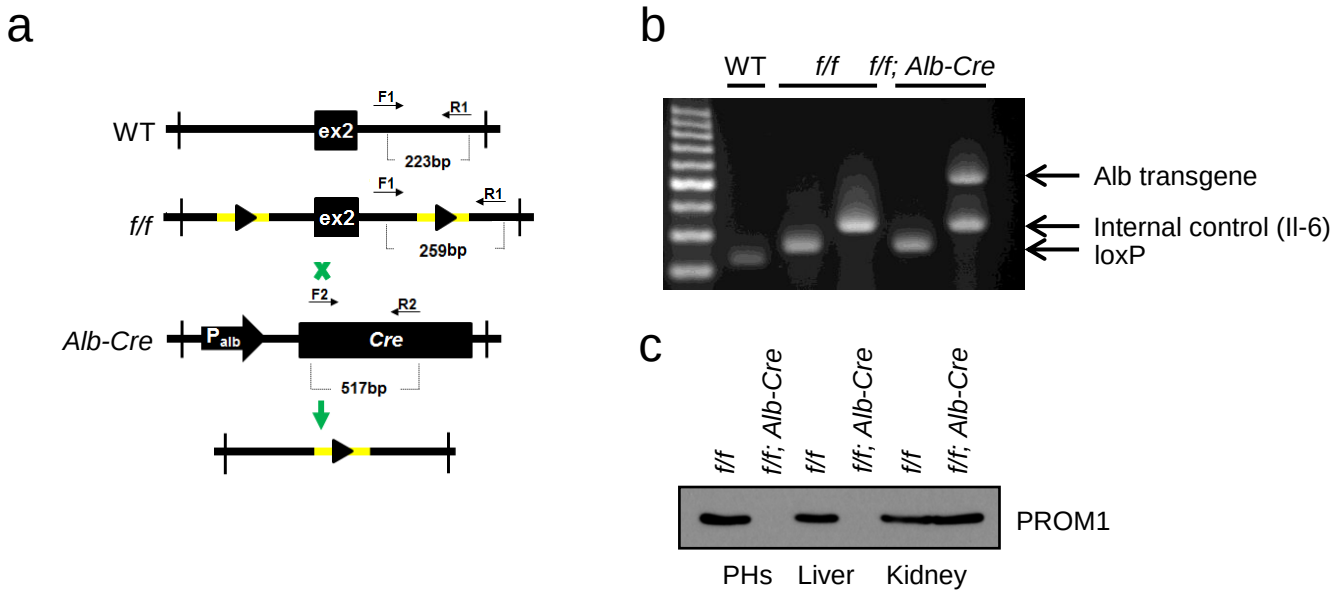

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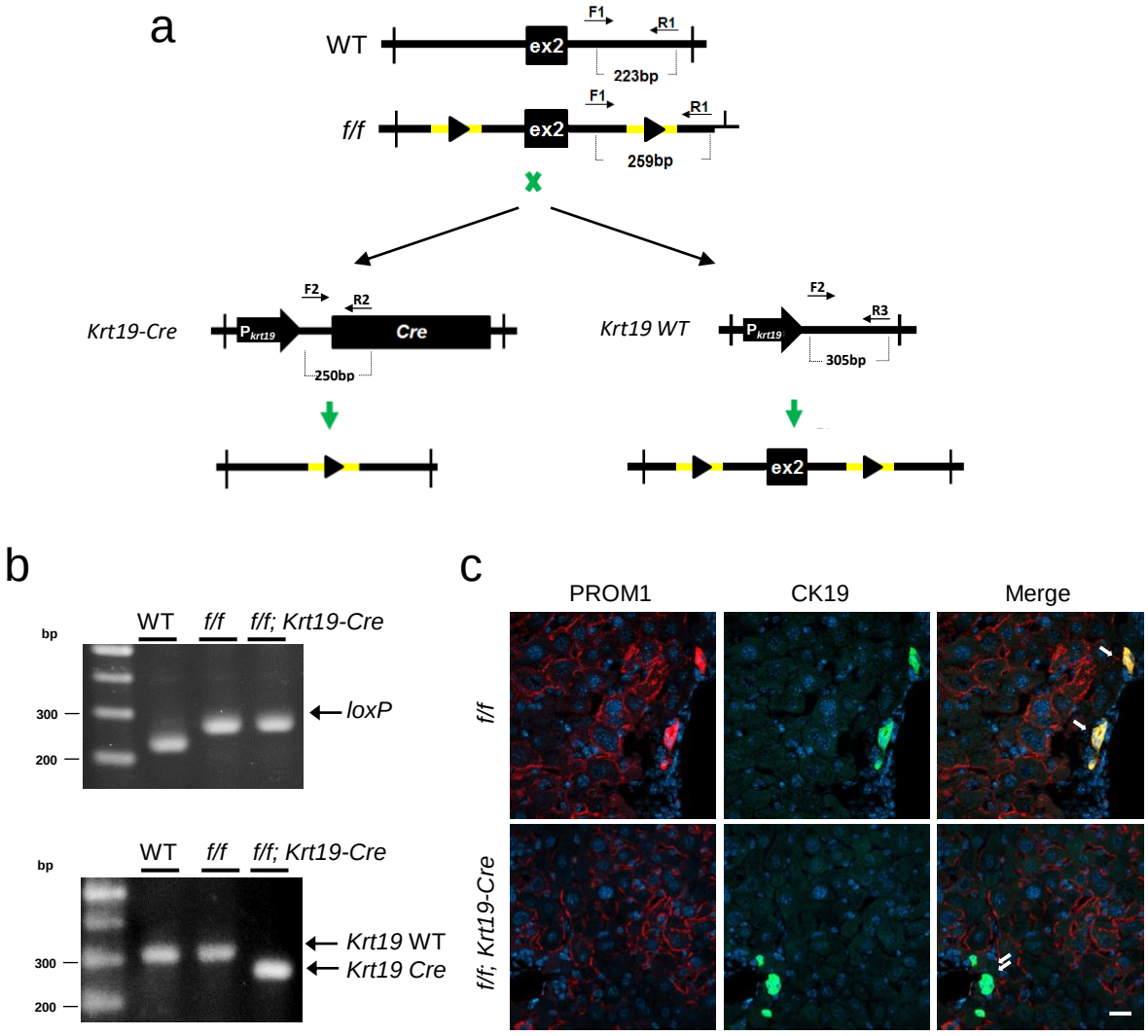

