Expanded View Figures

Figure EV1. DUB cDNA screening identified USP4 as the most potent activator of activin signaling.

A This graph depicts the activin (5 ng/ml)-induced ARE-Luc transcriptional reporter activity compared to control cells and the effects of a representative set of DUB cDNAs, $n = 3$. The top activator USP4 is indicated with an arrow. The data are presented as means ± SD.

B List of the results of screen performed in Appendix Fig S1A.

C Left panel: effect of USP4 and USP9x on the ARE-Luc transcriptional response induced by activin A (50 ng/ml) in HepG2 cells. Right panel (mentioned in the discussion): USP4, but not the USP9x rescued the defective ARE-Luc response caused by sh-USP4 that target USP4 3'UTR. The data are presented as means ± SD. Co.vec, empty vector; Co.sh, non-targeting shRNA. * indicates statistical significance ($P < 0.05$). Two-tailed, unpaired t-test. Experiments were performed in triplicate.

D Q-PCR analysis of *USP4* mRNA level in HepG2 cells infected with lentivirus encoding control (Co.sh) or USP4 shRNA (#1). Experiments were performed in triplicate. Two-tailed, unpaired t-test. $^{**}P < 0.01$ and $^{***}P < 0.001$. The data are presented as means ± SD.

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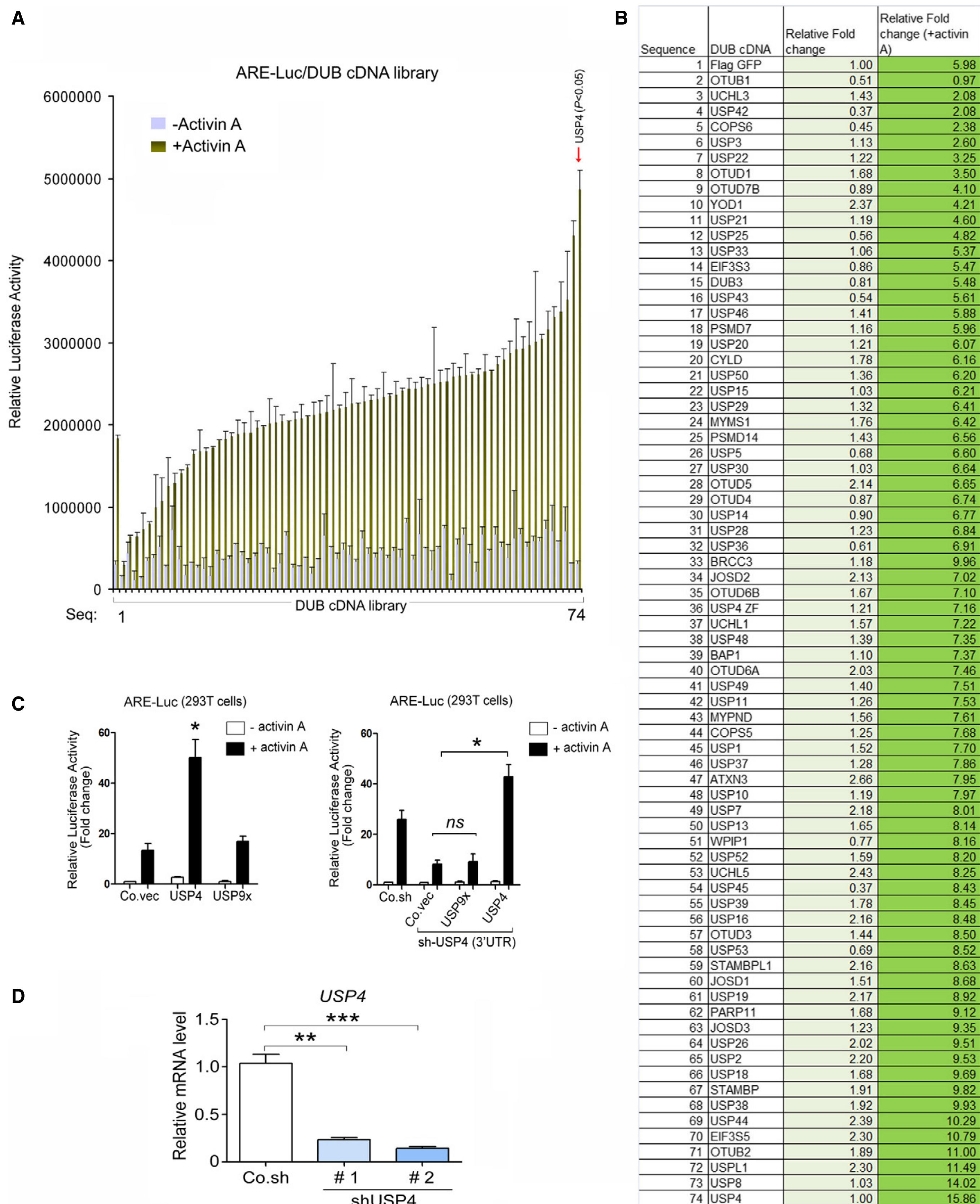

