Appendix

Supplementary Information

USP4 inhibits SMAD4 monoubiquitination and promotess activin and BMP signaling

FangFang Zhou, Feng Xie, Ke Jin, Zhengkui Zhang, Marcello Clerici, Rui Gao, Maarten van Dinther, Titia K. Sixma, Huizhe Huang, Long Zhang, and Peter ten Dijke

Appendix Supplemental Information Inventory

Appendix Materials and Methods

Immunoprecipitation and Immunoblot analysis

The cells were lysed with 1 ml lysis buffer (20 mM Tris-HCl pH 7.4, 2 mM EDTA, 25 mM NaF, and 1% Triton X-100) containing protease inhibitors (Sigma) for 10 min at 4°C. After centrifugation at 12×10^3 for 15 min, the protein concentrations were measured, and equal amounts of the lysates were used for immunoprecipitation. Immunoprecipitation was performed using various antibodies and protein A-Sepharose (GE Healthcare Bio-Sciences AB) for 3 h at 4°C. Next, the precipitants were washed three times with washing buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% SDS), and the immune complexes were eluted with sample buffer containing 1% SDS for 5 min at 95°C. The immunoprecipitated proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Western blotting was performed with specific primary antibodies and secondary anti-mouse or anti-rabbit antibodies conjugated to horseradish peroxidase (Amersham Biosciences). Visualization was achieved with chemiluminescence.

Transcription Reporter Assay

The transcriptional reporter plasmids ARE-luc and BRE-luc have been previously described (Labbé et al., 1998; Korchynsky and ten Dijke, 2002). HEK293T cells were seeded in 24-well plates and transfected with the indicated plasmids using calcium phosphate. Twenty-four hours after the transfection, the cells were treated with activin A or BMP (concentrations are indicated in the figure legends) overnight or left untreated, and then harvested. The luciferase activity was measured with a luminometer (Perkin Elmer). An internal transfection control β-galactosidase expression plasmid (30 ng) was used to normalize the luciferase activity. Each experiment was performed in triplicate, and the data represent the mean ± SD of three independent experiments.

Immunofluorescence Microscopy

Cells were fixed for 10 min in 4% paraformaldehyde in phosphate-buffered saline (PBS), permeabilized with 0.2% TritonX-100 in PBS, and then blocked with 3% bovine serum albumin (BSA) in PBS for 30 min at room temperature. The specific primary antibodies were diluted in PBS with 3% BSA and incubated with the slides for 3 h at room temperature. Secondary AlexaFluor 488-labeled anti-mouse antibody (Molecular Probes) or AlexaFluor 593-labeled anti-rabbit antibody (Molecular Probes) was added at a dilution of 1:200 in PBS with 3% BSA and incubated for 1 h at room temperature. The coverslips were mounted with Vectashield mounting medium (Vector Laboratories, Inc.). Fluorescence images were acquired with a Zeiss Axioplan microscope.

Lentiviral Transduction and Generation of Stable Cell Lines

We produced lentiviruses by transfecting HEK293T cells with shRNA-targeting plasmids as well as the helper plasmids pCMV-VSVG, pMDLg-RRE (gag/pol), and pRSV-REV. The cell supernatants were harvested 48 h after transfection and were used to infect cells or stored at -80°C.

To obtain stable cell lines, the cells were infected at low confluence (20%) for 24 h with lentiviral supernatants diluted 1:1 with normal culture medium in the presence of 5 ng ml^{-1} polybrene (Sigma). Forty-eight hours after infection, the cells were subjected to puromycin selection for 1 week and then passaged before use. Puromycin was used at 1 µg/ml to maintain the infected cells. The lentiviral shRNAs were obtained from Sigma (MISSION® shRNA). Typically, five shRNAs were identified and tested, and the two most effective shRNAs were used for the experiment. We used TRCN0000004040 (# 1), 5′-CCGGCCCAACTGTAAGAAGCATCAACTCGAGTTGATGCTTCTTACAGTTGGGTTTTT-3′, TRCN0000004041 (# 2), 5′-CCGGGCCCAGAATGTGCTAAGGTTTCTCGAGAAACCTTAGCACATTCTGGGCTTTTT-3′ for human USP4 knockdown. We used TRCN0000030740 (# 1), 5’-

CCGGCCGCGTAAAGAAGAAGCCTTACTCGAGTAAGGCTTCTTCTTTACGC GGTTTTTG-3', TRCN0000030743 (# 2), 5'-CCGGGCCTGGAATAAATTGCTGAATCTCGAGATTCAGCAATTTATTCCAG GCTTTTTG-3' for mouse USP4 knockdown.

