

Supplementary Information

TGFβ1 and BMP9 signal laterally through direct type I receptor cross-activation on a type II receptor scaffold

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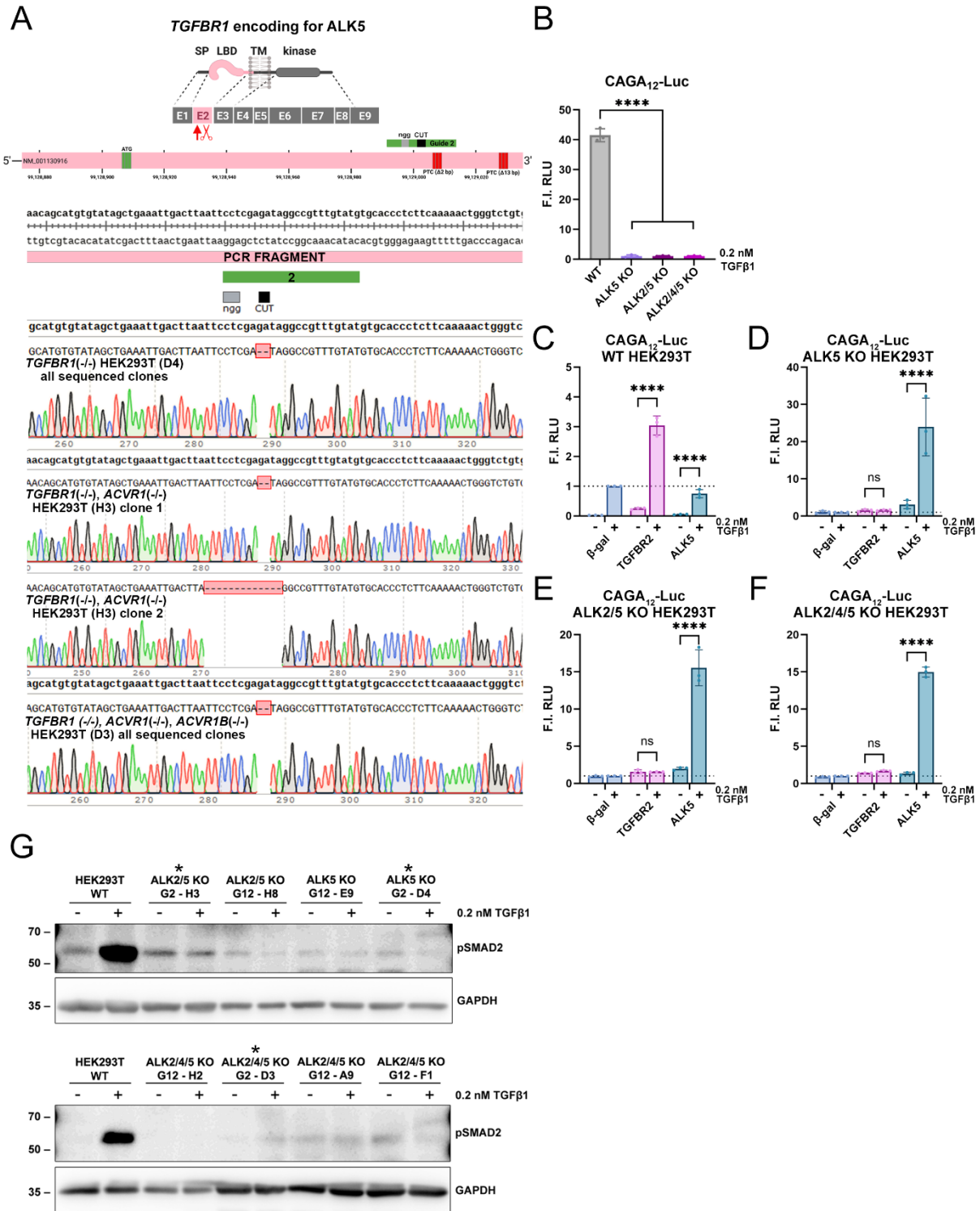
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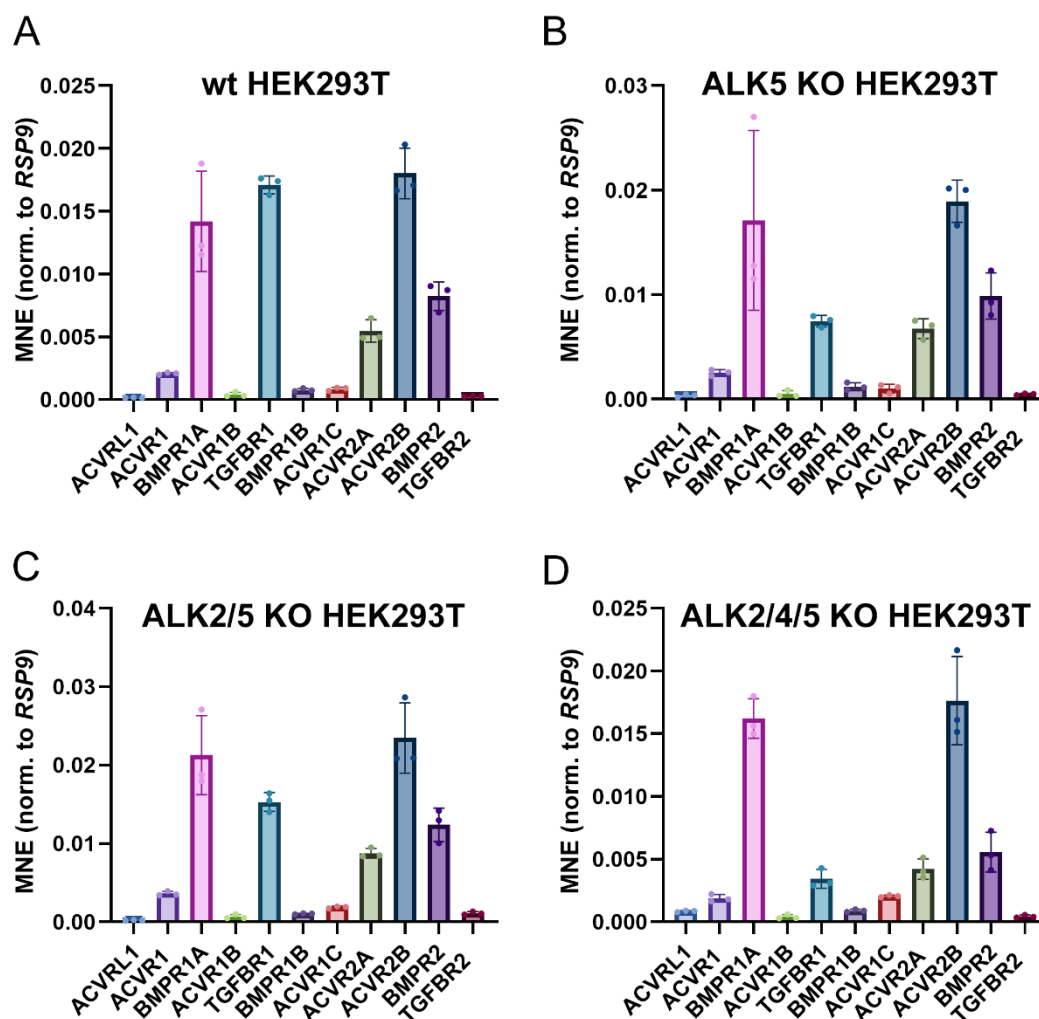
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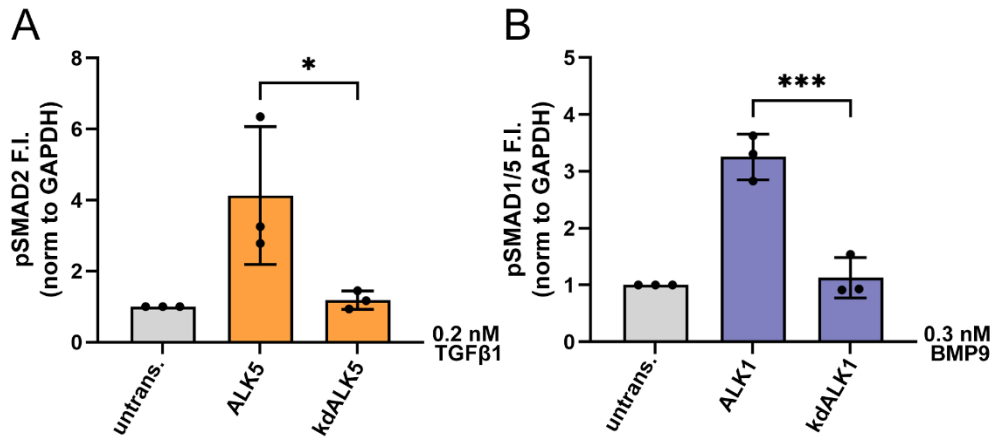


