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Supplemental Material to:

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Context-dependent function of the deubiquitinating enzyme USP9X in pancreatic ductal adenocarcinoma

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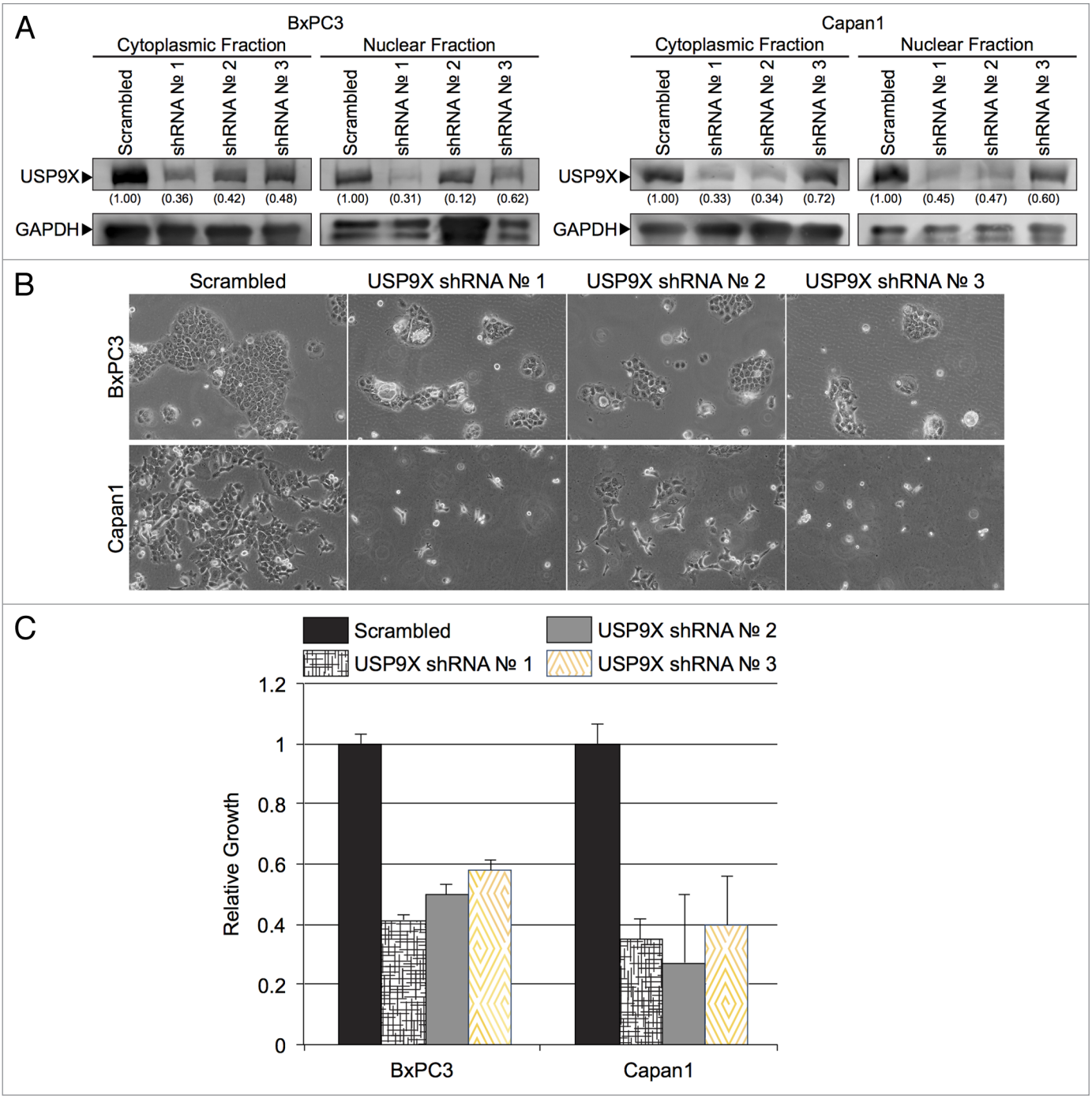