Real-Time RT-PCR (qRT-PCR)

The total RNAs was prepared using the NucleoSpin® RNA II Kit (BIOKÉ, Netherlands). One microgram of RNA was reverse transcribed using the RevertAid™ First Strand cDNA Synthesis Kit (Fermentas). qRT-PCR was conducted with SYBR Green (Applied Bioscience) using StepOne Plus (Applied Bioscience). All target gene expression levels were normalized to *GAPDH*. The primer sequences are listed below.

Primers and Reagents

We used the following primer sequences to detect target genes using qRT-PCR:

mPAI-1 forward, 5'- GCCAACAAGAGCCAATCACA-3'

mPAI-1 reverse, 5'- AGGCAAGCAAGGGCTGAAG-3'

mSMAD7 forward, 5'-TGGATGGCGTGTGGGTTTA-3'

mSMAD7 reverse, 5'-TGGCGGACTTGATGAAGATG-3'

mp21 forward, 5'-CCTCATCCCGTGTTCTCCTTT-3'

mp21 reverse, 5'-GTACCACCCAGCGGACAAGT-3'

mp15 forward, 5'-AAGCTGAGCCCAGGTCTCCTA-3'

mp15 reverse, 5'-CCACCGTTGGCCGTAAACT-3'

mGAPDH forward, 5'-AACTTTGGCATTGTGGAAGG-3'

mGAPDH reverse, 5'-ACACATTGGGGGTAGGAACA-3'

The following primer sequences were used for qRT-PCR to analyse the expression of zebrafish genes:

- *sqt*-F, 5'-TGCCGAGCACTCCAAGTATG-3'
- *sqt*-R, 5'-CATCAAGTTATCCAGGTGCC-3'

- *tbx6*-F, 5’- CAAGCTGGATTTGACTGCAA-3’
- *tbx6*-R, 5’- GGGGTTTGTGAAGGCTGATA-3’
- *gapdh*-F, 5’-GATACACGGAGCACCAGGTT-3’
- *gapdh*-R, 5’-GCCATCAGGTCACATACACG-3’
- *vox*-F, 5’-TGGTAATCGAATGCTGATGG-3’
- *vox*-R, 5’-GCTCTGCGGTGGATATGAGT-3’
- *vent*-F, 5’-CACCAAACCGACAATGTGACG-3’
- *vent*-R, 5’-CAAGTTACCTGTCCAAGTGTCC-3’

Myc-USP4 wt and Myc-USP4 C311S expression constructs were cloned and verified by DNA sequencing. The SMURF constructs were kindly provided by Dr. Hongrui Wang (Xiamen University, China). Expression constructs for hemagglutin-tagged ubiquitin (HA-Ub) WT, HA-Ub K63 (which forms only K63 polyubiquitin (polyUb) linkages because all other lysines have been mutated to arginines) and HA-Ub K48 (which forms only K48 polyUb linkages because all other lysines have been mutated to arginines) were kindly provided by Dr. Maréne Landström.

The MG132 proteasome inhibitor was purchased from Sigma. TGF-β3 was generously provided by Dr. K.K. Iwata (OSI Pharmaceuticals, New York, USA). Activin A was purchased from Prospec, and BMP6 was purchased from Peprotech.

The MO *Usp4* splicing blocker (MO2) 5’-TGAACATCTTTGTATACCTGGAGGG-3’ and its MO2 mismatch control, 5’-TCAAGATGTTTCTATACCTGGAGGG-3’ were previously validated (Zhou et al, 2012; Zhang et al., 2012). We have also used a *usp4* ATG (translation) targeting MO (MO1) 5’-CTGCACGATGGCGGAGGAGGCGGACC-3’ and its MO1 mismatch control 5’-CTGCACGATCCCGGTCGTGGCGGACC-3’.