Supplementary Figure 1. Generation and validation of TGF β type I receptor knockout HEK293T cell lines. (A) Schematic of exon 2 (partial) of *TGFR1*, encoding ALK5, showing the guide 2 target site (green) and the resulting premature termination codons (PTC, red). Below, Sanger sequencing chromatograms of the targeted region for representative clones of each CRISPR/Cas9-generated line, with the wild-type guide 2 target sequence shown on top as reference. Deletions relative to the wild-type sequence are highlighted in red. Knockout lines carry deletions in *TGFR1* (ALK5), *ACVR1* (ALK2) and *ACVR1B* (ALK4) respectively, and are named accordingly (ALK5 KO, ALK2/5 KO, ALK2/4/5 KO). **(B)** Luciferase reporter assay of TGF β -induced pSMAD2 signaling in WT and knockout HEK293T lines, using the pSMAD2-sensitive CAGA₁₂-luciferase reporter and RLTK-LUC. **(C–F)** Luciferase reporter assays in **(C)** WT, **(D)** ALK5 KO, **(E)** ALK2/5 KO and **(F)** ALK2/4/5 KO HEK293T cells co-transfected with TGFBR2, ALK5 or β -gal as control and stimulated with TGF β 1 (0.2 nM). **(G)** pSMAD2

