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**The Fibrodysplasia Ossificans Progressiva (FOP) mutation p.R206H in ACVR1 confers an altered ligand response**

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## Abstract

Patients with Fibrodysplasia Ossificans Progressiva (FOP) suffer from ectopic bone formation, which progresses during life and results in dramatic movement restrictions. Cause of the disease are point mutations in the Activin A receptor type 1 (ACVR1), with p.R206H being most common.

In this study we compared the signalling responses of $ACVR1^{WT}$ and $ACVR1^{R206H}$ to different ligands. $ACVR1^{WT}$, but not $ACVR1^{R206H}$ inhibited BMP signalling of BMP2 or BMP4 in a ligand binding domain independent manner. Likewise, the basal BMP signalling activity of the receptor BMPR1A or BMPR1B was inhibited by $ACVR1^{WT}$, but enhanced by $ACVR1^{R206H}$. In comparison, BMP6 or BMP7 activated $ACVR1^{WT}$ and caused a hyper- activation of $ACVR1^{R206H}$. These effects were dependent on an intact ligand binding domain. Finally, the neofunction of Activin A in FOP was tested and found to depend on the ligand binding domain for activating $ACVR1^{R206H}$. We conclude that the FOP mutation $ACVR1^{R206H}$ is more sensitive to a number of natural ligands. The mutant receptor apparently lost some essential inhibitory interactions with its ligands and co-receptors, thereby conferring an enhanced ligand-dependent signalling and stimulating ectopic bone formation as observed in the patients.

**Keywords:**

FOP, BMPR1A, BMPR1B, BMP, Activin A

## 1 Introduction

Fibrodysplasia Ossificans Progressiva (FOP, MIM #135100) is a rare genetic disease, which manifests through progressive heterotopic ossification, replacing skeletal muscles, tendons and ligaments [1]. Patients appear to be healthy at birth, except for shortened, malformed big toes [2]. Symptoms start in childhood being triggered by viral infections, traumas, surgical interventions or intra-muscular injections [3, 4]. FOP is caused by different gain-of-function mutations in the Activin A receptor type 1 (ACVR1). ACVR1 (also known as ALK2) is part of the bone morphogenetic protein (BMP) signalling and consists of an extracellular ligand binding domain, a transmembrane domain, an intracellular Glycine/Serine rich (GS) domain and a kinase domain. The majority of FOP patients shows a specific mutation in the GS domain at position 206 (p.R206H) [5].

BMP signalling plays a pivotal role in development and homeostasis of cartilage and bone [6]. During BMP signalling, two type I and two type II receptors form a hetero-tetrameric complex upon ligand binding. The GS domains of the type I receptors are thereby trans- phosphorylated by the constitutive kinase activity of type II receptors. ACVR1, a type I receptor, is known to complex with the type II receptors BMPR2, ACVR2A and ACVR2B upon activation [7-9]. Phosphorylation of the GS domain of type I receptors results in activation of its own kinase domain, which typically entails downstream phosphorylation of Regulatory Mothers against decapentaplegic homolog (R-SMAD) 1/5/8, which then recruit the co-SMAD4. The resulting complex of R-SMADs and SMAD4 translocates into the nucleus and functions as a transcription factor by directly binding to the DNA [10]. This can lead to the activation of target genes controlling chondrogenic or osteogenic differentiation [11].

However, the exact signalling mechanism of ACVR1 still needs to be elucidated. ACVR1 was first identified as an TGFβ type 1 receptor [12] and subsequently as an TGFβ / Activin

interchangeable type 1 receptor depending on partnering type 2 receptors referring to its binding specificity [13]. Later it was shown that ACVR1 in combination with ACVR2A or ACVR2B inhibits rather than activates Activin A signalling. Activin A also inhibits BMP6 or BMP9 signalling by ACVR1 in combination with ACVR2A or ACVR2B [14]. To date, only direct binding of BMP6 and BMP7 towards ACVR1 has been shown [15-17]. A significant increase in affinity of BMP7 to ACVR1 in the presence of ACVR2A has been reported [18]. In accordance, it was shown that the type II receptor is crucial for activation of ACVR1 via a scaffolding function in addition to its enzymatic function [9].

Recently, it had been shown that in FOP Activin A has a novel function in activating BMP signalling via the ACVR1 receptor. There is only limited knowledge of the mechanisms involved and the two studies existing raise contradictory statements about the binding mechanism of Activin A towards $ACVR1^{R206H}$ [40,41].