The antibodies used for immunoprecipitation (IP), immunoblot (IB), and Chromatine IP (ChIP) experiments were raised against the following proteins and used in the

following dilutions: β-actin 1:10,000 (IB; A5441, Sigma); c-Myc 1:1000 (IB; a-14, sc-789, Santa Cruz Biotechnology); HA 1:1000 (IB; Y-11, sc-805, Santa Cruz Biotechnology); HA 1:10,000 (IB; 12CA5, produced in-house); Flag 1:10,000 (IB; M2, Sigma), USP4 1:2000 (IB) or 1:200 (IP; U0635, Sigma); USP15 1:5000 (IB) or 1:200 (IP; BETHYL); USP11 1:5000 (IB; BETHYL A301-613A); Ub 1:1000 (IB; P4D1 Santa Cruz); SMURF2 (H-50, sc-25511, Santa Cruz Biotechnology); c-SKI (G8, sc-33693, Santa Cruz Biotechnology); SMAD4 1:1000 (IB) or 1:50 (IP; B8, Santa Cruz), SMAD2-3 1:2500 (IB) or 1:500 (IP; 610842 BD); phospho-SMAD2 1:5000 (IB; no. 3101, Cell Signaling); Phospho-SMAD1/5 (ser463/465) (41D10, #9516, Cell Signaling for immunoblots or produced in-house for ChIP), SMAD1/5/8 (A4, sc-7965, Santa Cruz Biotechnology).

DNA Oligonucleotide Precipitation

The biotinylated SMAD binding element (SBE) oligonucleotide was synthesized by Sangon (Shanghai, China): Bio–5′-TCGATAGCCAGACAGGTAGCCAGACAGGTAGCCAGACAGGTAGCCAGACAGG-3′. A volume of 750 μl whole-cell lysate from HEK293T cells was incubated with 10 μg biotinylated oligonucleotide and streptavidin beads (Pierce) at 4°C overnight in lysis buffer containing 100 mM KCl, 10 mM HEPES (pH 7.9), 10% glycerol, 1 mM dithiothreitol, 5 mM $MgCl_2$, 0.5% NP-40, 10 mM NaF, 20 mM β-glycerophosphate, and proteinase inhibitors (Roche). After being extensively washed with lysis buffer, the DNA-bound protein was subjected to SDS-PAGE, followed by immunoblot analysis using appropriate antibodies.

Appendix Supplementary References:

Korchynskyi O, ten Dijke P. (2002) Identification and functional characterization of distinct critically important bone morphogenetic protein-specific response elements in the Id1 promoter. *J Biol Chem* 277: 4883-491

Labbé E, Silvestri C, Hoodless PA, Wrana JL, Attisano L. (1998) Smad2 and Smad3 positively and negatively regulate TGFβ-dependent transcription through the forkhead DNA-binding protein FAST2. *Mol Cell* 2: 109-120

Zhang L, Zhou F, Drabsch Y, Gao R, Snaar-Jagalska BE, Mickanin C, Huang H, Sheppard KA, Porter JA, Lu CX, ten Dijke P. (2012) USP4 is regulated by AKT phosphorylation and directly deubiquitylates TGF-β type I receptor. *Nat Cell Biol* 14: 717-726

Zhou F, Zhang X, van Dam H, ten Dijke P, Huang H, Zhang L (2012) Ubiquitin-specific protease 4 mitigates Toll-like/interleukin-1 receptor signaling and regulates innate immune activation. *J Biol Chem* 287: 11002-11010

Appendix Figure Legends

Appendix Figure S1. USP4 colocalization with SMAD4.

Immunofluorescence analysis and 4, 6-diamidino-2-phenylindole (DAPI) staining of HeLa cells that had been transfected with Myc-tagged USP4 and Flag-SMAD4.