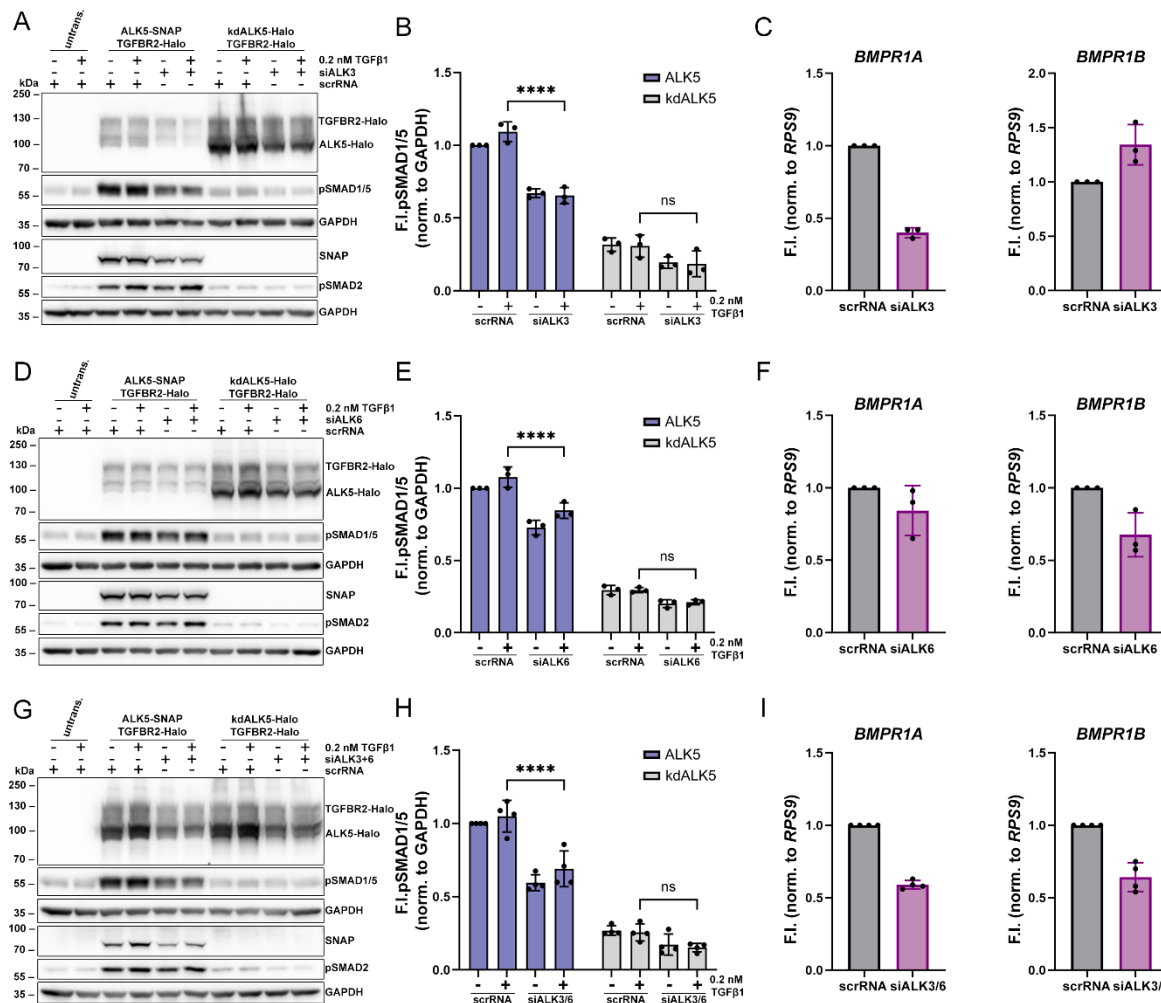
immunodetection confirming loss of TGF β 1-induced signaling in CRISPR/Cas9-generated clones. WT and all transfected clones were starved for 3 h and stimulated for 30 min with TGF β 1 (0.2 nM). pSMAD2 and GAPDH (loading reference) were detected by immunoblotting. Asterisks mark the clones used in this study. Data in (B–F) are expressed as relative luminescence units (RLU), mean fold induction $\pm$ SD ($n = 3$ independent experiments). In (B), significance was calculated by one-way ANOVA with Dunnett's multiple comparisons test relative to the WT control. In (C–F), values are normalized to the stimulated β -gal control and significance was calculated by two-way ANOVA with Tukey's multiple comparisons test comparing unstimulated and stimulated conditions for each construct; **** $p < 0.0001$ (ns, not significant). Schematics created in BioRender (<https://BioRender.com/dkvma5w>).



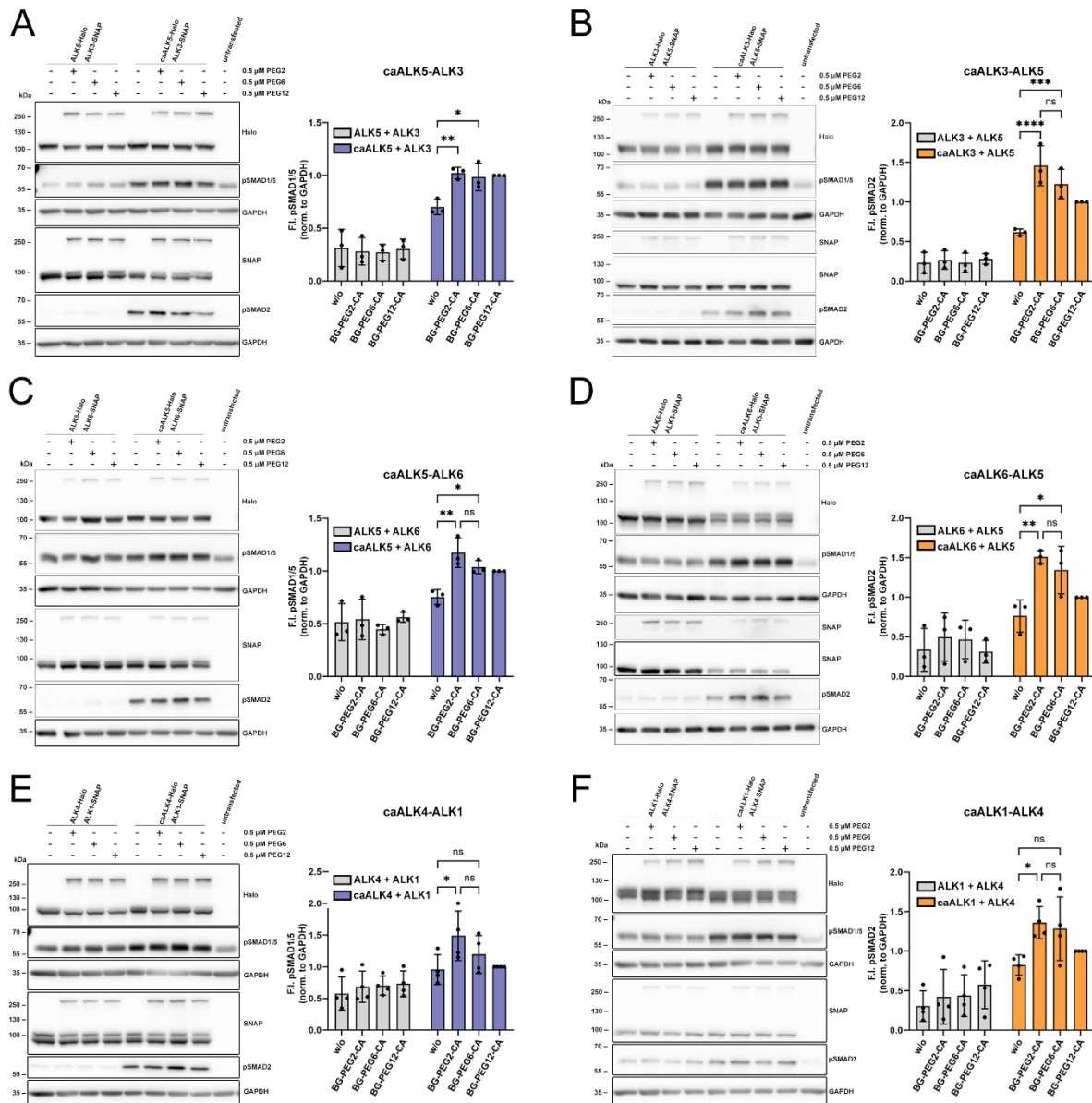
Supplementary Figure 2. Expression profile of TGF β and BMP receptors in WT and knockout HEK293T cell lines. (A–D) Quantitative RT-PCR analysis of the indicated type I and type II TGF β /BMP receptors in HEK293T cells: (A) WT, (B) ALK5 KO, (C) ALK2/5 KO and (D) ALK2/4/5 KO. Expression is shown as mean normalized expression (MNE) relative to the housekeeping gene *RPS9*. Data are mean $\pm$ SD of $n = 3$ biological replicates, each averaged from three technical replicates.