Figure S1. Stable knockdown of USP9X in pancreatic tumor cell lines BxPC3 and Capan1. (**A**) Western blot analysis to verify the knockdown of USP9X in pancreatic tumor cell lines (BxPC3 and Capan1) following transduction with lentiviruses to introduce constitutively active shRNA against USP9X transcripts. The non-specific Scrambled shRNA was used as a control. Twenty-four hours after transduction, cells were selected by culturing in medium supplemented with puromycin for 48 h. Nuclear and cytoplasmic protein fractions were prepared 3 d after transducing cells with lentiviruses. USP9X levels were quantified, normalized against GAPDH, and levels in the Scrambled control were set to 1.00. (**B**) Photomicrographs of pancreatic tumor cells (BxPC3 and Capan1) following knockdown of USP9X using lentiviral delivered shRNA constructs against USP9X transcripts. Cells were treated as described above. Three days after transduction, cells were subcultured into fresh culture flasks, and photos were taken 3 d later (day 6 after transduction). (**C**) MTT assay examining the growth of either Capan1 or BxPC3 cells transduced with lentiviral constructs to knockdown USP9X. Cells were treated as above. Three days after subculture (day 6 after transduction), MTT assay was conducted as described in the Materials and Methods. MTT values in Scrambled control were set to 1.0.