In this study, we investigate the influence of different ligands on ACVR1 wildtype ($ACVR1^{WT}$) and the FOP variant $ACVR1^{R206H}$. We show a dual role for $ACVR1^{WT}$, namely as inhibitor of BMP2 or BMP4 induced BMP signalling and activator of BMP6 or BMP7 induced BMP signalling. In contrast, in presence of the FOP variant $ACVR1^{R206H}$, BMP signalling is stimulated by BMP2 or BMP4 and even hyperactivated by BMP6 or BMP7. Moreover, we show that in response to Activin A stimulation $ACVR1^{R206H}$ switches from inhibiting to activating BMP signalling in a ligand binding domain dependent manner.

## 2 Material and Methods

### 2.1 Cell culture

C2C12 cells (ATCC) and NIH/3T3 cells (ATCC) were cultured in growth medium (DMEM 4.5g/l glucose (Lonza), 10 % FCS superior (Biochrom), 2mM/ml L-Glutamine (Biochrom)) and routinely passaged before reaching confluency using Trypsin-Versene (EDTA) Mixture (Lonza) every 2-3 days at a splitting ratio of ~1:10.

### 2.2 Expression plasmids

For luciferase experiments the following expression plasmids were used: Firefly SBE reporter [19], BRE reporter [20] and CAGA reporter [21] all in pGL3-Basic and Renilla in pRL-TK (Promega); mAcvr1$^{WT}$, mAcvr1$^{R206H}$, mAcvr1$^{WT\ dLBD}$, mAcvr1$^{R206H\ dLBD}$ [22], mBmpr1a (also known as Alk3) and mBmpr1b (also known as Alk6) all in pCS2+ [23]. For generation of the mTgfβr1$^{c.a.}$ (also known as Alk5$^{c.a.}$), mBmpr1b$^{c.a.}$ or mBmpr2 expression plasmids, the cds of the wildtype receptors were PCR amplified and cloned into the shuttle vector pSLAX-13. In vitro mutagenesis were performed to obtain the constitutive active version of mTgfβr1 (p.T204D) and the constitutive active version of mBmpr1b (p.Q203D). In addition, a silent mutation c.T1365C was introduced into the mBmpr1b$^{c.a.}$ cds to delete an internal ClaI restriction site. All constructs were subcloned into pCS2+ via ClaI.

### 2.3 Generation of C2C12$^{TVA+}$ cells

C2C12 cells were transfected with a PiggyBac transposon (pPB-CAG.EBNXN plasmid 14) carrying a myc-tagged TVA950 receptor driven by the human EF1α promoter and a puromycin resistance cassette [24].

Transfection was carried out in a 24-well-plate using Lipofectamine2000 (Life Technologies)

following the manufacturer´s instructions. Briefly, $4x10^4$ cells were transfected with 1.6 µg total DNA consisting of PB TVA950 transposon and PB transposase 14 (ratio of 10:1 (w/w)). Two days later, selection of stably transfected cells was done by adding 2 µg/ml of puromycin (Invivogen) to the culture medium. To obtain single cell clones, limited dilution on a 96-well-plate was performed. TVA receptor expression on the cellular surface of one clonal lineage was confirmed via positive immunofluorescence staining for myc-tag (9B11 Mouse mAb, Cell Signaling) (data not shown). $C2C12^{TVA+}$ cells were infected with an RCASBP(A) GFP virus [24] at a MOI (multiplicity of infection) of 50. Subsequent immunofluorescence staining and fluorescence activated cell sorting (FACS) revealed more than 90% transduction efficiency (supplementary figure 1). It was previously shown that about 15-20% of the viral mRNA is spliced into the gene of interest transcript [25].

### 2.4 RCASBP(A) cloning and production

RCASBP(A) vector was provided by Stephen Hughes. Cloning of cds encoding for $mACVR1^{WT}$ and $mACVR1^{R206H}$, both with HA tag, was performed by using the restriction site ClaI. RCASBP(A) viruses were produced in DF1 cells (ATCC) after transfection of the viral plasmids with PEI (Polysciences Inc.), following manufacturer´s instructions. On three consecutive days viral particles were harvested by collecting the supernatant, followed by ultracentrifugation for high-titer concentrates. Titers were determined as plaque forming units (PFU/ml) one day after DF1 cell infection [26].

### 2.5 Overexpression of ACVR1 in $C2C12^{TVA+}$ cells

For RCAS infection, $1x10^4$ (96-well) or $6x10^4$ (24-well) $C2C12^{TVA+}$ cells were seeded and

transduced with a MOI of 50. Two days after seeding, medium was changed to depletion medium containing 2% FCS supplemented with 2 nM (quantitative ALP assay) or 10 nM (qualitative ALP assay and myosin staining) of recombinant human BMP2 (InductOS, Medtronic), human BMP7 (Olympus) or human/mouse/rat Activin A (RnD). Medium was changed every 2-3 days. 7 days after seeding, cells were analyzed for ALP production or myosin expression.