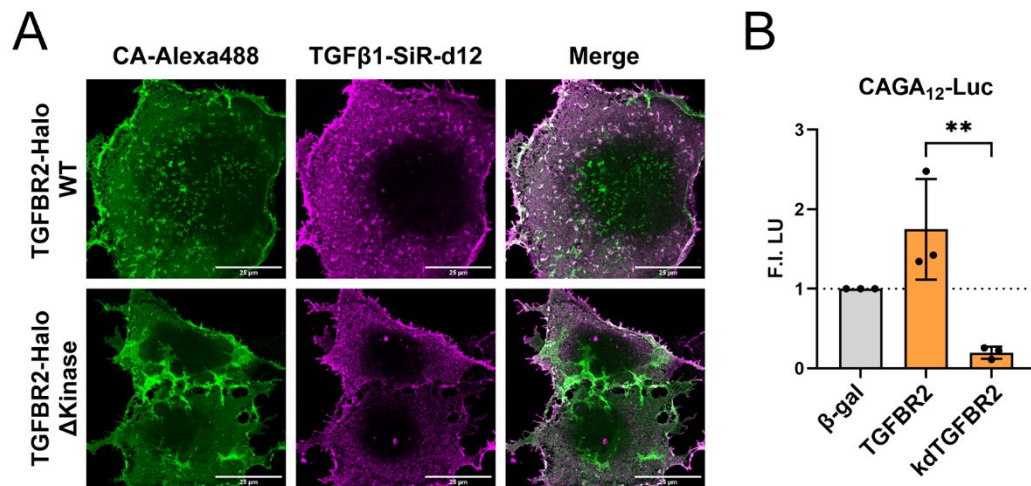
Supplementary Figure 3. Validation of kinase-dead ALK5 and ALK1 constructs. Densitometric quantification of the canonical SMAD readouts from the immunoblots shown in Figure 2, confirming loss of kinase activity in the kinase-dead (kd) receptor constructs. ALK2/5KO cells were transiently transfected with wild-type or kinase-dead ALK5 or ALK1, or left untransfected, and stimulated with ligand. GAPDH serves as loading reference throughout. **(A)** Quantification of pSMAD2 in cells expressing wild-type or kinase-dead ALK5, stimulated with 0.2 nM TGFβ1 for 30 min. **(B)** Quantification of pSMAD1/5 in cells expressing wild-type or kinase-dead ALK1, stimulated with 0.3 nM BMP9 for 60 min. Data are normalized to GAPDH and expressed as fold induction relative to the untransfected, stimulated condition. Significance was calculated by one-way ANOVA with Tukey's multiple comparisons test comparing wild-type and kinase-dead receptor; **(A)** * $p < 0.05$, **(B)** *** $p < 0.001$ ($n = 3$ independent experiments).