Sequence	DUB cDNA	Relative Fold change	Relative Fold change (+activin A)
1	Flag GFP	1.00	5.98
2	OTUB1	0.51	0.97
3	UCHL3	1.43	2.08
4	USP42	0.37	2.08
5	COPS6	0.45	2.38
6	USP3	1.13	2.60
7	USP22	1.22	3.25
8	OTUD1	1.68	3.50
9	OTUD7B	0.89	4.10
10	YOD1	2.37	4.21
11	USP21	1.19	4.60
12	USP25	0.56	4.82
13	USP33	1.06	5.37
14	EIF3S3	0.86	5.47
15	DUB3	0.81	5.48
16	USP43	0.54	5.61
17	USP46	1.41	5.88
18	PSMD7	1.16	5.96
19	USP20	1.21	6.07
20	CYLD	1.78	6.16
21	USP50	1.36	6.20
22	USP15	1.03	6.21
23	USP29	1.32	6.41
24	MYMS1	1.76	6.42
25	PSMD14	1.43	6.56
26	USP5	0.68	6.60
27	USP30	1.03	6.64
28	OTUD5	2.14	6.65
29	OTUD4	0.87	6.74
30	USP14	0.90	6.77
31	USP28	1.23	6.84
32	USP36	0.61	6.91
33	BRCC3	1.18	9.96
34	JOSD2	2.13	7.02
35	OTUD6B	1.67	7.10
36	USP4 ZF	1.21	7.16
37	UCHL1	1.57	7.22
38	USP48	1.39	7.35
39	BAP1	1.10	7.37
40	OTUD6A	2.03	7.46
41	USP49	1.40	7.51
42	USP11	1.26	7.53
43	MYPND	1.56	7.61
44	COPS5	1.25	7.68
45	USP1	1.52	7.70
46	USP37	1.28	7.86
47	ATXN3	2.66	7.95
48	USP10	1.19	7.97
49	USP7	2.18	8.01
50	USP13	1.65	8.14
51	WPIP1	0.77	8.16
52	USP52	1.59	8.20
53	UCHL5	2.43	8.25
54	USP45	0.37	8.43
55	USP39	1.78	8.45
56	USP16	2.16	8.48
57	OTUD3	1.44	8.50
58	USP53	0.69	8.52
59	STAMBPL1	2.16	8.63
60	JOSD1	1.51	8.68
61	USP19	2.17	8.92
62	PARP11	1.68	9.12
63	JOSD3	1.23	9.35
64	USP26	2.02	9.51
65	USP2	2.20	9.53
66	USP18	1.68	9.69
67	STAMBP	1.91	9.82
68	USP38	1.92	9.93
69	USP44	2.39	10.29
70	EIF3S5	2.30	10.79
71	OTUB2	1.89	11.00
72	USPL1	2.30	11.49
73	USP8	1.03	14.02
74	USP4	1.00	15.86

Figure EV1.

Figure EV2. USP4 is required for BMP-SMAD signaling.

A qRT–PCR analysis of activin target genes *SMAD7*, *PAI-1*, *P21*, and *P15* in $USP4^{+/+}$ and $USP4^{-/-}$ MEFs in the absence or presence of ectopically expressed USP4-WT or USP4-CS and treated with activin A (50 ng/ml) for 1 h. The values and error bars represent the mean ± SD of triplicate assays and at least two independent experiments.

