

Supplementary Data

Supplementary Figure Legends

Supplementary Figure 1 FHL-mediated TGF β -responsive reporter transcription and the promoter occupancy of Smad proteins. **(A)** HepG2 cells were co-transfected with the wwp-Luc reporter, and FLAG-tagged FHL1, FHL2, or FHL3, either singly or in combination, as indicated, in the absence of TGF β . Cells were harvested and analyzed for luciferase activity. **(B)** HepG2 cells were co-transfected with the wwp-Luc reporter, and FHL1 siRNA, FHL2 siRNA, or FHL3 siRNA, either singly or in combination, as indicated. Cells were treated and analyzed, as in (A). **(C)** Soluble chromatin was prepared from HepG2 cells treated with 5 ng/ml TGF β 1 and subjected to immunoprecipitation with normal serum (IgG) or indicated antibodies. Immunoprecipitated DNA was PCR-amplified with primers that annealed to the proximal region of the *PAI-1*, *p21*, or *c-myc* promoter, or to the region approximately 2 kb upstream of these promoters. **(D)** Soluble chromatin was prepared from HepG2 cells transiently transfected with FLAG-tagged FHL1 or CLIM-1, and were analyzed, as in (C). **(E)** Soluble chromatin was prepared from HepG2 cells stably transfected with FHL1 siRNA, and were analyzed, as in (C).

Supplementary Figure 2 Interaction of FHL1 and Smad4 is required for enhancement of TGF β -responsive reporter activity. **(A)** Mapping of the FHL1 interaction region in Smad4. FLAG-tagged FHL1 and lac-tagged full-length Smad4 or its deletion mutants were co-transfected into 293T cells. Cell lysates were immunoprecipitated by anti-FLAG and the precipitates were then immunoblotted with anti-lac antibody. Also shown are schematic diagrams of the constructs used in this study. **(B)** Mapping of the Smad4 interaction region of FHL1. 293T cells were co-transfected with lac-tagged Smad4 and FLAG-tagged full-length FHL1 or its mutants. Lysates from the transfected cells were immunoprecipitated with anti-FLAG antibody, and the immunoprecipitates were assayed with anti-lac antibody. Also shown are schematic diagrams of the constructs used in this study. **(C)** Luciferase assay with 293T cells co-transfected with p3TP-Lux and FLAG-tagged FHL1, FHL1(1-169), or FHL1(56-280), as indicated. **(D)** FLAG-tagged FHL1 and lac-tagged Smad4, Smad4(V370D), or Smad4(D493H) were co-transfected into 293T cells. Co-immunoprecipitation was performed, as in (A). Densitometric quantitation of lac-Smad4, lac-Smad4(D493H), and lac-Smad4(V370D) bands normalized to the corresponding α -lac (Input) and α -FLAG (IP: α -FLAG, IB: α -FLAG) bands from three independent experiments is shown at bottom (means $\pm$ SD). **(E)** Luciferase assays with 293T cells co-transfected with lac-Luc, FHL1, and lac-tagged Smad4,

Smad4(V370D), or Smad4(D493H). (F) Luciferase assays with MDA-MB-468 cells co-transfected with p3TP-Lux and FHL1, in the absence and presence of Smad4.

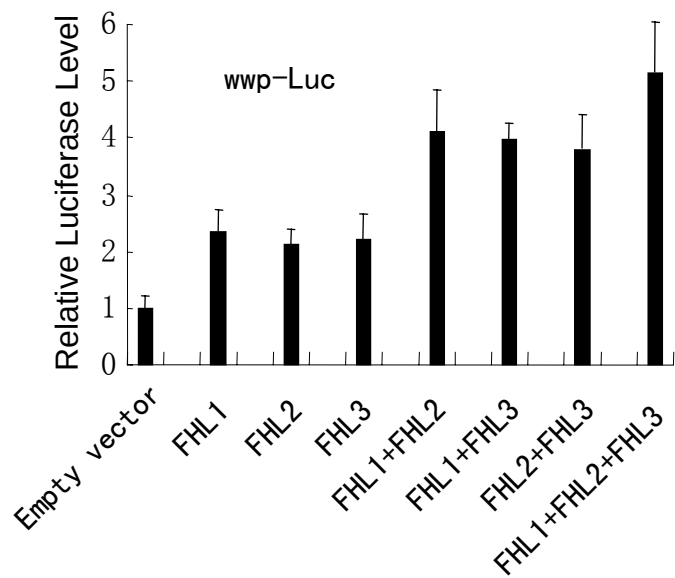
Supplementary Figure 3 CK1 δ -mediated Smad3 phosphorylation at the linker region is required for the increased interaction with Smad4, the nuclear accumulation of Smad proteins, and increased expression of p21 gene. (A) HepG2 cells were co-transfected with HA-tagged CK1 δ and FLAG-tagged Smad3 or Smad3(S207,212A) (Smad3Lm). Cell lysates were immunoprecipitated with anti-FLAG antibody, and the precipitates immunoblotted with anti-phosphoserine (p-Ser) antibody. (B) HepG2 cells were co-transfected with HA-CK1 δ , lac-Smad4, and FLAG-tagged Smad3 or Smad3(S207,212A). Cell lysates were immunoprecipitated with anti-FLAG antibody, followed by immunoblotting with the indicated antibodies. (C) HepG2 cells co-transfected with HA-CK1 δ and FLAG-tagged Smad3 or Smad3(S207,212A) were fractionated into cytoplasmic and nuclear fractions. The lysates were immunoblotted with the indicated antibodies. (D) HepG2 cells were co-transfected with HA-CK1 δ and FLAG-tagged Smad3 or Smad3(S207,212A). The cells were harvested and used for immunoblotting with anti-p21 antibody.