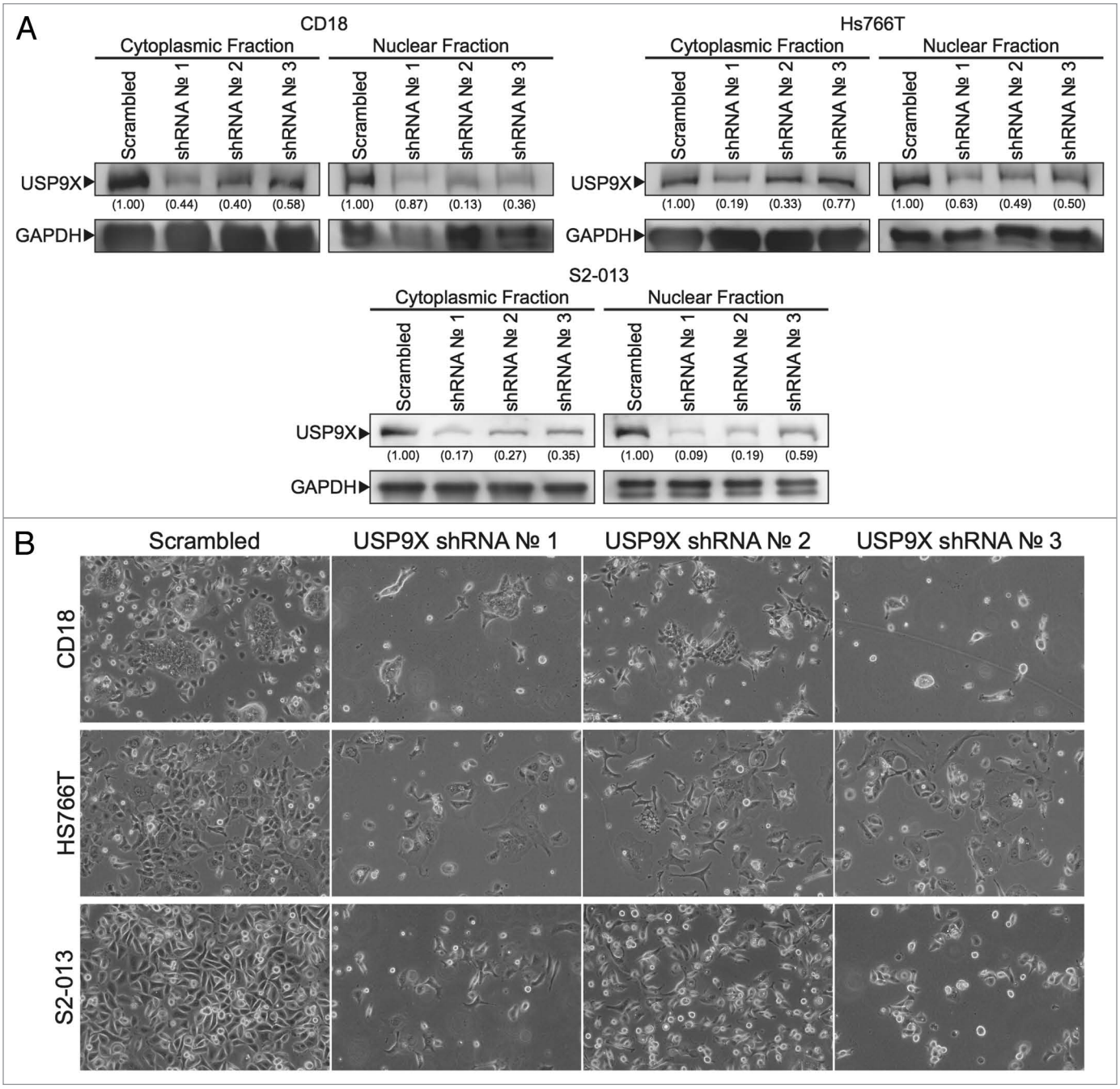

Figure S2. Stable knockdown of USP9X in additional pancreatic tumor cell lines. (**A**) Western blot analysis was conducted to determine the knockdown of USP9X in pancreatic tumor cell lines (CD18, Hs766T, and S2-013) following transduction with lentiviruses to introduce constitutively active shRNA against USP9X transcripts. The non-specific Scrambled shRNA was used as a control. Twenty-four hours after transduction, transduced cells were selected by culturing in medium supplemented with puromycin for 48 h. Nuclear and cytoplasmic protein fractions were prepared 3 d after transducing cells with lentiviruses. Quantified USP9X levels, normalized against GAPDH and relative to the Scrambled control, are indicated in parentheses. (**B**) Photomicrographs of pancreatic tumor cells (CD18, Hs766T, and S2-013) following knockdown of USP9X using lentiviral delivered shRNA constructs against USP9X transcripts. Cells were passaged into fresh culture flasks 3 d after transduction. Photomicrographs were taken 6 d after transduction.