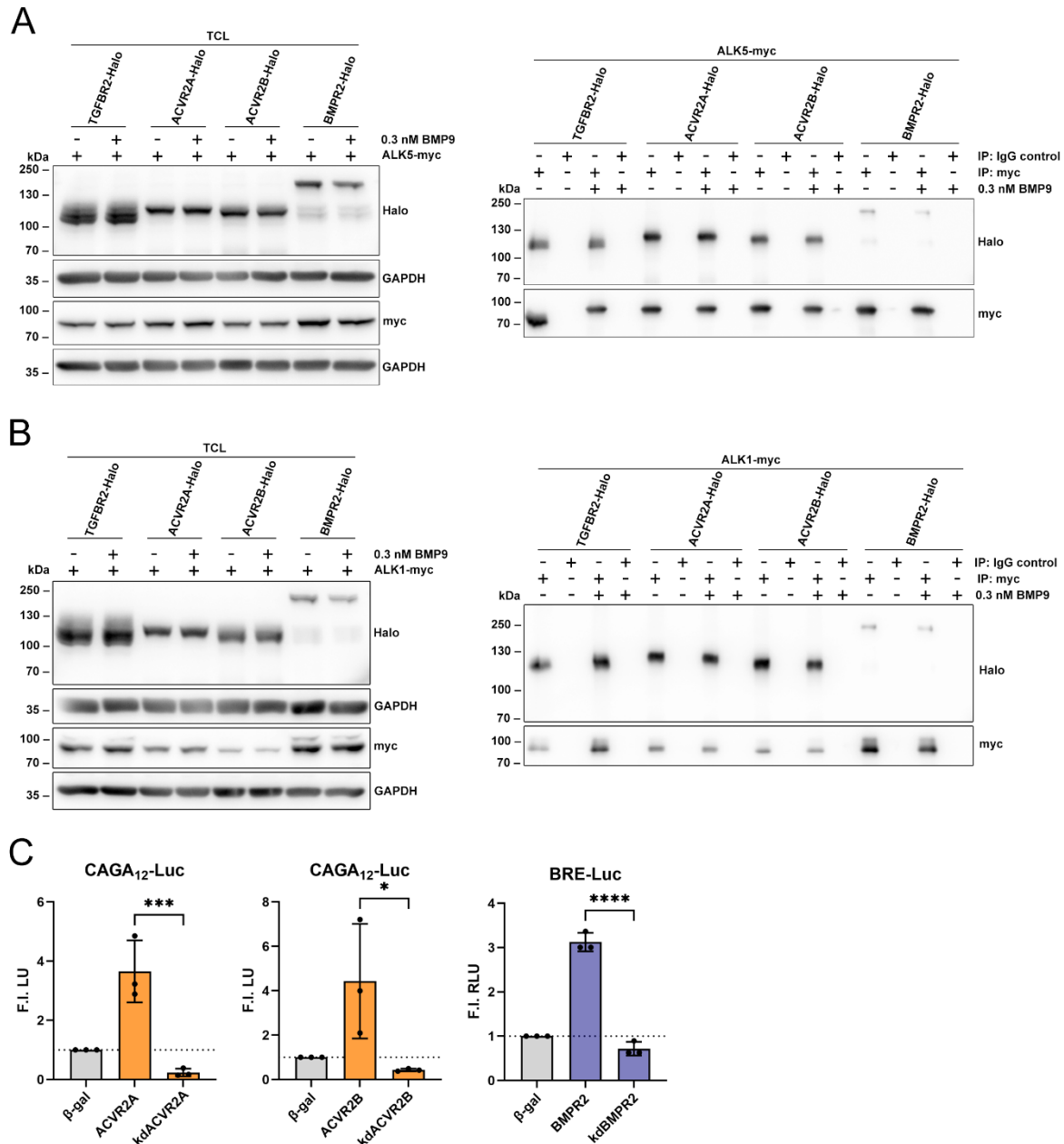
Supplementary Figure 4. siRNA-mediated knockdown of ALK3 and ALK6 and its effect on lateral pSMAD1/5 signaling. Full immunoblots of the siRNA experiments in Figure 3 and additional combined ALK3/6 knockdown, with the corresponding transcript levels measured by qPCR. Cells were transfected with ALK5-SNAP (or kdALK5-SNAP) and TGFBR2-Halo together with scrambled (scrRNA) or target-specific siRNA against ALK3 (BMPR1A), ALK6 (BMPR1B), or both, and stimulated with 0.2 nM TGFβ1. GAPDH serves as loading reference throughout. (A) Full immunoblot and (B) pSMAD1/5 quantification for siALK3 knockdown. (C) BMPR1A and BMPR1B transcript levels following siALK3 knockdown. (D) Full immunoblot and (E) pSMAD1/5 quantification for siALK6 knockdown. (F) BMPR1A and BMPR1B transcript levels following siALK6 knockdown. (G) Full immunoblot and (H) pSMAD1/5 quantification for combined siALK3+6 knockdown. (I) BMPR1A and BMPR1B transcript levels following combined siALK3+6 knockdown. pSMAD1/5 data are normalized to the unstimulated scrRNA condition in ALK5-transfected cells and expressed as fold induction; significance was assessed relative to the stimulated scrambled control. qPCR data are expressed as fold change relative to the scrambled control (scrRNA), normalized to the housekeeping gene RPS9, with each data point averaged from three technical replicates. Data are mean ± SD (single-knockdown rows, n = 3; combined siALK3+6 knockdown, n = 4 independent experiments). Significance for the pSMAD1/5 quantifications was calculated by two-way ANOVA with Tukey's multiple comparisons test; ****p < 0.0001 (ns, not significant).



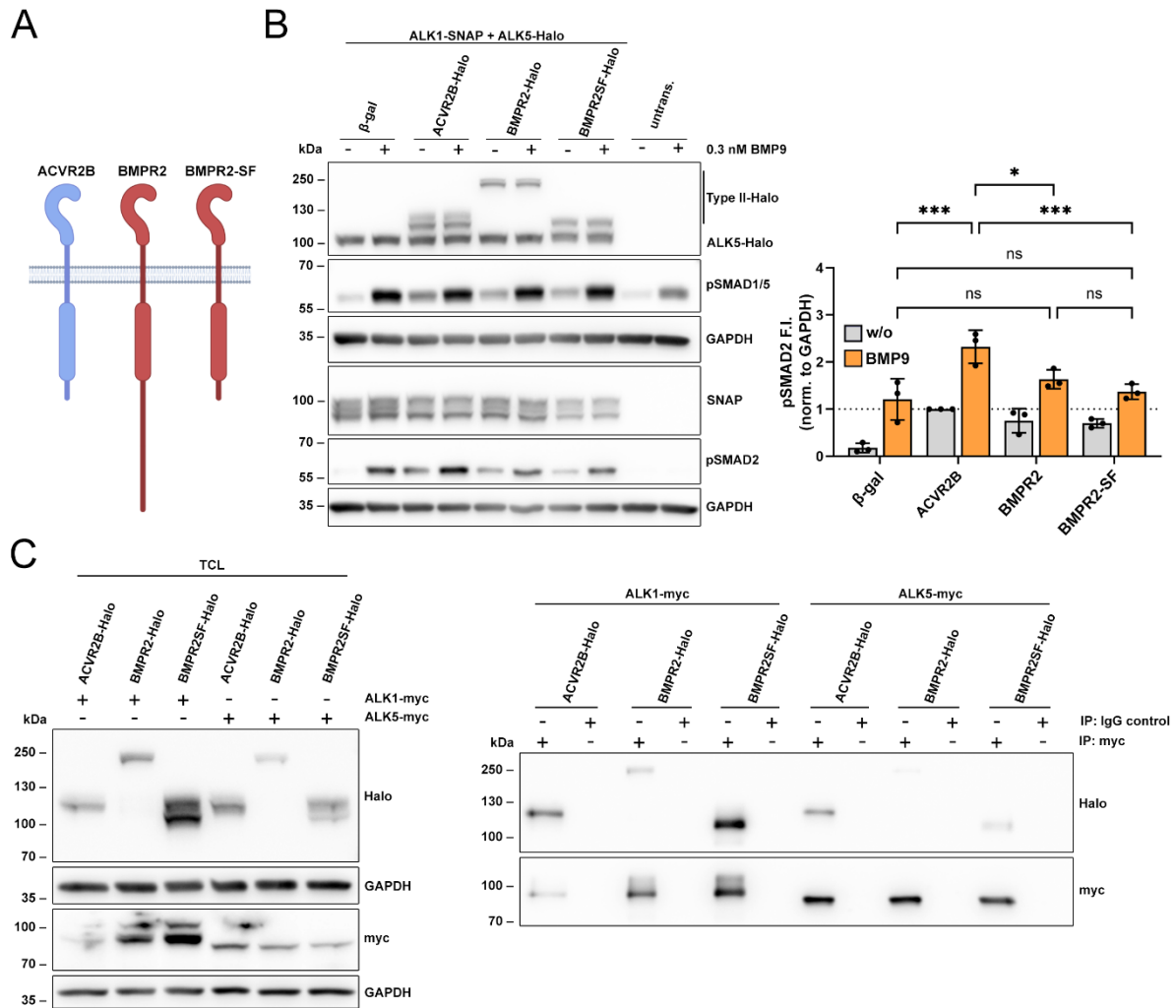
Supplementary Figure 5. Full immunoblots for the Figure 3 dimerizer experiments and lateral activation between ALK1 and ALK4. Uncropped immunoblots corresponding to the dimerizer panels of Figure 3, together with the reciprocal lateral activation of ALK1 and ALK4. All experiments were performed in ALK2/4/5 KO cells treated with 0.5 μ M of the indicated dimerizer for 30 min. Each panel shows a representative full immunoblot with densitometric quantification. GAPDH serves as loading reference throughout. **(A)** Lateral pSMAD1/5 induced by proximity of caALK5-ALK3. **(B)** Reciprocal lateral pSMAD2 induced by proximity of caALK3-ALK5. **(C)** Lateral pSMAD1/5 induced by proximity of caALK5-ALK6. **(D)** Reciprocal lateral pSMAD2 induced by proximity of caALK6-ALK5. **(E)** Lateral pSMAD1/5 induced by proximity of caALK4-ALK1. **(F)** Reciprocal lateral pSMAD2 induced by proximity of caALK1-ALK4. Data are mean $\pm$ SD and expressed as fold induction, normalized to the PEG₁₂-treated condition, with statistical comparisons assessed among the w/o, PEG₂, and PEG₆ conditions (ALK3 and ALK6 pairs, n = 3; ALK4 pairs, n = 4 independent experiments). Significance was calculated by two-way ANOVA with Tukey's multiple comparisons test; *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 (ns, not significant).