Supplementary Figure 4 Effects of over-expression of FHL1 or siRNA knockdown of endogenous FHL1 on Smad nuclear translocation. HepG2 cells stably expressing FHL1 (**A**) or FHL1 siRNA (**B**) were treated with or without 2 ng/ml TGF β 1, immunostained for FHL1 (red) and Smad2/3 or Smad4 (green), and counterstained for DNA with DAPI (blue). The images were captured by confocal immunofluorescence microscopy; original magnification, $\times 150$.

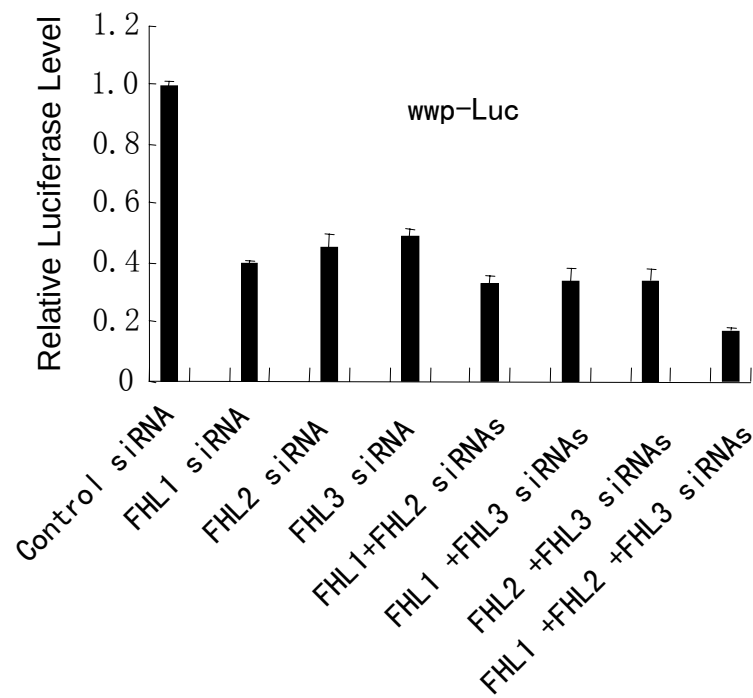
Supplementary Figure 5. CK1 δ and Smad4 are required for FHL1 inhibition of tumor cell growth. (**A**) HepG2 cells stably expressing CK1 δ siRNA, FHL1 or in combination were transiently transfected with CK1 δ or empty vector, as indicated. The transfection efficiency is approximately 70%. At the indicated times, cells were harvested, and the cell number was determined by crystal violet assay. (**B**) Smad4-negative MDA-MB-468 cells were transfected with EGFP-FHL1 in the absence or presence of the expression vector for Smad4. The transfection efficiency is 10%-20%. Cell cycle analysis was then performed by flow cytometry.