**2.6 Quantitative alkaline phosphatase (ALP) assay**

For quantification of ALP production, cells grown on a 96-well plate were once washed with PBS (Lonza) and afterwards lysed with 100 µl ALP1 buffer (0.1 M glycin pH 9.6 (Roth), 1% Niaproof 4 (Sigma Aldrich), 1 mM magnesium chloride (Roth) and 1 mM zinc chloride (Roth)) per well and incubated for 1 h on a plate shaker (Heidolph). Thereafter, 100 µl of ALP2 buffer (0.1 M glycin pH 9.6, 1 mM magnesium chloride (Roth) and 1 mM zinc chloride) containing 10% para-Nitrophenylphosphat (pNPP) dissolved in water buffered with 0.1 M Glycin, 1 mM magnesium chloride (Roth) and 1 mM zinc chloride were added to each well. When color reaction reached an optimal detection range (30 min– 5 h at room temperature), absorbance was determined at a wave length of 405 nm with an ELISA SpectraMax 340 PC (MDS).

**2.7 Qualitative alkaline phosphatase (ALP) assay**

$C2C12^{TVA+}$ cells were fixed with 4% Paraformaldehyde (PFA), followed by incubation with 500 µl/ 24 well of CHAP buffer (100 mM Tris pH 9.5, 100 mM NaCl, 50 mM $MgCl_2$ (all Roth)) for 30 min. Then 500 µl/well of the detection reagent consisting of 0.175 g/l BCIP (5- Bromo-4-chloro-3-indolyl phosphate) and 0.45 g/l NBT (NitroBlue Tetrazolium) in water were added. After incubating for 30 min in the dark, 500 µl of 10 mM Tris-EDTA buffer, pH 8 were added to stop the reaction. Results were analyzed in bright field with a Leica microscope.

### 2.8 Immunofluorescence staining for Myosin

$C2C12^{TVA+}$ cells were fixed with 4% PFA, washed with PBS and afterwards incubated with blocking buffer (10% FCS and 0.3% Triton X-100 (Sigma Aldrich) in PBS) for 1 h. Then, the primary antibody against fast skeletal myosin heavy chain (Clone MY-32, Sigma Aldrich) diluted 1:500 in antibody dilution buffer was kept on the cells for 2 h. After washing the cells with PBS, the secondary antibody AlexaFluor488 (1:2000, Invitrogen) was incubated for 1 h. Nuclei were stained with Hoechst 33342 (2.5 µg/ml in PBS). Microscopic analysis was performed with an Operetta imaging system (Perkin Elmer).

### 2.9 Luciferase reporter gene assay

NIH/3T3 cells were seeded in growth medium onto a 96-well plate at a density of $1x10^4$ cells/well. 24 h later, cells were transfected using Lipofectamine 2000 following the manufacturer´s instructions. The firefly luciferase reporter pGL3ti-SBE, pGL3ti-BRE or pGL3ti-CAGA, the Renilla luciferase normalization vector pRL-TK and pCS2+ plasmids containing the murine cds encoding for $ACVR1^{WT}$, $ACVR1^{WT\ dLBD}$, $ACVR1^{R206H}$, $ACVR1^{R206H\ dLBD}$, BMPR1A, BMPR1B, $TGF\beta R1^{c.a.}$, $BMPR1B^{c.a}$ or BMPR2 were transfected as indicated. Similar levels of expression of the ACVR1 constructs have been confirmed previously [22]. Upon transfection serum was reduced to 1% in growth medium. 18 h later, cells were stimulated with 2 nM of BMP2, human BMP4 (RnD), human BMP6 (RnD), BMP7 or Activin A. After 48 h cells were lysed in potassium phosphate buffer (9 mM potassium dihydrogen phosphate (Merck), 91 mM dipotassium phosphate (Sigma Aldrich), 0.2% Triton X-100) to determine relative luciferase activity using a Mithras LB 940 (Berthold Detection Systems), as described previously [27]. For normalization Renilla luciferase reporter measurements were used and calculated together with

Firefly. All measurements were normalized to NIH/3T3 cells expressing $ACVR1^{WT}$ without stimulation with a ligand.

### 2.10 Statistical analysis

Statistical analyses were performed using GraphPad Prism 6 (GraphPad Software Inc.). The Kolmogorov Smirnov test revealed that data sets were normally distributed. Statistical significance was tested with 1-way ANOVA and Bonferroni. Statistical significance was assigned, if $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***).