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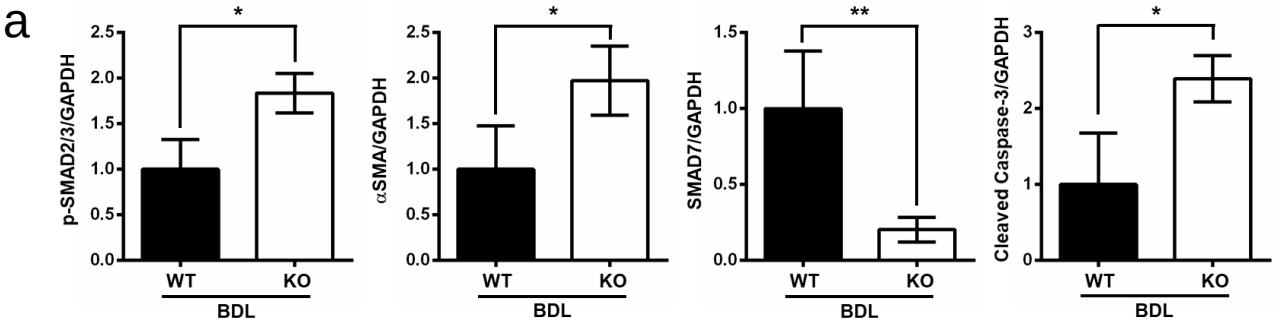
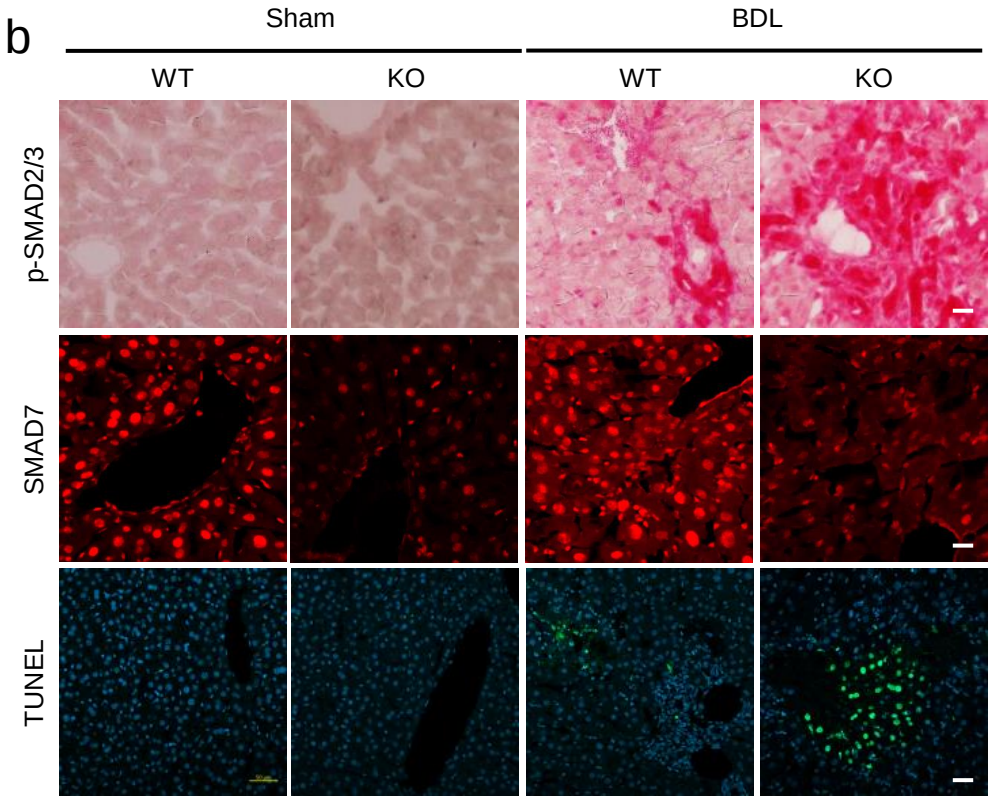