Appendix Figure S2. SMURF2 is required for TGF-β-induced c-SKI ubiquitination

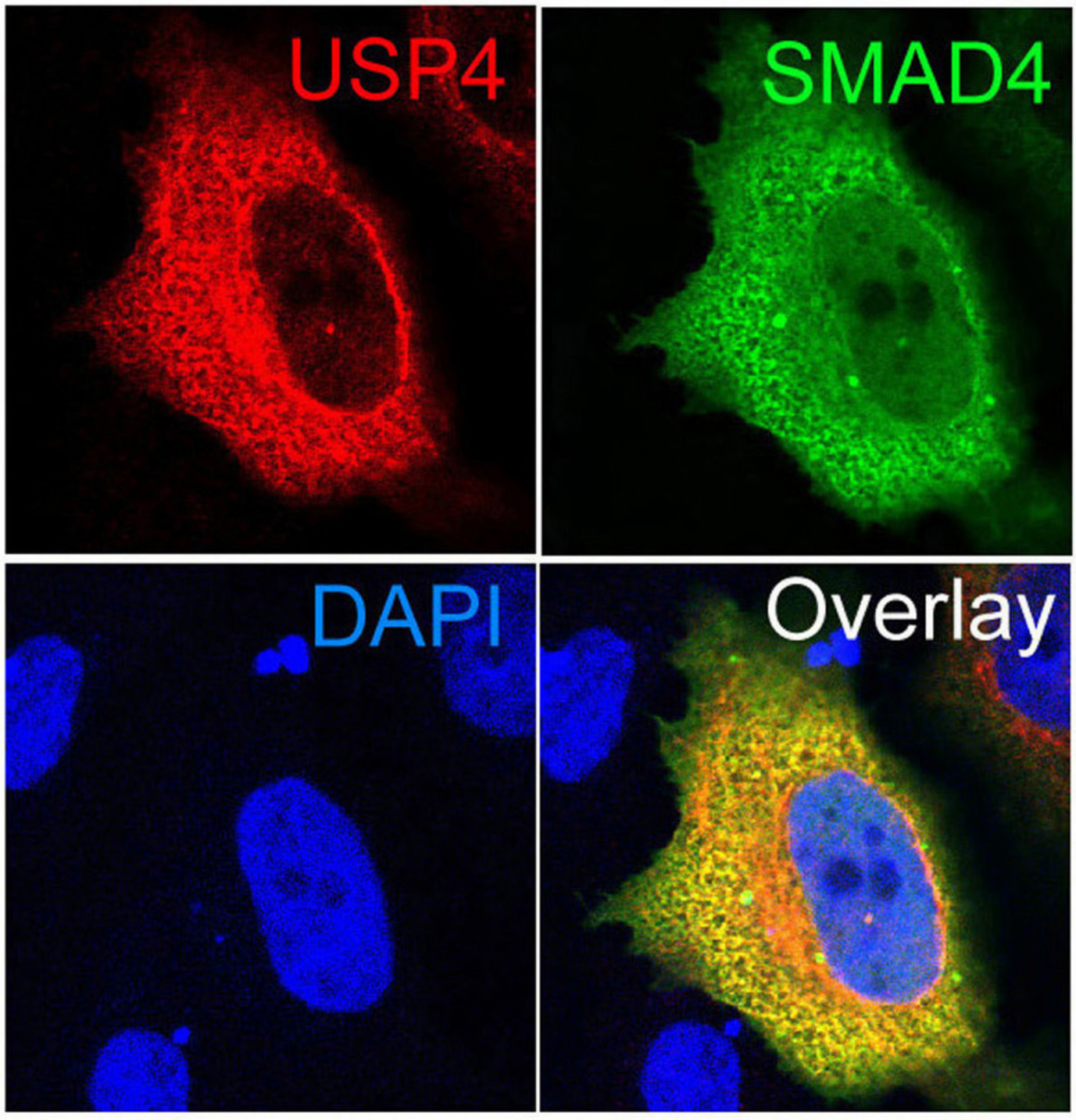
SMURF2 promoted the efficient ubiquitination of c-SKI following treatment of cells with TGF-β (5 ng/ml). IB analysis of the total cell lysate (TCL) and immunoprecipitates derived from control and SMURF2 deficient HEK293T cells transfected that had been with Flag-c-SKI.