Supplementary Figure 1

A

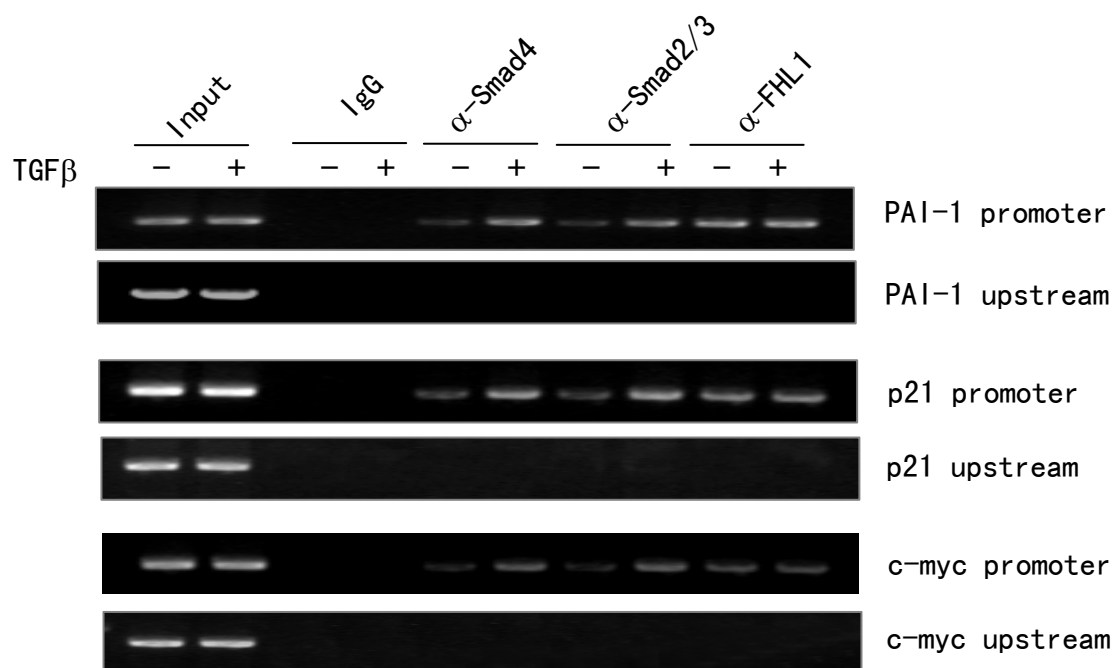


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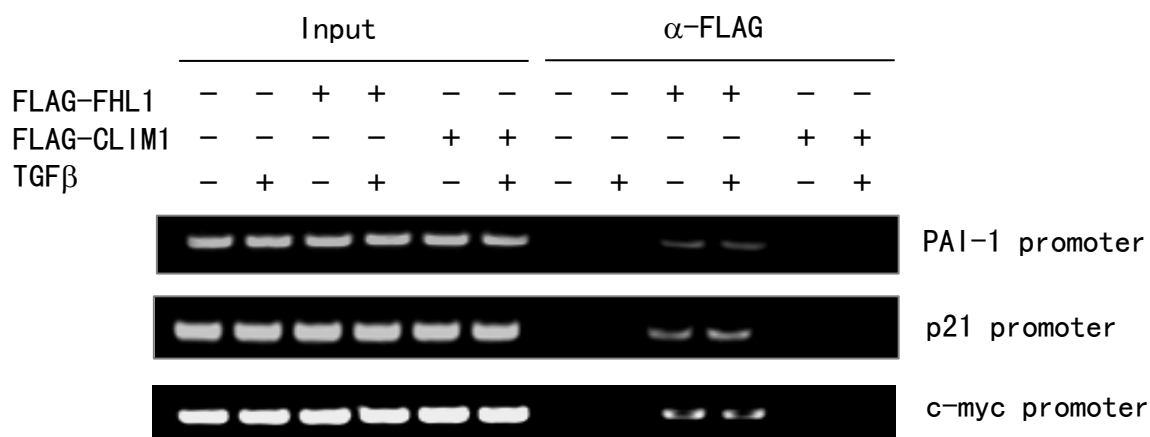


Supplementary Figure 1

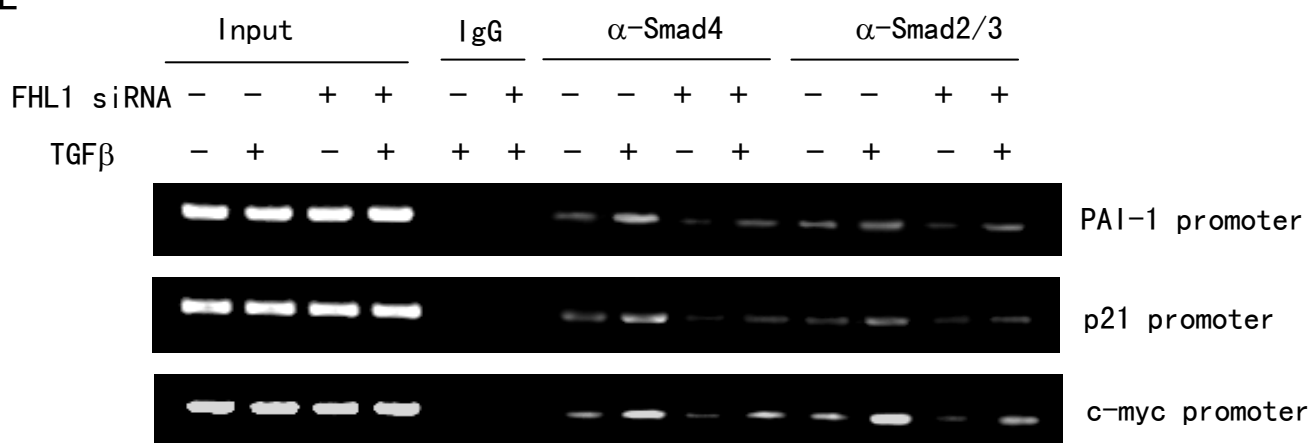
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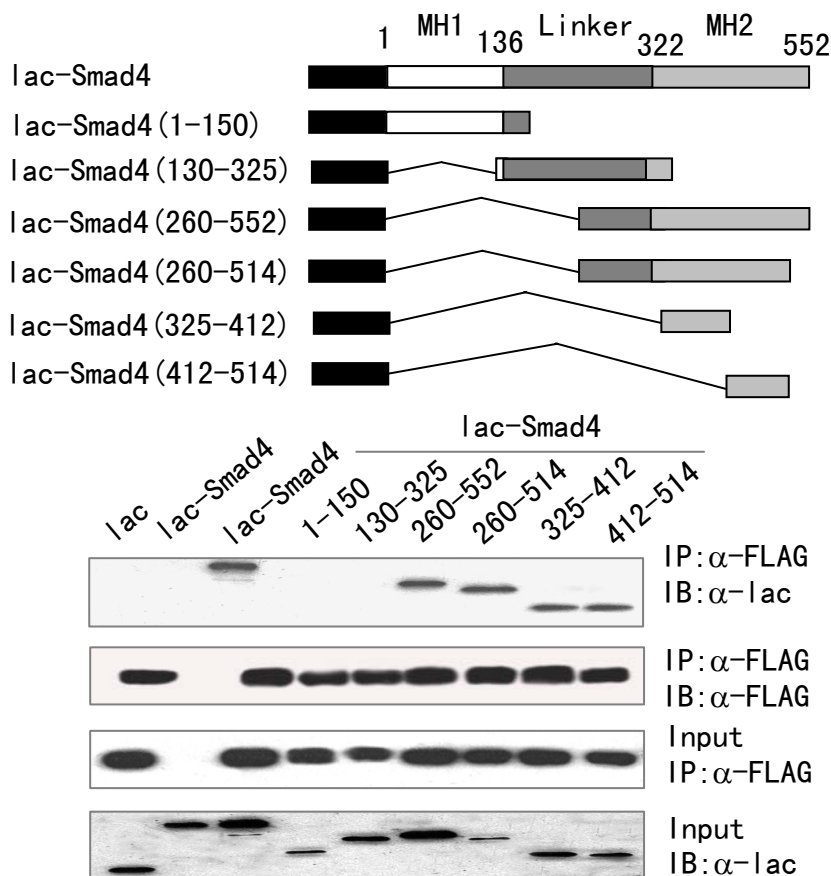