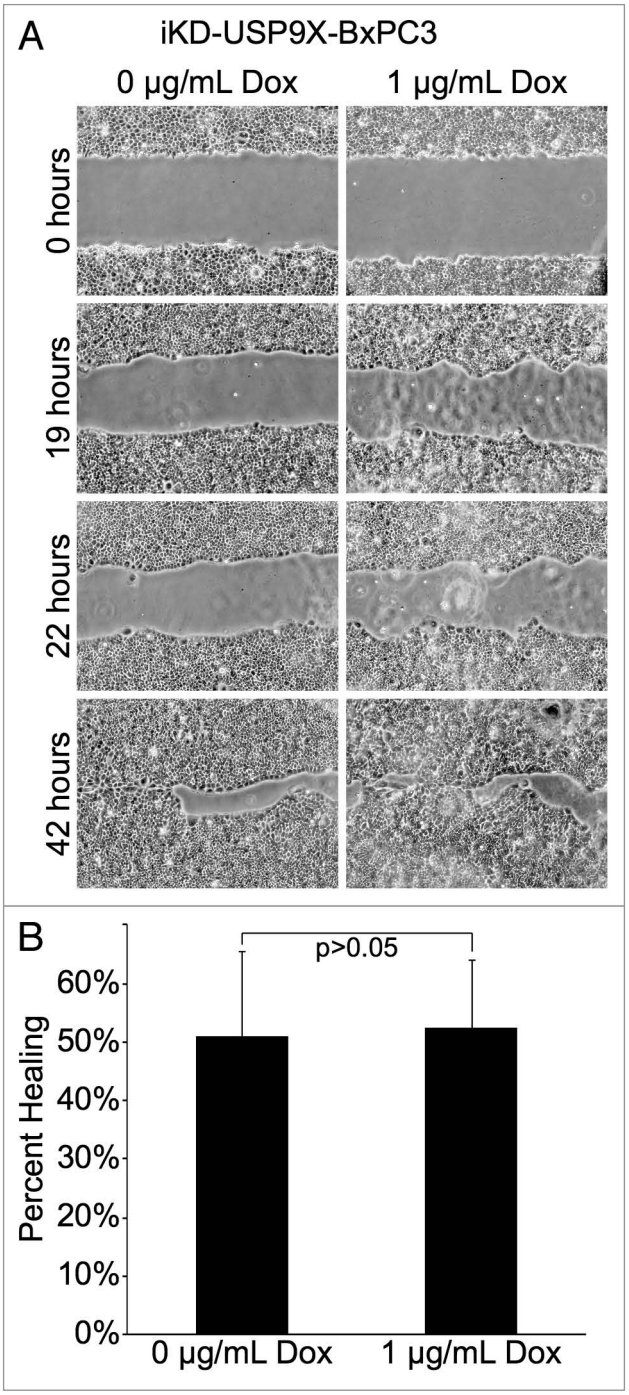

Figure S3. Wound-healing assay. (**A**) iKD-USP9X-BxPC3 cells grown in the absence or presence of Dox (1 µg/mL, 3 d) were wounded using a sterile pipette tip. Photomicrographs were taken at 0, 19, 22, and 42 h after wounding. (**B**) Percent healing of wounds in iKD-USP9X-BxPC3 cells from multiple experiments ($n = 6$); error bars represent standard deviation. The size of the wound area was calculated as indicated in the Materials and Methods.