## 3 Results

### 3.1 ACVR1$^{R206H}$ induces osteogenic differentiation in response to BMP2, BMP7 or Activin A in C2C12$^{TVA+}$ cells

The mouse myoblast cell line C2C12 is commonly used to monitor either myogenesis or osteogenesis in response to BMP stimuli. This can be done by assessing the expression of the myogenic marker myosin or the osteogenic marker alkaline phosphatase (ALP) [28]. The poor transfection rate of C2C12 cells is a challenge for overexpression studies. Therefore, we developed a C2C12 cell line, which expresses the avian tumor virus receptor A (TVA) ectopically (C2C12$^{TVA+}$) (Supplemental Figure 1). TVA expression renders cells susceptible for recombinant RCAS (replication competent avian leucosis virus (ALV) long terminal repeat (LTR) with a splice acceptor) virus infection, which can be used for overexpressing genes of interest with high efficiency.

Here, C2C12$^{TVA+}$ cells were infected with RCAS viruses holding the cds of either ACVR1$^{WT}$, ACVR1$^{R206H}$ or an empty vector, for assessing the influence of the individual ACVR1 variants on differentiation. Two days after seeding cells were stimulated with BMP2, BMP7 or Activin A and myogenic or osteogenic differentiation was monitored.

In C2C12$^{TVA+}$ cells transduced with the empty vector, differentiation without a ligand led to myogenesis and no osteogenesis. In the presence of BMP2 or BMP7, ALP production was activated and myotube formation inhibited (Figure 1A - C). During stimulation with Activin A, no ALP was produced and myotube formation was comparable to unstimulated control cells (Figure 1A and D).

In contrast, even without stimulating the C2C12$^{TVA+}$ cells, overexpression of ACVR1$^{R206H}$, but not ACVR1$^{WT}$ made the cells tend to favor differentiating into the osteogenic rather than

the myogenic lineage. Accordingly, without a ligand low levels of ALP were detected and myotube formation was slightly decreased for the FOP variant ACVR1$^{R206H}$ (Figure 1A). In contrast, no ALP was produced, but instead myotubes were formed in C2C12$^{TVA+}$ cells infected with ACVR1$^{WT}$.

The special properties of ACVR1$^{R206H}$ became even clearer once C2C12$^{TVA+}$ cells overexpressing this variant were stimulated with ligands.

During stimulation with BMP2, overexpression of ACVR1$^{R206H}$ led to a high ALP production, while ACVR1$^{WT}$ hardly shows any ALP positive staining, instead myotubes were formed (Figure 1A and B).

When C2C12$^{TVA+}$ cells overexpressing ACVR1$^{WT}$ or ACVR1$^{R206H}$ were stimulated with BMP7 both showed enhanced osteogenesis and decreased myogenesis. However, ACVR1$^{R206H}$ led to a tenfold higher ALP production than ACVR1$^{WT}$ (Figure 1 A and C).

Interestingly, when stimulating C2C12$^{TVA+}$ cells with Activin A, only expression of ACVR1$^{R206H}$, but not ACVR1$^{WT}$ resulted in ALP production (Figure 1 A and D).

ACVR1$^{R206H}$ showed a promoting effect on osteogenesis in response to all three tested ligands, which was not or on a much lower level seen during overexpression of ACVR1$^{WT}$. This highlights the strong effect of the FOP mutation on cellular differentiation, in a ligand dependent manner.

### 3.2 The inhibitory effect of ACVR1$^{WT}$ on BMP signalling in response to BMP2 or BMP4 is absent in ACVR1$^{R206H}$

To gain more insight into the mechanism leading to the ligand specific response of ACVR1 during differentiation, we measured direct downstream signalling using luciferase reporter gene assays.

ACVR1 is known to activate SMADs for downstream signalling, therefore the SMAD dependent luciferase reporter SMAD binding element (SBE) was used to monitor ACVR1 activity.

The mouse fibroblast cell line NIH/3T3 was transfected with the luciferase reporter plasmid pGL3ti-SBE, pRL-TK for normalization and an empty vector (pCS2+) or one of the indicated ACVR1 variants and stimulated with BMP2 or BMP4. It is of note that these variants also include constructs missing the ligand binding domain (deleted ligand binding domain = dLBD), in order to test for requirement of the ligand binding domain for downstream signalling.

In the absence of a BMP ligand, $ACVR1^{R206H}$ and $ACVR1^{R206H\ dLBD}$, but not $ACVR1^{WT}$ and $ACVR1^{WT\ dLBD}$ strongly induced basal levels of SMAD signalling, when compared to the empty vector (Figure 2). Interestingly, the leaky activation of $ACVR1^{R206H}$ is more clearly seen here than in the $C2C12^{TVA+}$ differentiation assay, which might be due to the different read outs used. SMAD-phosphorylation takes only few minutes, whereas ALP activity takes about 3 days after BMP stimulation and depends also on additional MAPK signalling.