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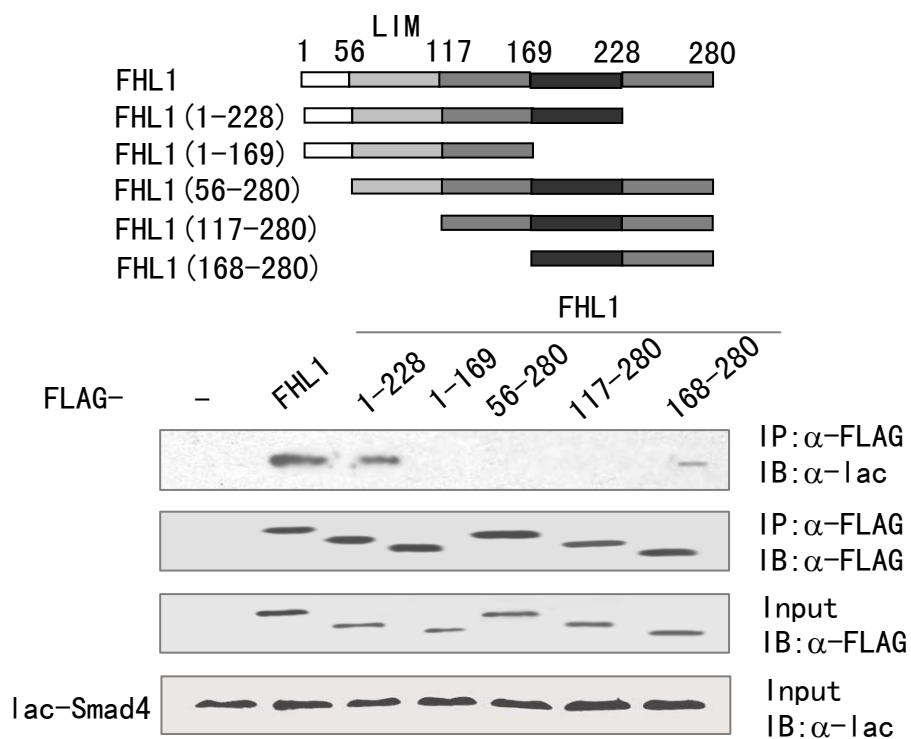


Supplementary Figure 2

A

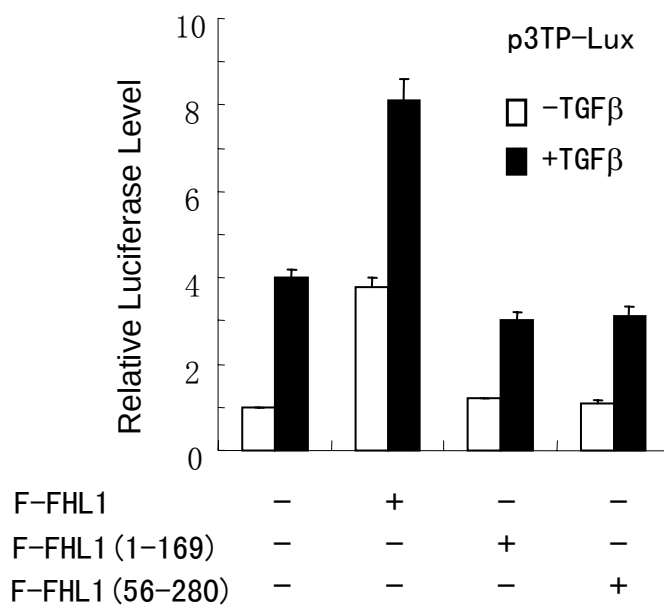


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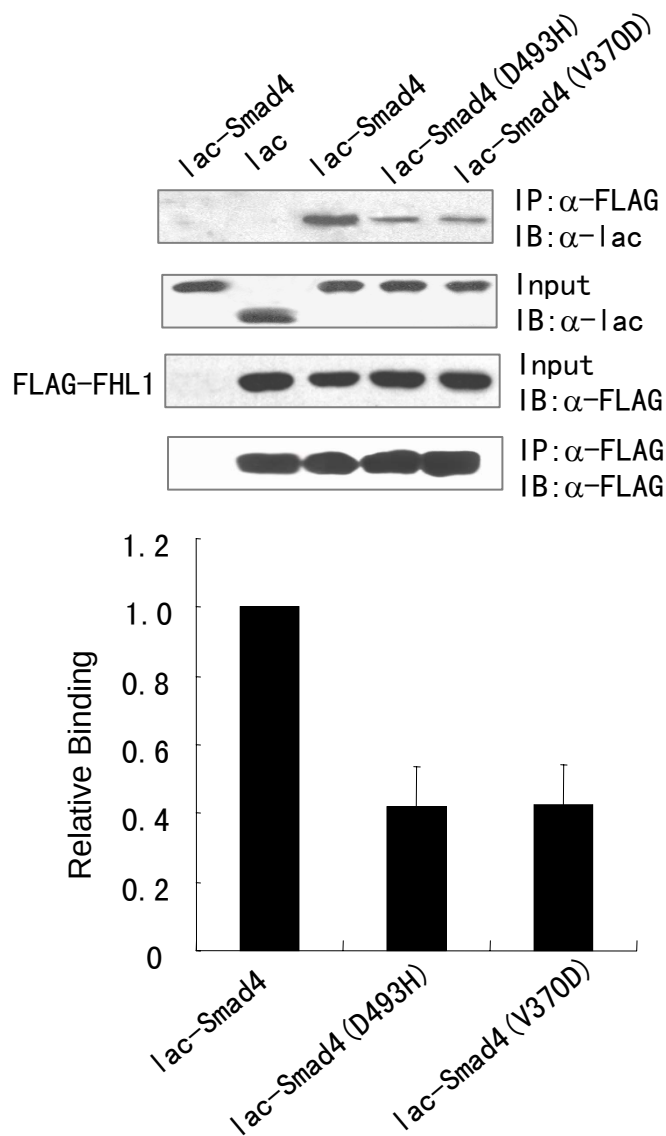