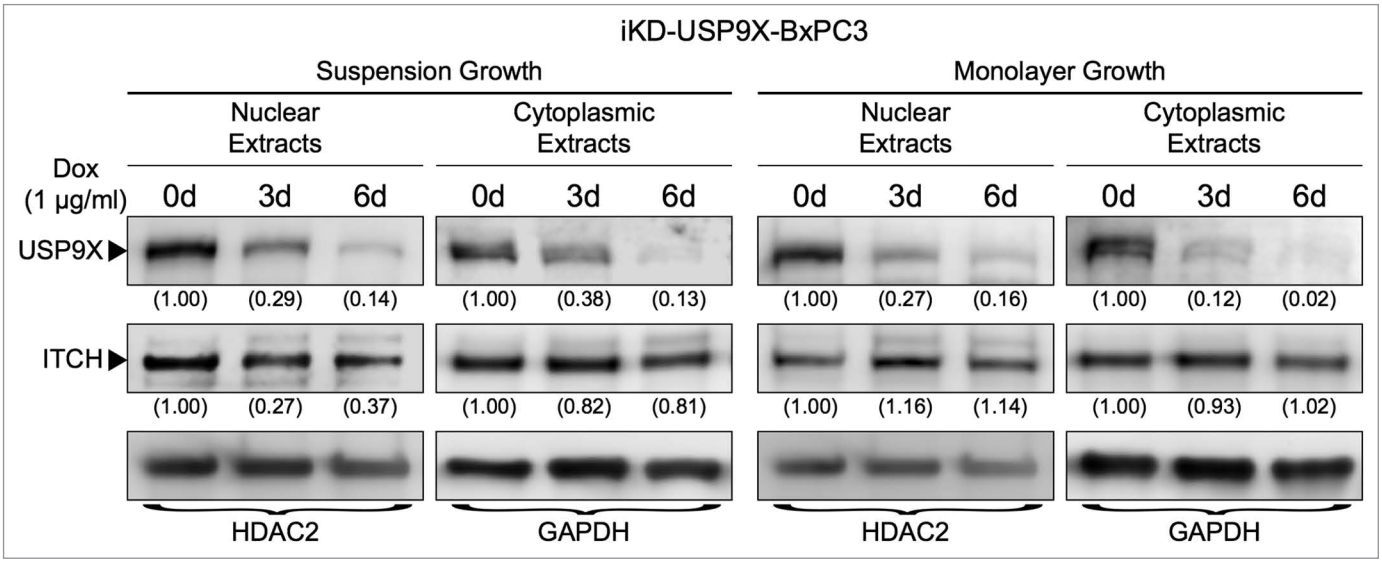

Figure S4. Western blot analysis of the USP9X target, ITCH. Nuclear and cytoplasmic proteins were isolated from iKD-USP9X-BxPC3 cells grown in the absence (0 d) and presence of Dox (1 μg/mL), for either 3 d (3 d) or 6 d (6 d). For suspension growth, monolayer cells were transferred to plates coated with 0.5% noble agar 3 d before harvesting cells for protein extracts. USP9X and ITCH levels were normalized against HDAC2 loading controls in nuclear extracts or GAPDH loading controls in cytoplasmic extracts. Relative levels are indicated in parentheses.