Appendix Figure S3. MO-induced *usp4* morphant phenotype is rescued by the ectopic expression of *usp4* mRNA

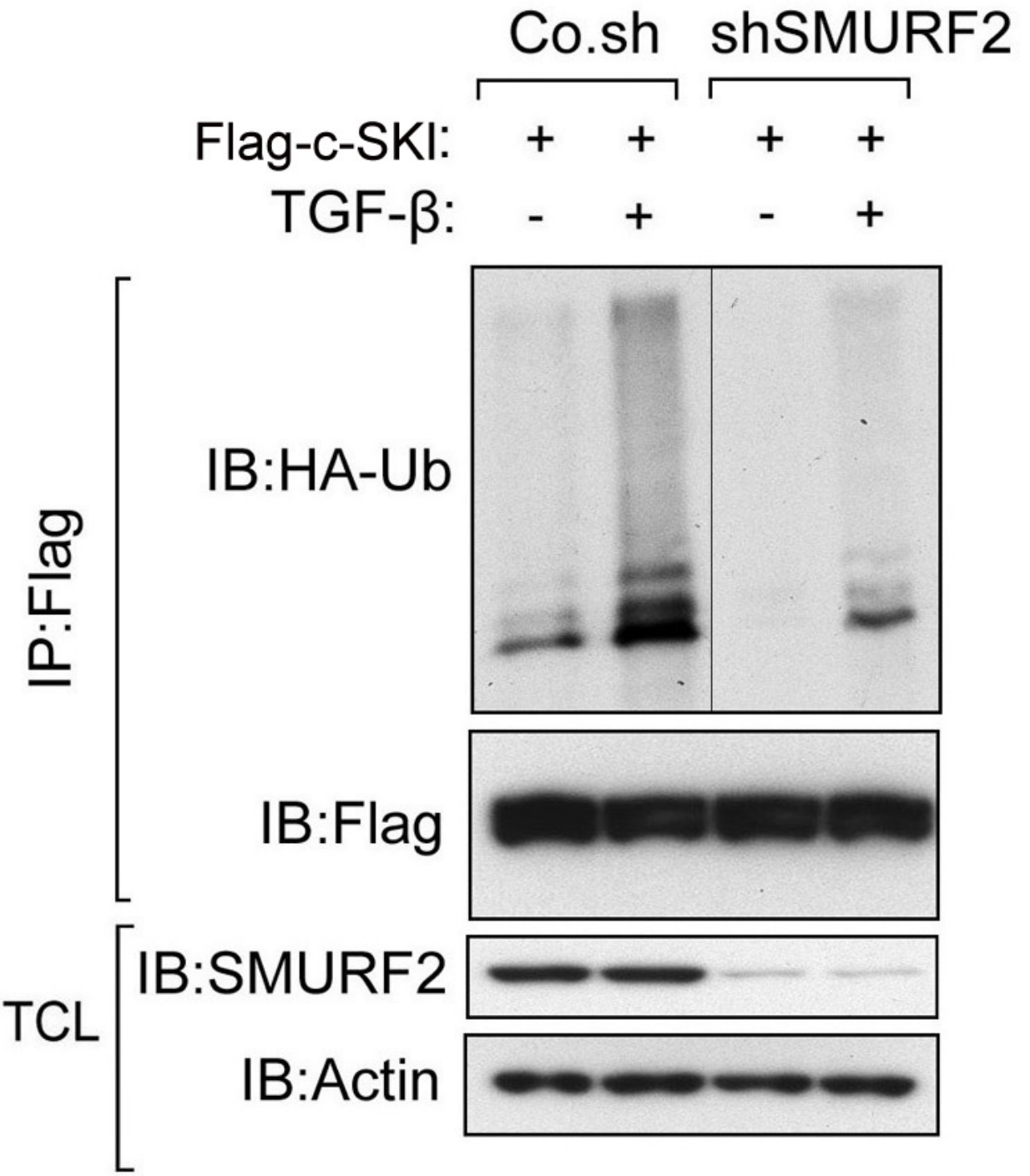
(**A**) Control MO targeting *p53* zebrafish phenotype.

(**B, D**) *usp4* MO1 (ATG *usp4* blocker) and MO2 (targeting *usp4* splicing) induced similar phenotypes.