Cells transfected with the empty vector showed a considerable activation of SMAD signalling after stimulation with BMP2 or BMP4 (Figure 2). During overexpression of $ACVR1^{WT}$ this activation was reduced by a half for BMP2 and by a fifth for BMP4. In contrast, $ACVR1^{R206H}$ stimulated with BMP2 or BMP4 led to a considerable activation of SMAD signalling (Figure 2). Deletion of the LBD of $ACVR1^{WT}$ still led to an inhibition of SMAD signalling when stimulated with BMP2 or BMP4. Similarly, $ACVR1^{R206H\ dLBD}$ showed an activation of SMAD signalling

comparable to ACVR1$^{R206H}$ in response to BMP2 or BMP4 (Figure 2). ACVR$^{R206H}$ shows a three times higher sensitivity towards BMP4 than BMP2 (Figure 2B).

These results show that ACVR1$^{WT}$ has an inhibitory function on BMP signalling in response to BMP2 or BMP4. This function is vanished for the FOP mutation R206H.

### 3.3 ACVR1$^{R206H}$ loses its inhibitory effect on BMPR1A or BMPR1B function

BMP2 or BMP4 bind with high affinity to BMPR1A or BMPR1B. To elucidate, if the inhibitory function of BMP2 or BMP4 on ACVR1$^{WT}$ is mediated by BMPR1A or BMPR1B, we investigated the effect of ACVR1$^{WT}$, but also ACVR1$^{R206H}$, on BMPR1A or BMPR1B.

NIH/3T3 cells were transfected with pGL3ti-SBE, pRL-TK for normalization and the empty vector (pCS2+) or one of the indicated Acvr1 variants and co-transfected with Bmpr1a or Bmpr1b.

In control, ACVR1$^{WT}$ and ACVR1$^{WT\ dLBD}$ slightly repressed BMP signalling, while ACVR1$^{R206H}$ and ACVR1$^{R206H\ dLBD}$ strongly induced it (Figure 3A).

When BMPR1A or BMPR1B were overexpressed, a high basal BMP activity, particularly for BMPR1B, was observed. This basal activity of both receptors is inhibited by half for BMPR1A and even by about 80% for BMPR1B, when co-expressed with ACVR1$^{WT}$ or ACVR1$^{WT\ dLBD}$ (Figure 3A).

Contrasting, co-expression of BMPR1A or BMPR1B and ACVR1$^{R206H}$ or ACVR1$^{R206H\ dLBD}$ led to no inhibition, but to an activation of BMP signalling (Figure 3A).

Next we investigated, whether the inhibition of BMP signalling of BMPR1A and BMPR1B by ACVR1$^{WT}$ can be rescued by BMPR2 overexpression.

Overexpression of BMPR2 in NIH/3T3 cells led to a considerable activation of BMP signalling (Figure 3B). When BMPR1A or BMPR1B were co-expressed with BMPR2 this activation was even further enhanced.

Interestingly, co-expression of BMPR1A or BMPR1B and BMPR2 with ACVR1$^{WT}$ led to a rescue of the inhibition of BMP signalling and restored the activating function of BMPR1A or BMPR1B on BMP signalling almost to the initial levels (Figure 3B).

In contrast, co-expression of BMPR1A or BMPR1B and BMPR2 with ACVR1$^{R206H}$ resulted in an increase of BMP signalling that was even exceeding the activation gained from co-transfection of BMPR1A or BMPR1B alone with ACVR1$^{R206H}$.

These results imply that ACVR1$^{WT}$ has an inhibitory function on BMPPR1A and BMPR1B, independent of its LBD, due to the competition for the shared type II receptor BMPR2. This inhibitory function is not present for the FOP mutation R206H.

### 3.4 ACVR1$^{R206H}$ shows a higher sensitivity towards BMP6 or BMP7 than ACVR1$^{WT}$

BMP6 or BMP7 are direct ligands of ACVR1. Differentiation of C2C12$^{TVA+}$ cells overexpressing ACVR1$^{R206H}$ led to a much higher ALP production than ACVR1$^{WT}$, when stimulated with BMP6 or BMP7. To analyze these differences in activation of ACVR1 further, we used a luciferase reporter gene assay.

In NIH/3T3 cells transfected with an empty vector, stimulation with BMP6 or BMP7 activated SMAD signalling (Figure 4). This activation was enhanced by approximately 50 % for BMP6 (Figure 4A) and even fourfold for BMP7 (Figure 4B), when cells overexpressed ACVR1$^{WT}$.

Interestingly, ACVR1$^{R206H}$ overexpression led to an even higher activation of SMAD signalling

than ACVR1$^{WT}$ (Figure 4). However, the increase of SMAD activation by ACVR1$^{R206H}$ after stimulation with BMP7 was not comparable to the strong effect of ACVR1$^{R206H}$ on ALP production in C2C12$^{TVA+}$ cells.