B, C The effect of USP4 wild-type (USP4-WT) and the USP4 mutant in which the de-ubiquitinating enzyme activity is inactive (USP4-CS) (B) or USP4 knockdown (shUSP4 #1 and #2) (C) on the BRE-Luc transcriptional response induced by BMP6 (25 ng/ml) in HepG2 cells. These data are presented as the means ± SD (n = 3). Co.vec, empty vector; Co.sh, non-targeting shRNA.

D, E Immunoprecipitation (IP) and immunoblotting (IB) analyses of SMAD1/5-SMAD4 complex formation and SMAD1/5 phosphorylation in HepG2 cells that stably overexpressed USP4-WT or USP4-CS (D) or in which stable knockdown of USP4 by shRNA (shUSP4 #1 and #2) (E) was achieved and after stimulation with BMP6 (25 ng/ml) for the indicated times. IB for actin on TCL is included as loading control.

F, G Effect of morpholino-mediated depletion of *usp4* on *activin*- and *bmp4*-induced phenotype as measured by expression of *gata5* and *sox32* (F) and *eve1* and *bmp2* (G). These figures relate to Fig 1I and J.

H The relative expression levels of zebrafish *vox* and *vent* as determined by qRT–PCR. The quantified mRNA levels were normalized to *β-actin* mRNA level and are presented relative to control embryos. * indicates statistical significance (P < 0.05), and ** indicates statistical significance (P < 0.005). Two-tailed, unpaired t-test. These data are presented as the means ± SD (n = 3).

Source data are available online for this figure.

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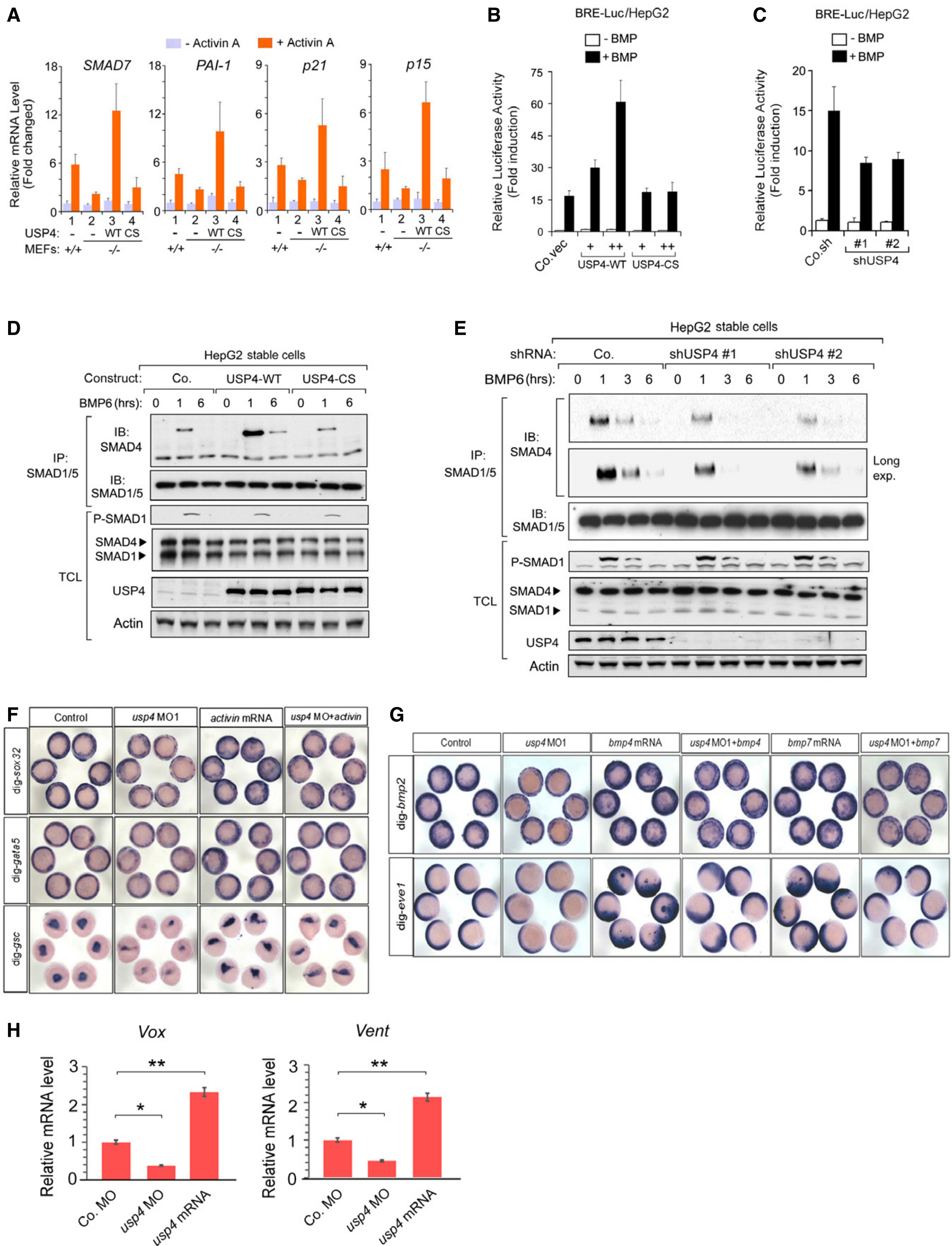