Supplementary Figure 2

C

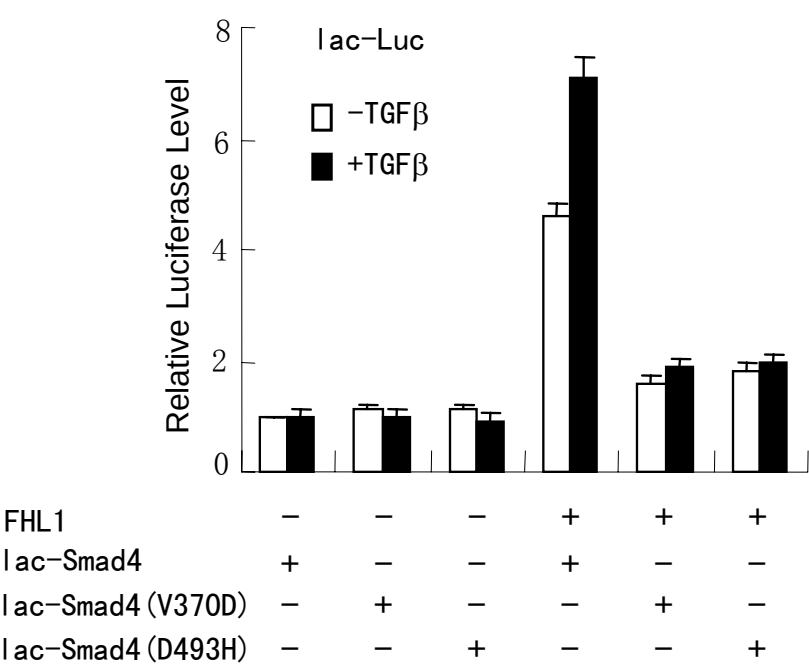


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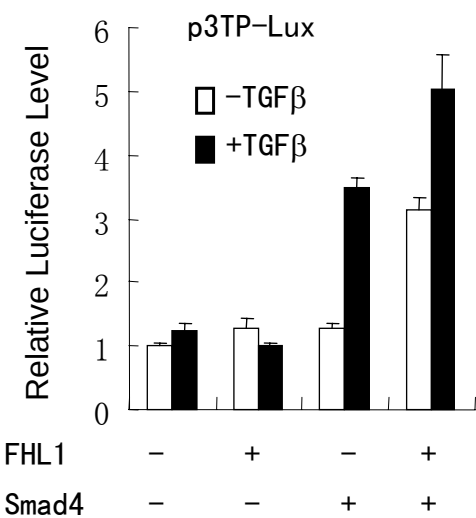


Supplementary Figure 2

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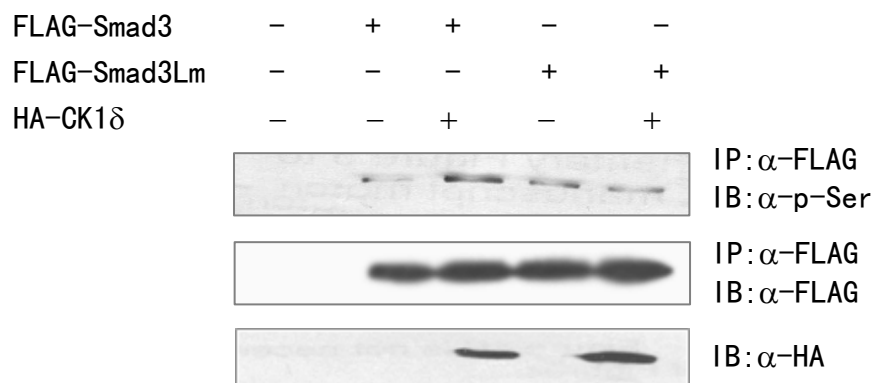


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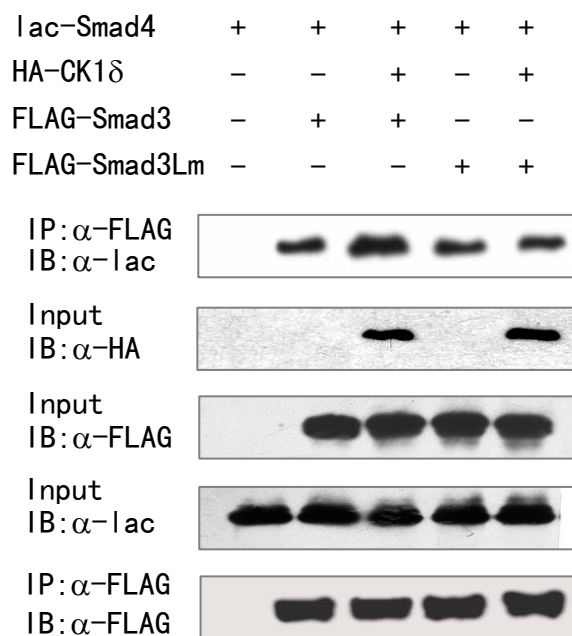