When the LBD of ACVR1$^{WT}$ or ACVR1$^{R206H}$ was deleted, the strong activation of SMAD signalling by BMP6 as well as BMP7 was prevented (Figure 4), indicating that the LBD is essential for this activation and confirming direct binding of BMP6 or BMP7 towards ACVR1.

### 3.5 ACVR1$^{R206H}$ activates BMP signalling in response to direct binding of Activin A

Recently, it has been described that Activin A acts as an activator of BMP signalling in FOP. We confirmed these results during differentiation of C2C12$^{TVA+}$ cells overexpressing ACVR1$^{R206H}$. To shed more light on the process involved, we first analyzed overall SMAD activation by Activin A using the SBE reporter in a luciferase reporter gene assay.

The mouse fibroblast cell line NIH/3T3 was transfected with pRL-TK for normalization, the luciferase reporter pGL3ti-SBE and the empty vector control (pCS2+) or one of the indicated ACVR1 variants and stimulated with Activin A.

In cells expressing the empty vector as well as ACVR1$^{WT}$ or ACVR1$^{WT\ dLBD}$ no activation following stimulation with Activin A was seen. However, similar to the results obtained during differentiation of C2C12$^{TVA+}$ cells, overexpression of ACVR1$^{R206H}$ led to an increase in SMAD activity. This increase was strongly reduced for ACVR1$^{R206H\ dLBD}$, indicating that the LBD is essential for BMP signalling (Figure 5A).

As the used SMAD reporter SBE shows overall SMAD activity, we then analyzed TGFβ signalling via SMAD2/3 activity with the luciferase reporter CAGA and BMP signalling via SMAD1/5/8 activity with the BMP responsive element reporter BRE. TGFβR1 constitutive

active (c.a.) served as positive control for the CAGA reporter and BMPR1B c.a. for the BRE reporter.

Overexpression of $ACVR1^{WT}$ or $ACVR1^{R206H}$ led to an inhibition of Activin A induced SMAD2/3 signalling (Figure 5B). Contrary, BMP signalling induced by $ACVR1^{R206H}$, but not $ACVR1^{WT}$ reached levels twice as high when stimulated with Activin A (Figure 5B and C). These results propose that the binding of Activin A towards ACVR1, which normally leads to inhibition of BMP signalling, gets misinterpreted by the cell in the presence of the FOP mutation R206H.

## 4 Discussion

Mutations in ACVR1, which are associated with the disease FOP, activate BMP signalling and lead to heterotopic ossification in patients. Several experimental studies show that despite the gain of function mutation, ACVR1 is still ligand sensitive and cannot be considered as constitutive active [22]. This might also contribute to the episodic nature of disease progression. In order to understand the ligand dependency, this study aims to clarify the mechanisms underlying signalling of $ACVR1^{WT}$ as well as the ACVR1 variant R206H, which is present in most FOP patients. Therefore, we analyzed the effect of BMP2 or BMP4, BMP6 or BMP7 and Activin A on $ACVR1^{WT}$ and $ACVR1^{R206H}$ in differentiation assays using $C2C12^{TVA+}$ cells as well as in luciferase reporter gene assays to quantify immediate downstream signalling.

No direct binding of BMP2 or BMP4 towards ACVR1 has been described in the literature [16, 17]. However, the increased osteogenic activity observed in FOP patients and elevated levels of BMP4 in FOP lesions and lymphoblastic cells made them subject to intense research in the context of this disease [29, 30]. Here, we show that $ACVR1^{WT}$ has an inhibitory effect on BMP signalling and osteogenic differentiation in $C2C12^{TVA+}$ cells after stimulation with BMP2 or BMP4. BMP2 or BMP4 bind to the type I receptors BMPR1A or BMPR1B and signal in complex with the type II receptor BMPR2, which is also a co-receptor of ACVR1 [31]. As we also show that expression of BMPR1A or BMPR1B led to an upregulation in BMP signalling, but co-expression of $ACVR1^{WT}$ and BMPR1A or BMPR1B to a downregulation, it seems likely that $ACVR1^{WT}$ has an inhibitory effect on BMPR1A or BMPR1B function caused by competition for BMPR2. This hypothesis is supported by the observation that the inhibitory effect of $ACVR1^{WT}$ is independent from its LBD, which means that $ACVR1^{WT}$ does not bind BMP2 or BMP4 directly, but inhibits BMP signalling on a receptor level (Figure 6, left, upper

panel). In line with this, co-transfection of BMPR2 led to a rescue of this inhibition, which supports the hypothesis that ACVR1$^{WT}$ is competing for BMPR2 with BMPR1A or BMPR1B. Interestingly, BMPR2 ablation in pulmonary artery smooth muscle cells is reported to having a similar effect as our overexpression studies of ACVR1$^{WT}$ and results in a decreased signalling via BMP2 or BMP4 and to an increased signalling via BMP6 or BMP7 [32].