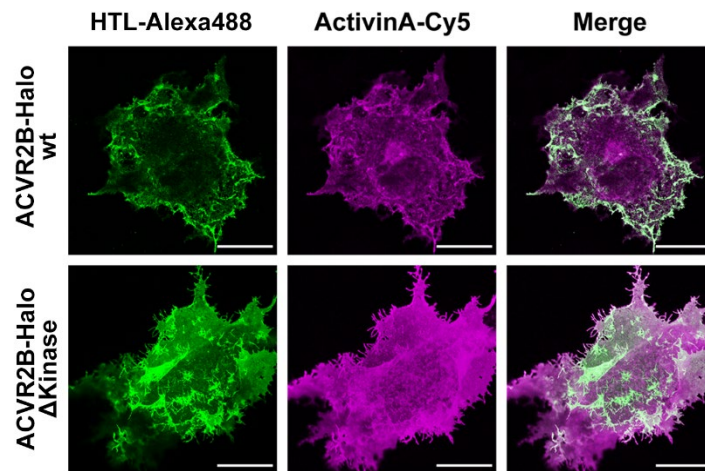
Supplementary Figure 6. Verification of TGFBR2 kinase mutant expression and function. (A) Fluorescence microscopy of COS-7 cells transiently expressing Halo-tagged wild-type TGFBR2 or TGFBR2 Δ kinase, labeled with HTL-Alexa488 (HaloTag ligand, green) and TGFβ1-SiR-d12 (magenta); merge at right. Both the wild-type and Δ kinase receptor localize to the plasma membrane and retain TGFβ1 binding. Scale bar, 20 μ m. **(B)** CAGA₁₂-luciferase reporter assay of canonical signaling in wild-type HEK293T cells co-transfected with the reporter and TGFBR2, kdTGFBR2 (kinase-dead), or β -gal (control for endogenous signaling), without ligand stimulation. CAGA₁₂-luciferase reporter activity is expressed as fold induction relative to the β -gal control (mean $\pm$ SD, n = 3 independent experiments); values were not normalized to Renilla. Significance was calculated by one-way ANOVA with Dunnett's multiple comparisons test relative to wild-type TGFBR2; **p < 0.01.