Supplementary Figure 3

A

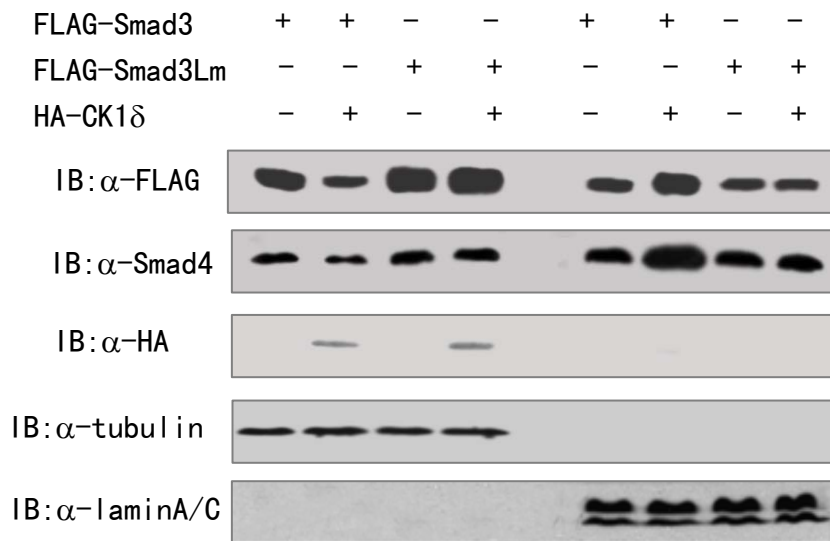


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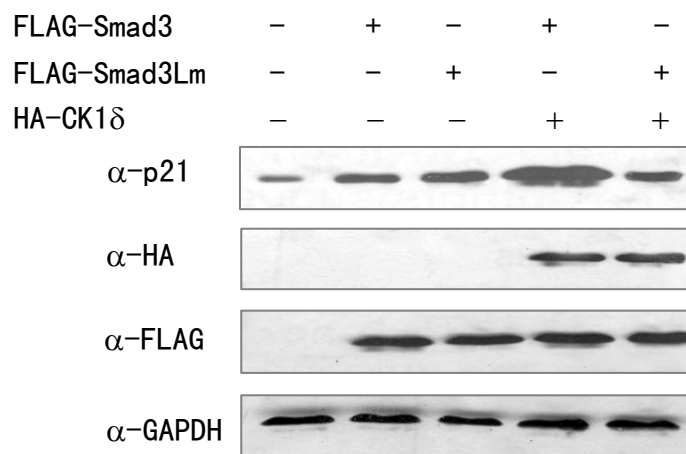