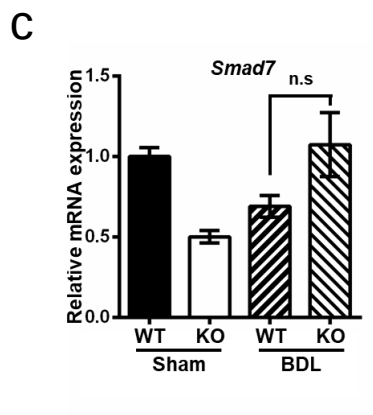
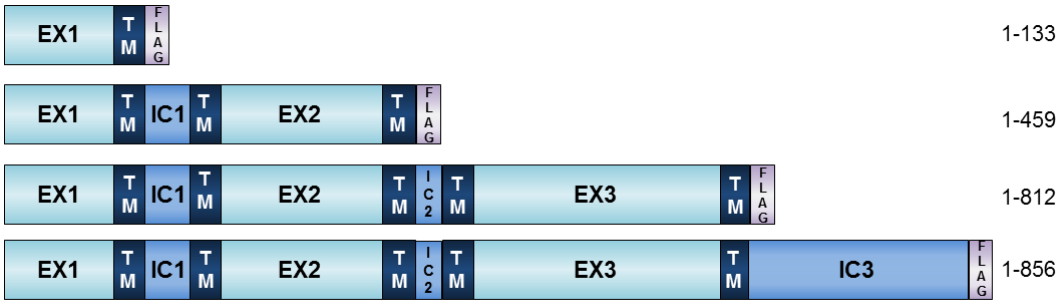






Supplementary Fig. 4

a