Figure EV2.

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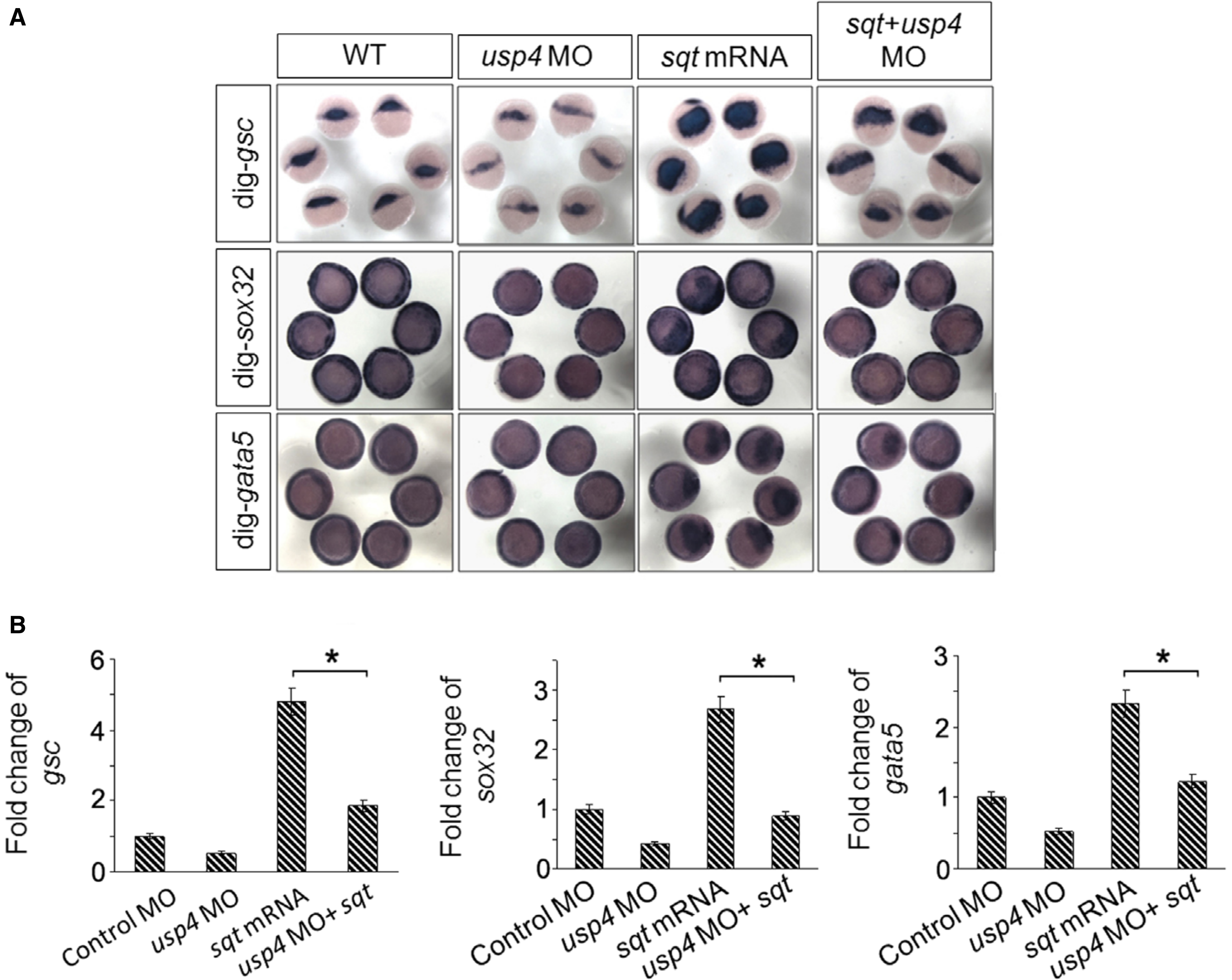