Several ACVR1 knock-out studies are published that report different outcomes regarding BMP signalling or its effect on differentiation. For example, in calvarial preosteoblasts ACVR1 knockout does not affect levels of BMP-SMAD signalling [33] and a chondrocyte-specific knockout of Acvr1 results in slight decrease of pSmad immuno-signals [34]. In contrast, an osteoblast specific ACVR1 deficiency led to an increase in bone mass, which argues for an inhibitory function of ACVR1 [35]. These heterogeneous results are in line with our data that show a dual function of ACVR1$^{WT}$, stimulating BMP signalling in response to BMP6 or BMP7 on the one hand and inhibiting BMP signalling in response to BMP2 or BMP4 on the other hand, which is also underlined by previous studies in Drosophila melanogaster [36, 37]. Therefore, ACVR1 knockout can result in an increased as well as a decreased BMP signalling depending on the cell context.

However, the results were obtained using overexpression systems or knock out strategies. It can thus not be excluded that the results differ at endogenous receptor expression levels, which should be taken into consideration when trying to extrapolate the findings to living organisms. Nevertheless, ACVR1$^{R206H}$ overexpression does not lead to the inhibition of BMP signalling following BMP2 or BMP4 stimulation. Instead, an activation of excessive BMP signalling is seen (Figure 6, left, lower panel). This might be due to the overall “leaky” induction of BMP signalling by ACVR1$^{R206H}$ without any ligand [22] and the fact that ACVR1 and BMPR1A or

BMPR1B build heterodimeric structures by direct protein-protein interactions [38], which leads to a further increase in BMP signalling after stimulation with BMP2 or BMP4 in the case of FOP instead of an inhibition in the wildtype situation.

It is known that BMP6 or BMP7 bind directly towards ACVR1. Accordingly, in our study BMP signalling was increased in response to BMP6 or BMP7 and prevented by deletion of the LBD. Nevertheless, $ACVR1^{R206H}$ overexpression led to an even stronger activation of BMP signalling and osteogenic differentiation than seen for $ACVR1^{WT}$ (Figure 6, middle), which is in line with previous findings [39]. As the mutation R206H is not located in the ligand binding domain, this severe increase in BMP signalling might be due to other structural changes that enhance the responsiveness of ACVR1 [22].

Recently, a neofunction of $ACVR1^{R206H}$ on activating BMP signalling in the presence of the TGFβ ligand Activin A has been reported [40, 41]. Our results demonstrate that $ACVR1^{WT}$ has indeed such an inhibitory effect, in contrast to $ACVR1^{R206H}$ where it rather leads to an activation of SMAD signalling. Additionally, we showed that this effect is LBD dependent, as overexpression of $ACVR1^{R206H\ dLBD}$ led to no significant increase in SMAD signalling compared to its unstimulated control. Furthermore, by analyzing the differential activation of either SMAD2/3 or SMAD1/5/8 we showed that for $ACVR1^{R206H}$ the inhibitory effect on SMAD2/3 signalling remains, but an activating effect on BMP signalling is gained (Figure 6, right). It has been shown that in the presence of $ACVR1^{WT}$ Activin A inhibits BMP signalling, due to direct binding to ACVR1, which blocks the receptor for BMP signalling [14] (Figure 6). It is of note that Activin A still binds to ACVR2A with a higher affinity. Our results support the conclusion made by Hatsell et al. that in the case of FOP the inhibition of ACVR1 by Activin A is no longer possible owing to the mutation R206H [41]. However, our results are in contrast to Hino et al.,

who showed no necessity of the extracellular domain of ACVR1, but of ACVR2A or ACVR2B on activation of BMP signalling for FOP [40]. Our results show that deletion of the LBD of $ACVR1^{R206H}$ leads to a reduction of activation of BMP signalling to nearly a half.

## 5 Conclusion

Taken together this study demonstrates that due to the most prominent FOP mutation R206H, ACVR1 loses inhibitory functions and gains ligand responsiveness. Different to the artificial constitutive active mutation Q207D this activation of BMP signalling remains ligand dependent, which might explain the episodic nature of disease progression. It is known that the immune system plays a critical role in this process. It is likely that after an injury or viral infection cytokines are released, which normally have an inhibiting, no or weak effect on BMP signalling, but leads to misinterpretation in the body of FOP patients and thereby causing flare-ups. Understanding that the heterotopic ossification in FOP patients is caused by the sum of many effects rather than the result of one misinterpreted ligand is essential to develop suitable therapeutic approaches. To date, no cure for FOP has been found, even though a promising clinical trial, using a retinoic acid γ agonist for blocking chondrogenic differentiation, started recently [37].