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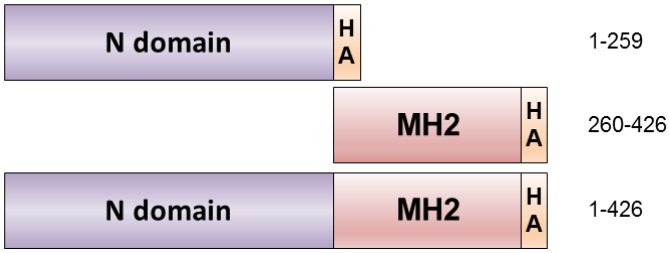

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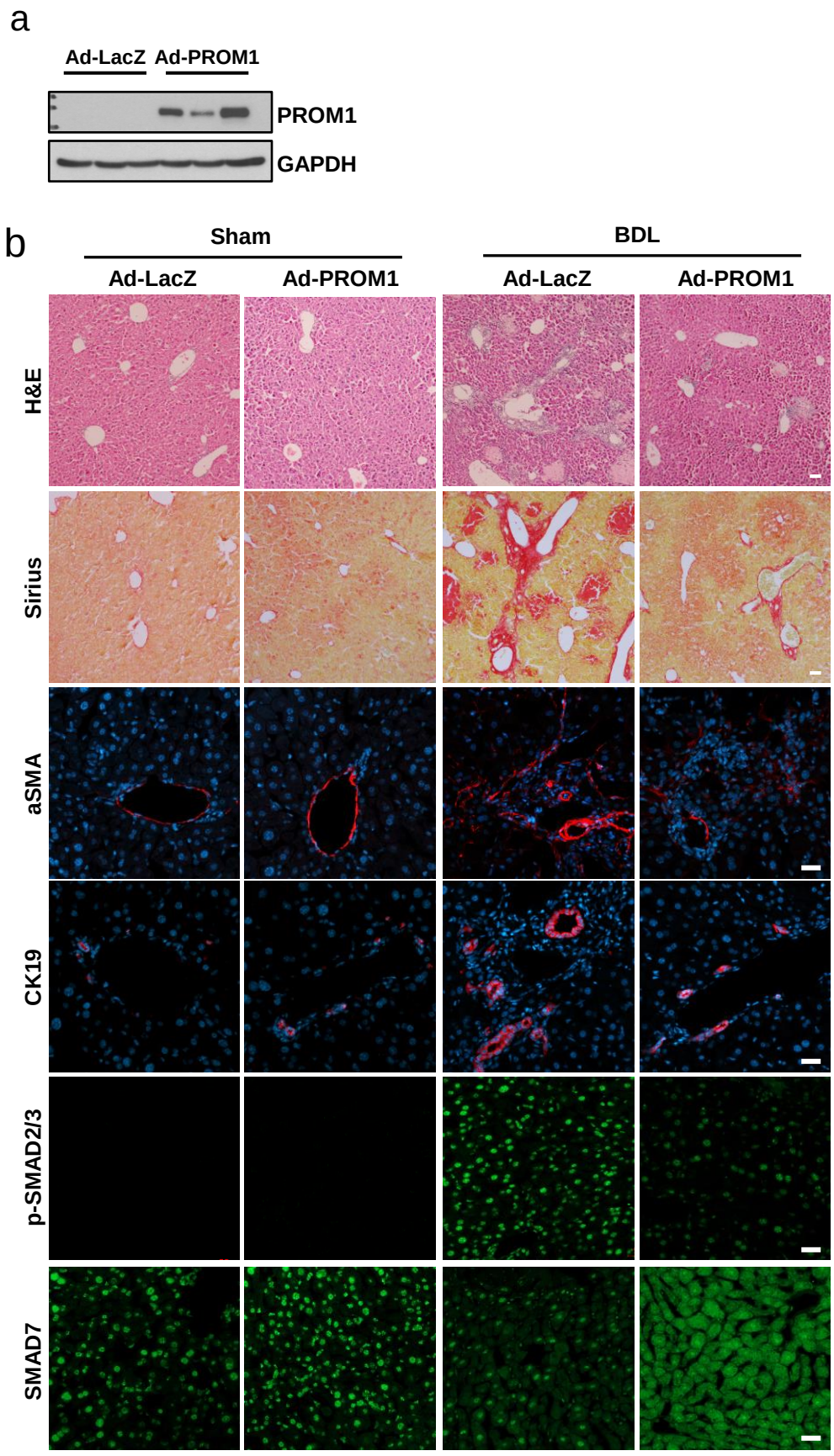

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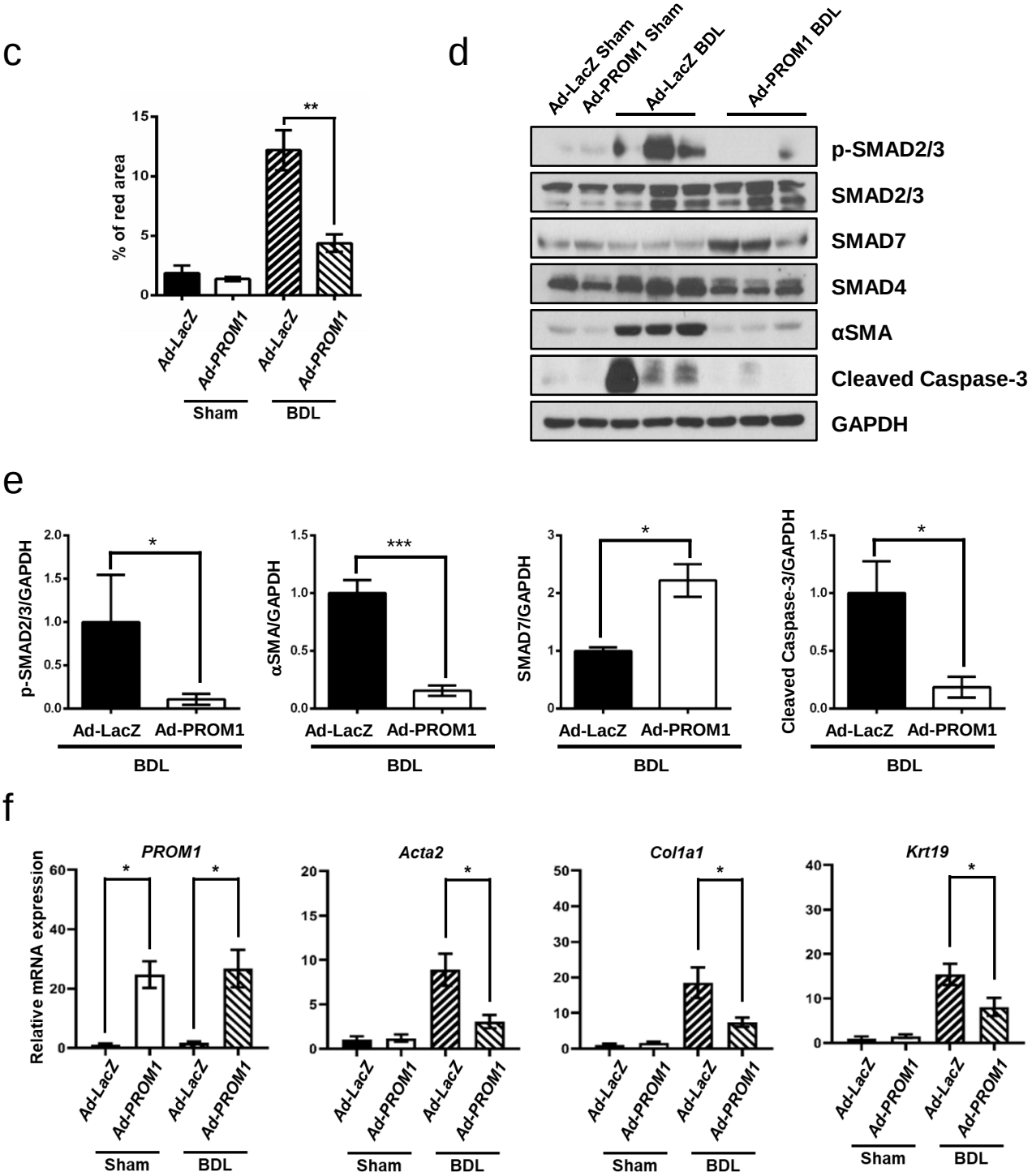