Figure EV3. Genetic interaction between zebrafish *usp4* and *sqt*.

A The depletion of *usp4* partially inhibited the *sqt*-induced phenotype. Single-cell embryos were injected with control MO, *usp4* MO, *sqt* mRNA, or their combination. Embryos at the shield stage or 70% epiboly stage were fixed and hybridized with probes for markers of the endoderm and mesoderm including *sox32*, *gata5*, and *goosecoid* (*gsc*). Each group of embryos is oriented differently: animal pole view for *sox32*, and *gata5* panels; organizer view for the *gsc* panel.

B The relative expression levels of zebrafish *goosecoid* (*gsc*), *sox32*, and *gata5* were monitored by quantitative real-time PCR (qRT–PCR). The quantified mRNA levels were normalized to *β-actin* and are presented relative to the control embryos. * indicates statistical significance ($P < 0.05$). Two-tailed, unpaired *t*-test. Experiments were performed on at least three independent injected embryos. These data are presented as the means ± SD.

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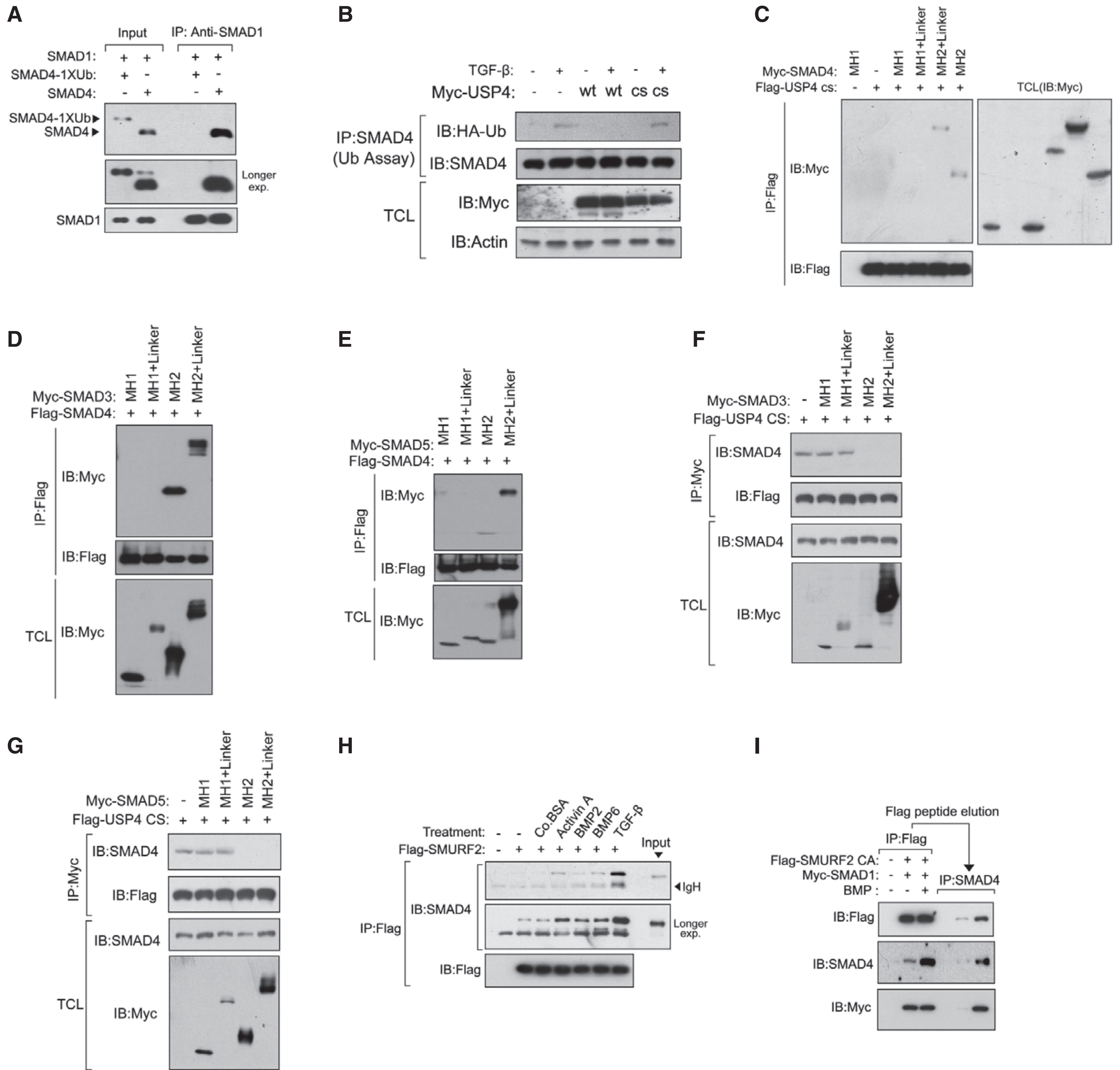