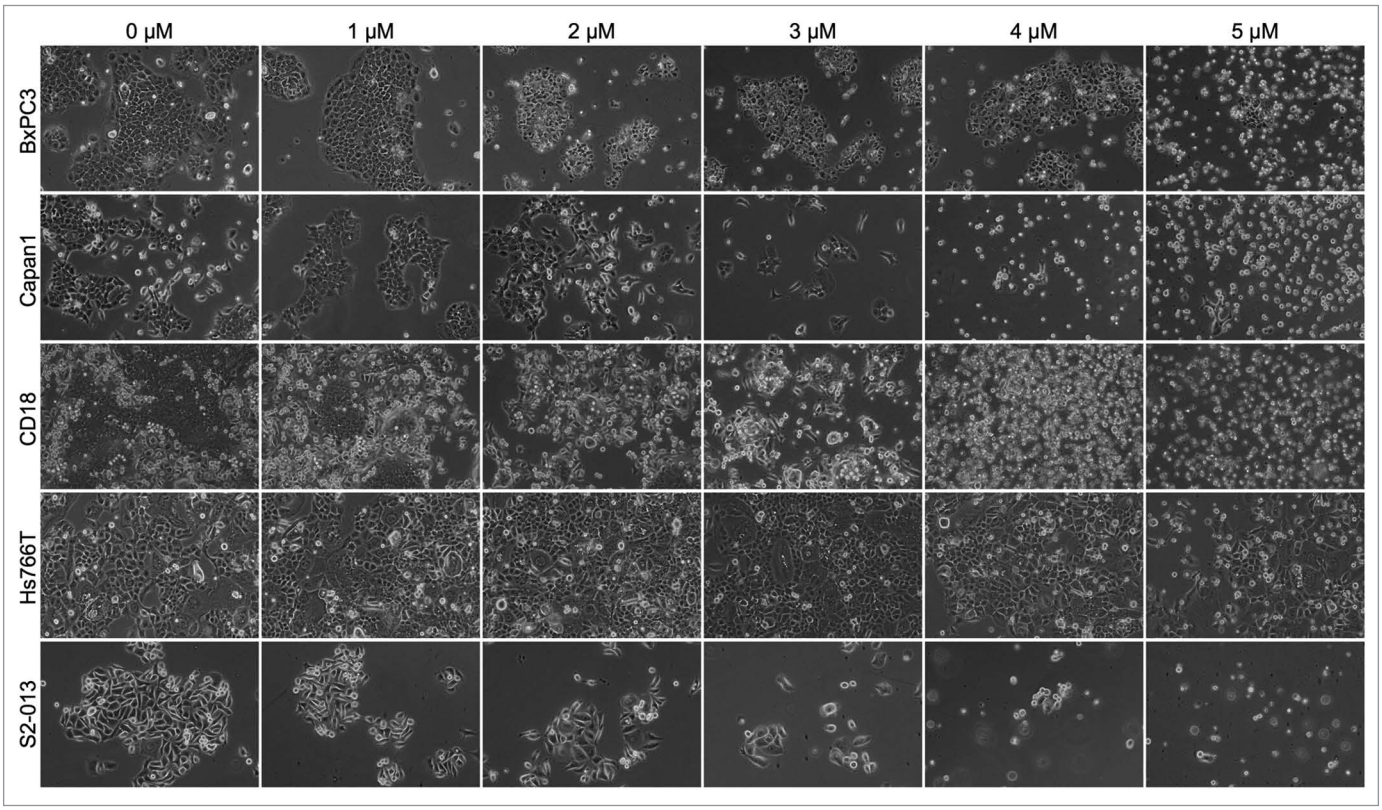

Figure S5. Photomicrographs of PDAC cells cultured in WP1130. Photomicrographs of BxPC3, Capan1, CD18, Hs766T and S2-013, PDAC cells, 48 h after the addition of the indicated concentrations of WP1130 to the cell culture medium. All conditions tested included the same amount of DMSO vehicle.

Table S1. shRNA sequences used for USP9X knockdown

Plasmid Name	Vendor	Catalog No.	Hairpin Sequence (SENSE LOOP ANTISENSE)	Mature Sense
USP9X shRNA #1	Open Biosystems	TRCN0000007361	CCGGGAGAGTTTATTCACTGTCTTACTCGAGTAAGACAGTGAATAAACTCTCTTTTT	GAGAGTTTATTCACTGTCTTA
USP9X shRNA #2	Open Biosystems	TRCN0000007362	CCGGCGATTCTTCAAAGCTGTGAATCTCGAGATTCACAGCTTTGAAGAATCGTTTTT	CGATTCTTCAAAGCTGTGAAT
USP9X shRNA #3	Open Biosystems	TRCN0000007363	CCGGCGACCCTAAACGTAGACATTACTCGAGTAATGTCTACGTTTAGGGTCGTTTTT	CGACCCTAAACGTAGACATTA
			Mature Antisense	
Inducible USP9X shRNA	Open Biosystems	V2THS-41521	TTCATTGGAAGAATCAGGC	

Table S2. Antibodies used for western blot analysis. (**A**) Primary antibodies used. (**B**) Secondary antibodies used. Following incubation with secondary antibodies, ECF substrate (GE Healthcare) and a Typhoon FLA7000 laser scanner (GE Healthcare) were used to visualize protein bands.

A

Target Protein	Animal Host	Antibody Type	Supplier	Supplier Location	Catalog No.
α-Gapdh	Mouse	Monoclonal IgG	Sigma-Aldrich	St. Louis, MO	G8795
α-Hdac1	Rabbit	Polyclonal IgG	Abcam	Cambridge, MA	ab7028
α-Hdac2	Rabbit	Polyclonal IgG	Abcam	Cambridge, MA	ab7029
α-ITCH	Mouse	Monoclonal IgG	BD Biosciences	San Jose, CA	611198
α-Usp9x	Mouse	Monoclonal IgG	Abcam	Cambridge, MA	ab56461

B

Target	Host	Conjugated Molecule	Supplier	Catalog No.	Dilution Used
α-Mouse IgG	Rabbit	AP	Sigma-Aldrich	A4312	1:10,000
α-Rabbit IgG	Goat	AP	Sigma-Aldrich	A3687	1:10,000