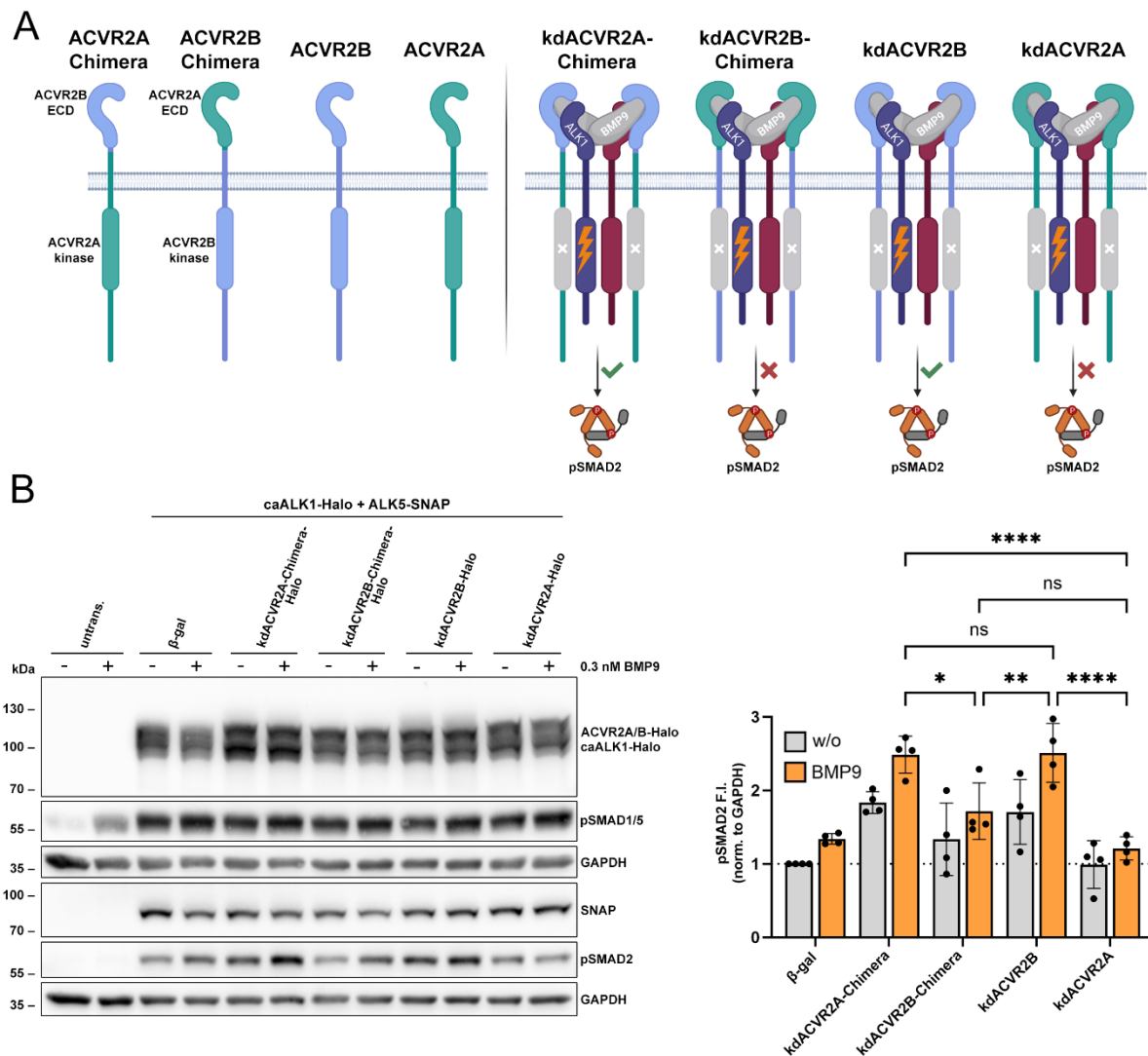
Supplementary Figure 8. Validation of kinase-dead type II receptors and interaction of ALK1 and ALK5 with type II receptors. (A, B) Co-immunoprecipitation of myc-tagged (A) ALK5 or (B) ALK1 with Halo-tagged TGFBR2, ACVR2A, ACVR2B or BMPR2 in COS-7 cells; total cell lysate (left) and myc-tag immunoprecipitate (right). Cells were starved for 24 h and analyzed with or without 0.3 nM BMP9 stimulation for 30 min. Blots were probed for Halo-tag and myc-tag, with GAPDH as loading control for the total cell lysate; normal mouse IgG serves as immunoprecipitation control. (C) Luciferase reporter assays of canonical signaling in WT HEK293T cells co-transfected with the indicated type II receptor or its kinase-dead variant and either the pSMAD2/3-sensitive CAGA₁₂-luciferase reporter (ACVR2A, ACVR2B) or the pSMAD1/5-sensitive BRE-luciferase reporter (BMPR2), without ligand stimulation; β -gal served as control for endogenous signaling. Luciferase activity is expressed as fold induction relative to the β -gal control (mean $\pm$ SD, $n = 3$ independent experiments); values were not normalized to Renilla. Significance was calculated by one-way ANOVA with Dunnett's multiple comparisons test relative to the respective wild-type type II receptor; * $p < 0.05$, *** $p < 0.001$, **** $p < 0.0001$.



Supplementary Figure 9. Truncation of the BMPR2 C-terminal tail enables ALK1 interaction but not lateral signaling. (A) Schematic of ACVR2B, full-length BMPR2, and the short-form BMPR2-SF, which lacks the long BMPR2 C-terminal cytoplasmic extension. (B) Immunoblot of ALK2/4/5 KO cells transiently expressing ALK5-Halo, ALK1-SNAP and one of the Halo-tagged type II receptors ACVR2B, BMPR2 or BMPR2-SF, stimulated with 0.3 nM BMP9 for 60 min; pSMAD2 (lateral) and pSMAD1/5 (canonical) as downstream readout. (right) Densitometric quantification of lateral pSMAD2. GAPDH serves as loading reference. (C) Co-immunoprecipitation of myc-tagged ALK1 or ALK5 with Halo-tagged ACVR2B, BMPR2 or BMPR2-SF in COS-7 cells; total cell lysate (left) and myc-tag immunoprecipitate (right). Cells were starved for 24 h and harvested without stimulation. Blots were probed for Halo-tag and myc-tag, with GAPDH as loading control for the total cell lysate; normal mouse IgG serves as immunoprecipitation control. Quantification in (B) is normalized to the unstimulated ACVR2B control and expressed as fold induction, with comparisons made between the stimulated receptor constructs. Significance was calculated by two-way ANOVA with Tukey's multiple comparisons test; *p < 0.05, ***p < 0.001 (ns, not significant) (n = 3 independent experiments). Schematics created in BioRender (<https://BioRender.com/dkvma5w>).



Supplementary Figure 10. ACVR2B Δ kinase retains membrane localization and ligand binding. Fluorescence microscopy of COS-7 cells transiently expressing Halo-tagged wild-type ACVR2B or ACVR2B Δ kinase, labeled with HTL-Alexa488 (HaloTag ligand, green) and Activin A-Cy5 (magenta), with the merge at right. Both the wild-type and Δ kinase receptor localize to the plasma membrane and retain Activin A binding. Scale bar, 20 μ m.



Supplementary Figure 11. The extracellular domain determines the difference between ACVR2A and ACVR2B in BMP9-induced lateral ALK5 activation. (A) Schematics of the four type II receptor constructs, comprising ACVR2A and ACVR2B and two chimeras in which the extracellular domains (ECD) were swapped (ACVR2A in cyan, ACVR2B in blue), together with the resulting pSMAD2 output in the BMP9-stimulated caALK1/ALK5 complex. The chimeras are named after the receptor providing the intracellular domain. Silenced kinase domains are shown in gray and receptors are depicted without their respective tags for simplicity. (B) Immunoblot of ALK2/4/5 KO cells transiently expressing caALK1-Halo, ALK5-SNAP and one of the Halo-tagged kinase-dead receptors kdACVR2A, kdACVR2B, or the kinase-dead ECD chimeras, stimulated with 0.3 nM BMP9 for 60 min; pSMAD2 (lateral) and pSMAD1/5 (canonical) as downstream readout. (right) Densitometric quantification of lateral pSMAD2. GAPDH serves as loading reference throughout. Quantification is normalized to the unstimulated β-gal control and expressed as fold induction, with comparisons made between the stimulated receptor constructs. Significance was calculated by two-way ANOVA with Tukey's multiple comparisons test; *p < 0.05, **p < 0.01, ****p < 0.0001 (ns, not significant) (n = 3 independent experiments). Schematics created in BioRender (<https://BioRender.com/dkvma5w>).