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Figure EV4. Regulation of SMAD4 monoubiquitination.

- A R-SMAD preferred to associate with free SMAD4 rather than monoubiquitinated SMAD4. IB analysis of the input and immunoprecipitates derived from the incubation of purified SMAD1 with SMAD4 or SMAD4 -1xUb.
- B USP4 prevented the monoubiquitination of SMAD4 upon stimulation of cells with TGF-β. IB analysis of total cell lysates (TCL) and immunoprecipitates derived from HA-Ub expressing HEK293T cells that were transfected with Myc-USP4 wild-type (WT) or Myc-USP4 C311S mutant (CS),with or without TGF-β treatment (5 ng/ml) for 1 h.
- C USP4 bound to the SMAD4 MH2 domain. HEK293T cells were transfected with Myc-tagged SMAD4 deletions and Flag-tagged USP4 CS. The cells were harvested for immunoprecipitation (IP) with anti-Flag affinity resin and subjected to IB analysis with indicated antibodies (left panel). TCL were analyzed by IB using myc antibodies (right panel).
- D, E The SMAD4 bound to the MH2 domain of SMAD3 or SMAD5. HEK293T cells were transfected with Myc-tagged SMAD3 deletions (D) or SMAD5 deletion (E) and Flag-tagged SMAD4. The cells were harvested for IP with anti-Flag affinity resin and IB with the indicated antibodies (upper two panels). TCL were analyzed by IB using myc antibodies (bottom panel).
- F, G The MH2 domain of SMAD3 or SMAD5 displaced USP4 from SMAD4. IB analysis of total cell lysate (TCL) and immunoprecipitates derived from HEK293T cells that were transected with Flag-USP4 CS and Myc-SMAD3 (F) or Myc-SMAD5 (G) deletions as indicated.
- H IB analysis of immunoprecipitates derived from Flag-SMURF2-transfected HEK293T cells treated with or without activin A (50 ng/ml), BMP2 (50 ng/ml), or TGF-β (5 ng/ml) for 1 h.
- I BMP6 promoted the formation of a trimeric complex of SMURF2, SMAD1, and SMAD4. Flag-SMURF2 CA and Myc-SMAD1 were co-expressed in HEK293T cells. The cells were treated with BMP6 (50 ng/ml) for 1 h and then harvested for immunoprecipitation with anti-Flag beads and elution with Flag-peptide. The second IP with anti-SMAD4 antibody was performed, and the resulting samples were subjected to IB with anti-Flag, anti-SMAD4, or anti-Myc antibodies.

Source data are available online for this figure.