Supplementary Figure 3

C

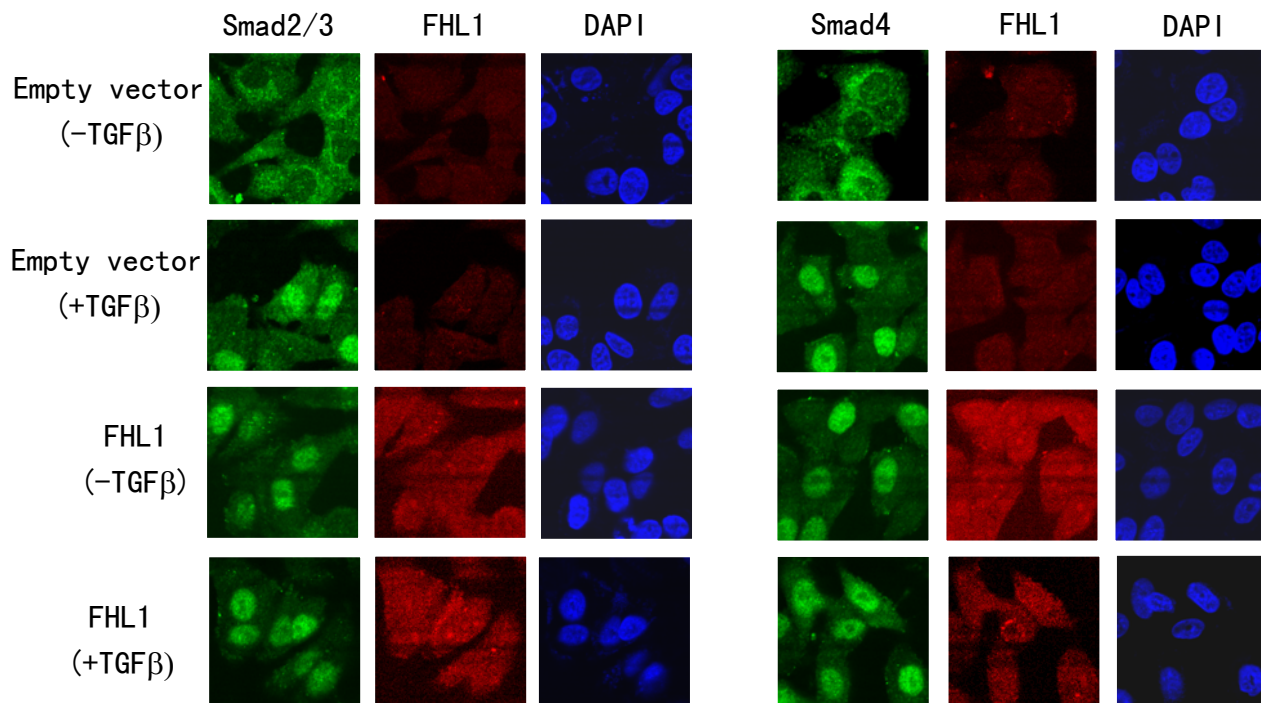


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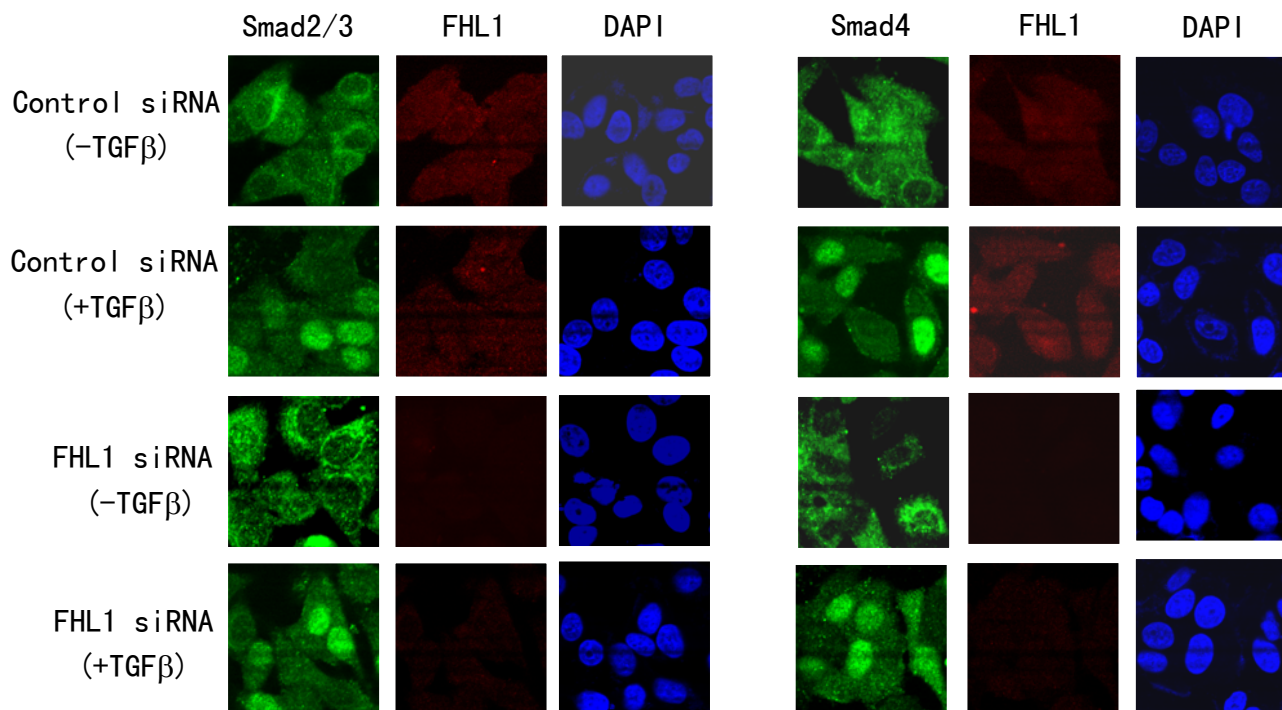