Supplementary Table 1: Oligonucleotides used for molecular cloning.

Name	Oligonucleotide-sequence (5'-3')	
	for	rev
Backbone primer	GCATTAATGAATCGGCCAACGCGC GGGGAGAGGCGGTTTGC	CGTTGGCCGATTCATTAATGCAGCTGGC ACGACAGGTTTCCCG
caALK1-Halo	GGCACGGGACGTTGCCTTGGTGG AGTGTGTGGGAAAAGGCCGC	CAAGGCAACGTCCCGTGCCACTGTCCT CTGCACCAGGAAGGGG
caALK3-Halo	CTATTGCCAAAGACATTTCAGATGGT CCGGCAAGTTGGTAAAGG	CATCTGAATGTCTTTGGCAATAGTTCGC TGAACCAATAAAGGTAGTCC
caALK4-Halo	GGCCCGAGACATCGTTTTACAAGA GATTATTGGCAAGGGTCG	GTAAACGATGTCTCGGGCCACTGTGC GCTGGACAAAGAGG
caALK5-Halo	GCGAGAGACATTGTGTTACAAGAA AGCATTGGCAAAGGTCG	TGTAACACAATGTCTCTCGCAATTGTTCT CTGAACAAGCAATGG
caALK6-Halo	GCTAAGGACATTTCAGATGGTGAAG CAGATTGGAAAAGGCCG	CCATCTGAATGTCTTAGCTATTGTCCTT TGGACCAGCAGAGG
kdALK1-Halo	GTGGCCGTCAGGATCTTCTCCTCC AGGGATGAACAGTCCTGG	GAGAAGATCCTGACGGCCACACTCTCAC CGTGCCACAAGCC
kdALK5-Halo	GTTGCTGTTAGGATATTCTCCTCTA GAGAAGAACGTTCTGTGG	GGAGAATATCCTAACAGCAACTTCTTCT CCCCGCCACTTTCC
ALK1 Δ ECD-Halo/myc	GAGGCCACCCAACCTCCTTCGGAG CAGCCG	GAATTCTCCACCTCCACCTCCGCCGGAA ATC
kdTBR2	GTGGCAGTCAGGATCTTTCCTAT GAGGAGTATGCCTCTTGG	GGGAAAGATCCTGACTGCCACTGTCTCA AACTGCTCTGAAG
TBR2 Δ kinase	GCAGAAGCTGCCTGAAGACGGCTC CCTAAACACTACCAAATAGG	CGTCTTCAGGCAGCTTCTGCTGCCGGTT AACGCGGTAGC
kdACVR2A	GTGGCTGTCAGAATATTTCCAATAC AGGACAAACAGTCCTGG	GGAAATATTCTGACAGCCACATATTCATT GAGCAACTGGGC
kdACVR2B	GTGGCTGTGAGGATCTTCCCACTT CAGGACAAGCAGTCGTGGC	GGGAAGATCCTCACAGCCACAAAGTCGT TCATGAGCTGAGCC
ACVR2B Δ kinase	CGGCCATGTGGTGGACCTGCTCCC TAAAGAGTCCAGC	GCAGGTCCACCACATGGCCGTAGGGAG GTTTCCG
kdACVR2A-Chimera	CCCTGCTCACGATTCTGCTGTATTC CTTGGTACCACTAATGTTAATTGC	CAGCAGAATCGTGAGCAGGGTGGGGGC TGTCGG
kdACVR2B-Chimera	CCTATTACAACGTGCTGGCCTACT CGCTGCTGCCCATTTGG	GGCCAGCACGTTGTAATAGGGTGGCTT CGGTGTAACAGG
kdBMPR2-Halo	GTTGCTGTAAGAGTGTTCCTTTG CAAACCGTCAGAATTTTATC	GGAAAACACTCTTACAGCAACTGGACGC TCATCCAAGGAGCC
guide 2 sgRNA	CACCGCATACAAACGGCCTATCTC G	AAACCGAGATAGGCCGTTTGTATGC
guide 12 sgRNA	CACCGTGGCAGAAACACTGTAACG C	AAACGCGTTACAGTGTTCCTGCCAC

Supplementary Table 2: Oligonucleotides used qPCR.

Name	Oligonucleotide-sequence (5'-3')	
	for	rev
ACVR1L	ACAACATCCTAGGCTTCATCGC	GGTTTGCCCTGTGTACCG
ACVR1	AAGCCTGGAGCATTGGTAA	TCACTGGGGTACTCGGAGA
BMPR1A	CATCTTGGAGGAGTCGTAAGAA	TTCTGTCCTTGAACACGAGAAA
ACVR1B	TGCAACAGGATCGACTTGAG	ATGATGCCTACCAGCTCCAC
TGFBR1	ACTGTAAAGTCATCACCTGGC	GTGAATGACAGTGC GGTTGT
BMPR1B	CTGCCATAAGTGAGAAGCAAAC	ACAACGCAAGACCTTTGGAC
ACVR1C	ACTTGTGCCATAGCGGACTTA	GGTTCCCACTTTAGGATTCTGAG
ACVR2A	CCTGACAGCTTGCAATTGCTGACTT	TCTGCGTCGTGATCCCAACATTCT
ACVR2B	TGAAGCACGAGAACCTGCTACAGT	GGCATACATGTCAATGCGCAGGAA
BMPR2	CATGGAGATGCGTAGCTGTC	GGTTCTGAGGAAGTGCGAGT
TGFBR2	G TTCAGAAAGTCGGATGTGGAA	TCTGGTTGTCACAGGTGGAA
RSP9	CTGCTGACGCTTGATGAGAA	CAGCTTCATCTTGCCCTCAT