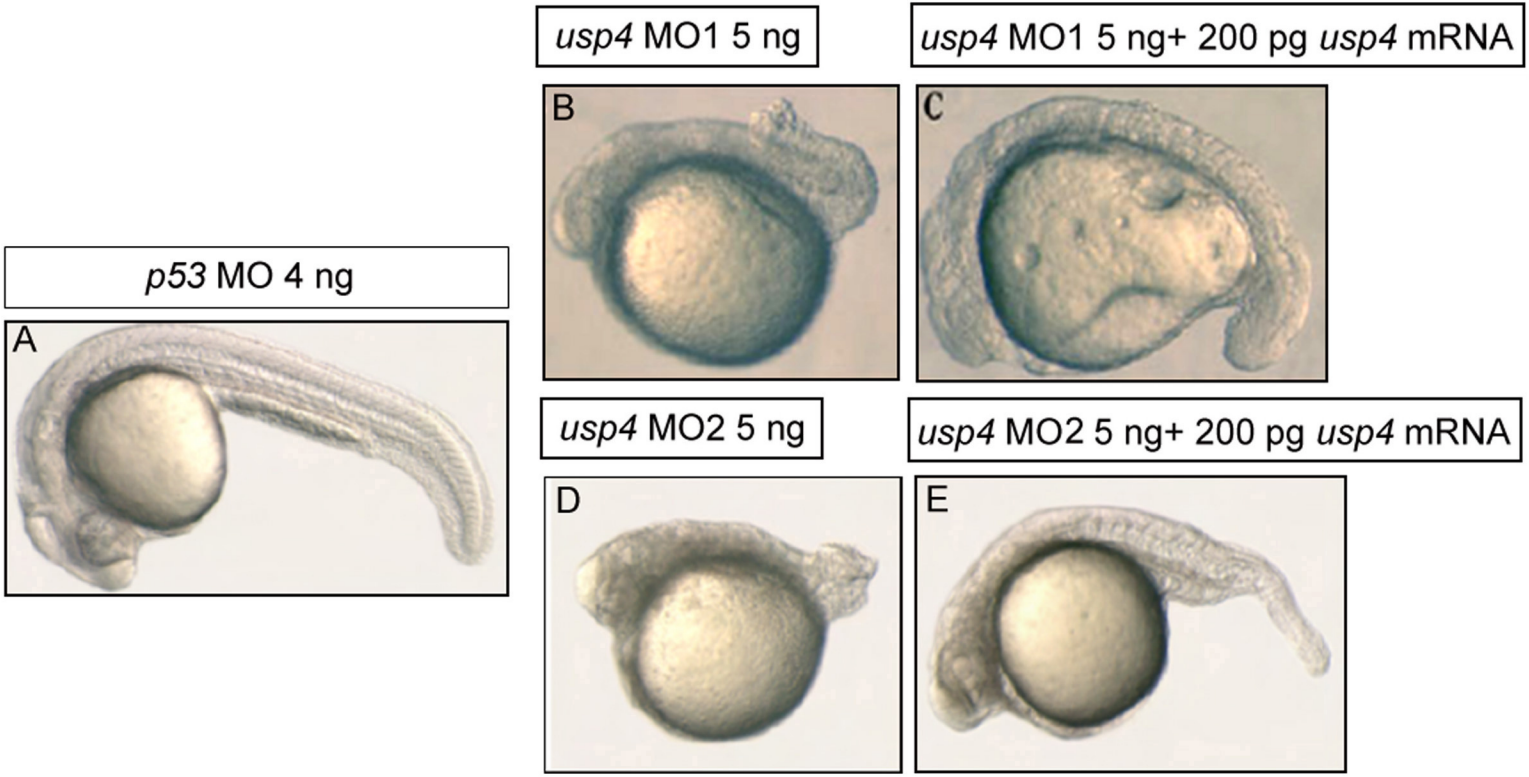
(**C, D**) The MO-induced *usp4* morphant phenotypes were partially rescued by 200 pg *usp4* mRNA co-injection. Effect of MO targeting *usp4* splicing are shown in Figure 1, Figure 7, Figure EV3 and Figure EV5 and indicated as "MO".



Appendix Figure S1



Appendix Figure S2



Appendix Figure S3