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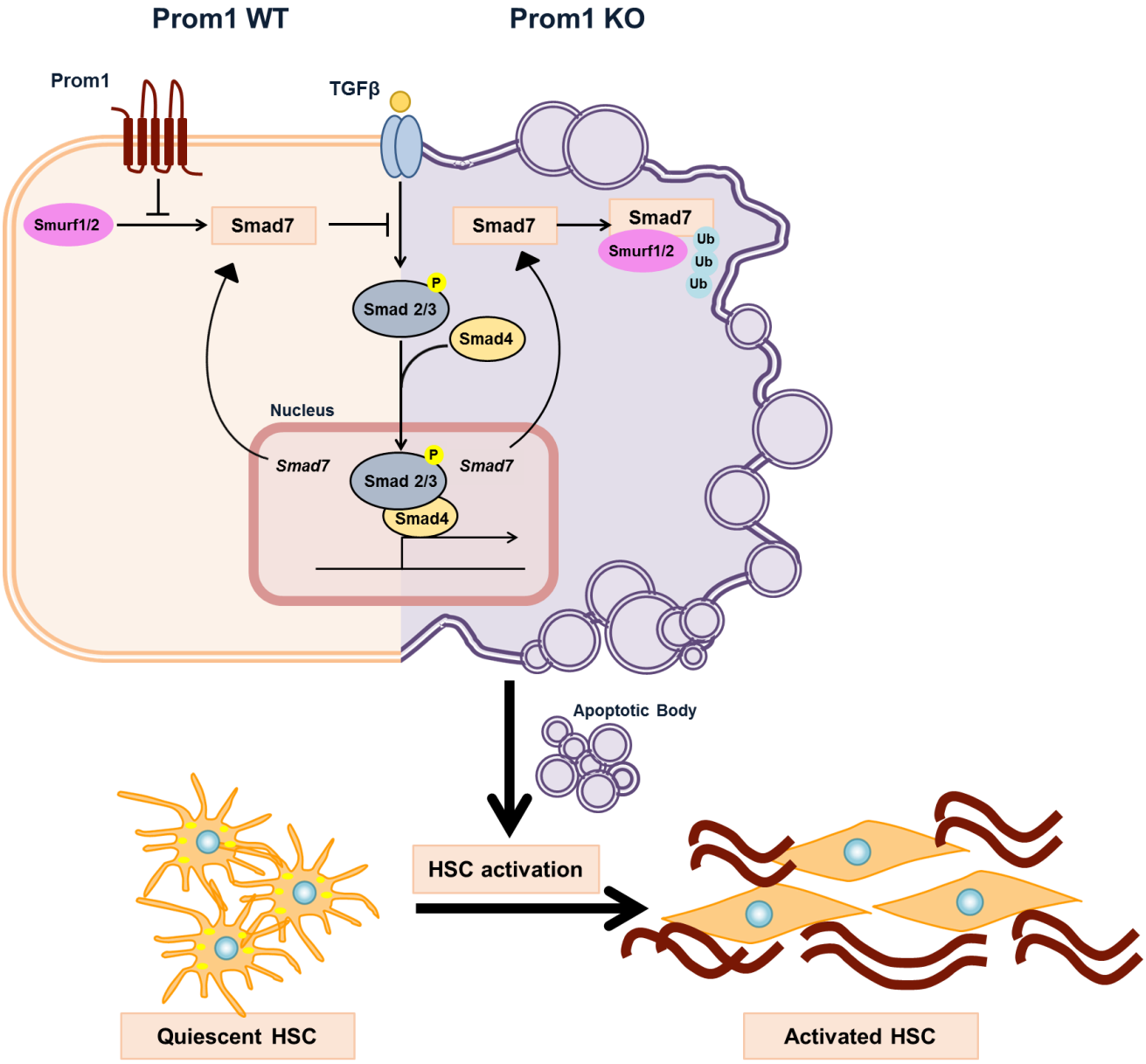

Supplementary Fig. 7