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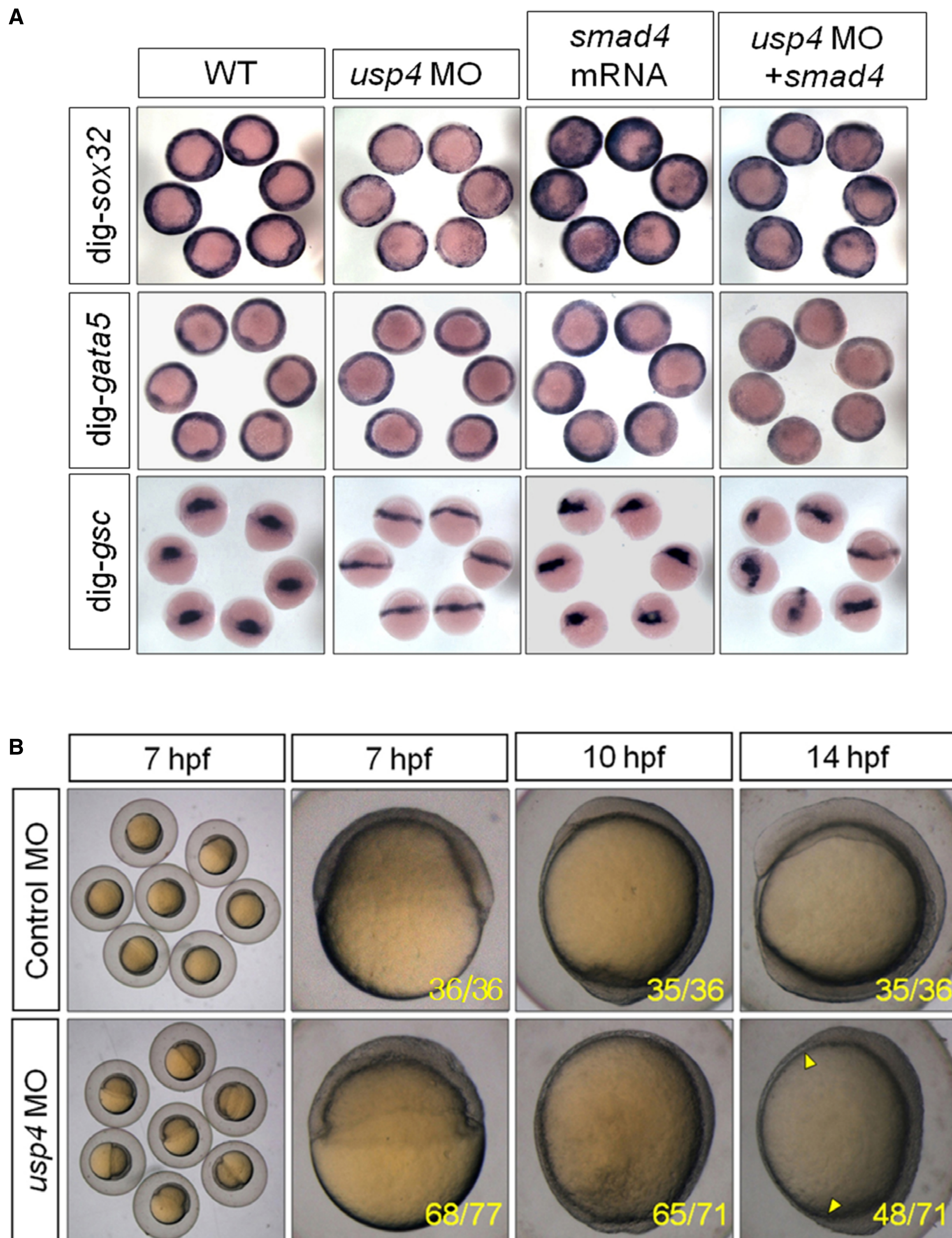

Figure EV5. Effect of usp4 morphants on embryonic cell movements.

A Related to Fig 7A, overview of zebrafish embryos. The *usp4* depletion partially inhibits *smad4*-induced phenotype. Single-cell embryos were injected with control morpholino (MO), *usp4* MO, *smad4* mRNA, or their combination (as indicated).

B Injection of *usp4* morpholino led to impaired epiboly and convergent extension (CE) movement. Single-cell embryos were injected with *usp4* MO or control morpholino (control MO). The stages of the embryos are shown, and the number of described embryos and total number are listed in the bottom.

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