Supplementary Figure 4

A

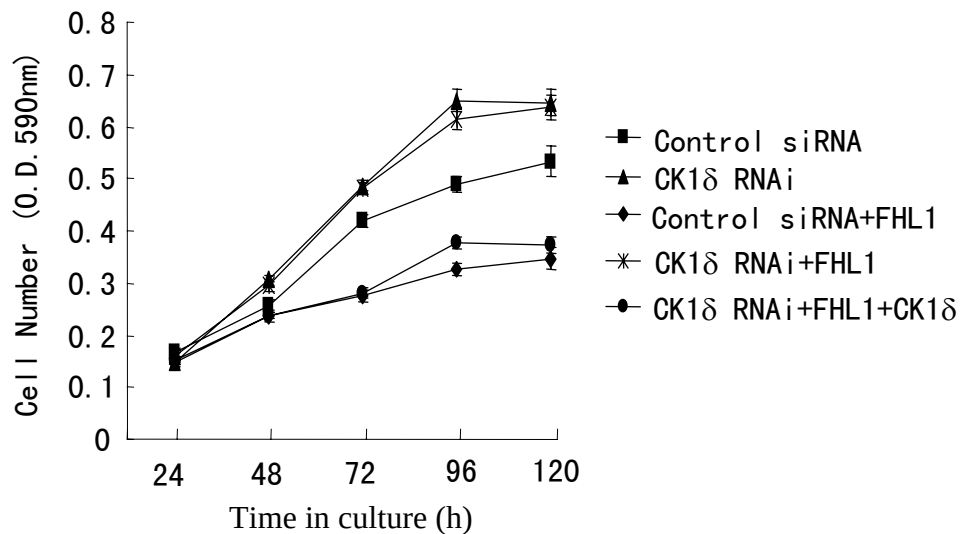


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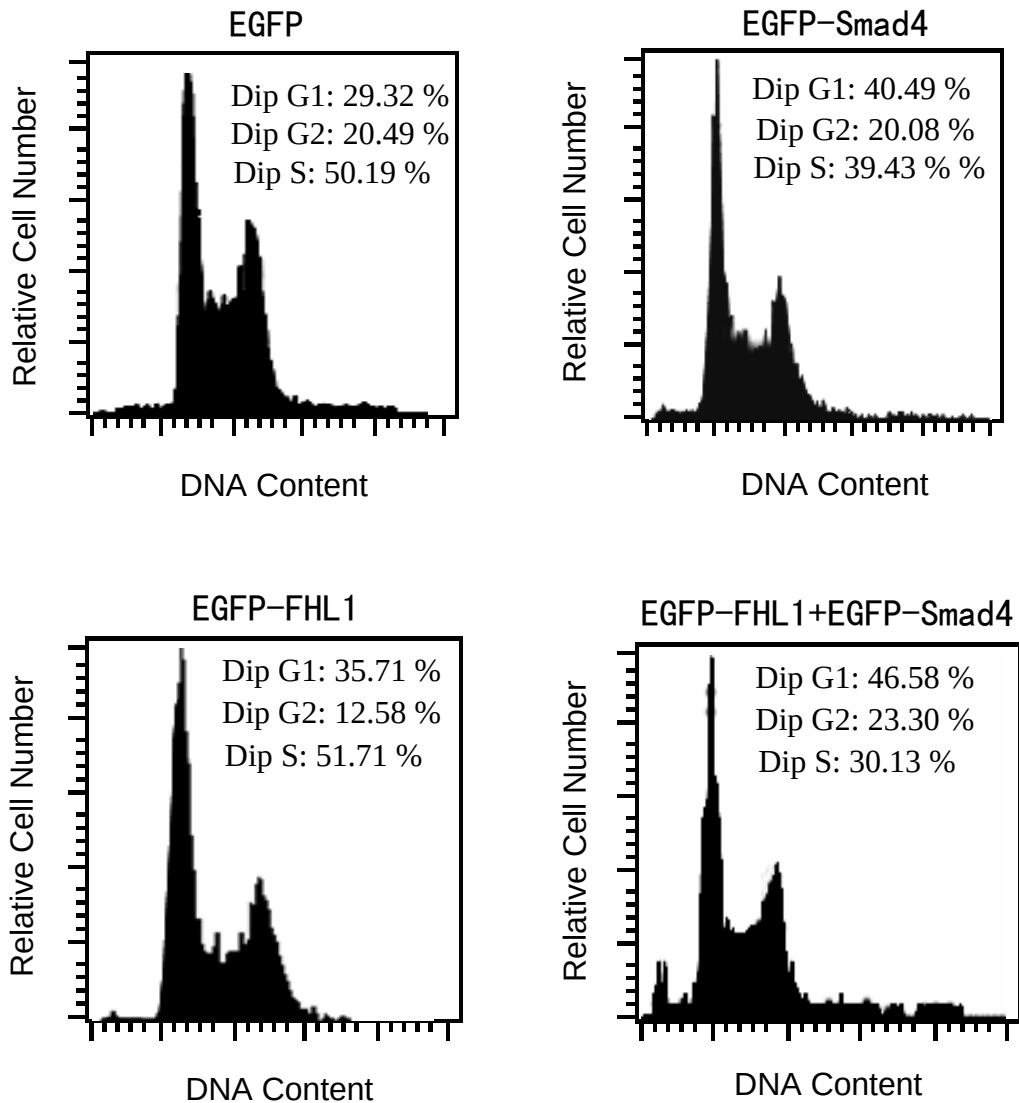


Supplementary Figure 5

A



B



Supplementary Methods

Plasmids. The entire coding sequences of FHL1, FHL2, FHL3, CLIM1, and CK1 δ were isolated by standard PCR from a mammalian two-hybrid cDNA library (Clontech), and those of Smad2, Smad3, and Smad4 were obtained by standard PCR with the corresponding plasmids as templates. Various FHL1, Smad2, and Smad4 fragments were amplified by standard PCR, and point mutations were introduced by recombinant PCR. Mammalian expression vectors encoding FLAG-tagged FHL1, FHL2, FHL3, CLIM1, and CK1 δ were constructed by inserting PCR products into the KpnI/XbaI, BamHI/XhoI, BamHI/XbaI, BamHI/EcoRI, and BamHI/XbaI sites, respectively, of pcDNA3 (Invitrogen) linked to FLAG at the amino terminus. Mammalian expression vectors for HA-tagged FHL1 were made by cloning PCR products into the KpnI/XbaI sites of pcDNA3 linked to HA at the amino terminus. HA-tagged CK1 δ expression vector were generated by inserting a PCR-amplified fragment into the EcoRI/BamHI sites of pIRESpuro2 (Clontech) linked to HA at the amino terminus. PCR products were cloned into the AscI site of pcDNA3 ligated to lac at the amino terminus for the construction of lac-tagged Smad2, Smad3 and Smad4 vectors. EGFP-tagged fusion protein constructs for Smad2 and Smad4 were made by ligating PCR-amplified cDNA fragments into the

HindIII/KpnI and EcoRI/KpnI sites, respectively, of pEGFP-C1 (Clontech). Plasmids encoding His-tagged FHL1, Smad2, Smad3, and Smad4 fusion proteins were prepared by cloning a PCR-amplified sequence into the EcoRI/XhoI sites of pET28a (Novagen). Vectors encoding GST-tagged FHL1, FHL2, FHL3, CLIM1, and CK1 δ fusion proteins were generated by inserting PCR products into the EcoRI/XhoI, BamHI/XhoI, BamHI/XbaI, BamHI/EcoRI, and BamHI/XbaI sites, respectively, of pGEX-KG (Amersham Pharmacia Biotech). For yeast two-hybrid assay, the bait plasmid, pGBKT7-Smad4 (260-514), was created by cloning the corresponding cDNA fragment into the EcoRI/BamHI sites of pGBKT7 (Clontech). Expression vectors for siRNA-resistant FHL1, FHL2, FHL3, and CK1 δ were prepared by inserting the corresponding PCR products into KpnI/XbaI, BamHI/XhoI, BamHI/XbaI, and BamHI/XbaI sites, respectively, of pcDNA3 fused to FLAG at the amino terminus.