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**Figures**

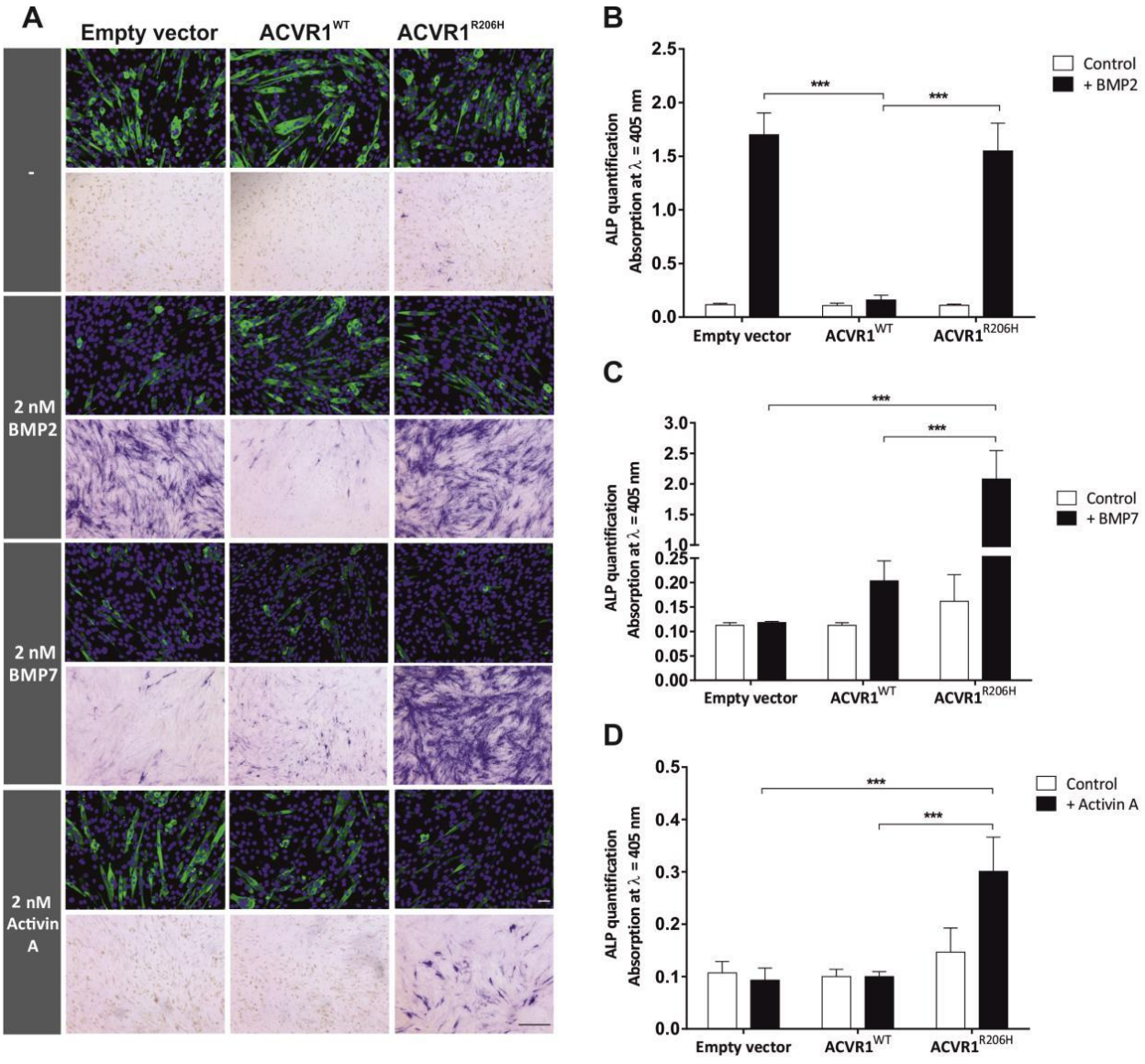


**Figure 1: ACVR1$^{R206H}$ overexpression leads to an increased ALP activity after BMP2, BMP7 or Activin A stimulation.**

C2C12$^{TVA+}$ cells were transduced with RCASBP(A) holding the cds information for ACVR1$^{WT}$ or ACVR$^{R206H}$ and grown in depletion medium for 5 days with repeated stimulation with BMP2, BMP7 and Activin A, followed by staining for ALP or Myosin. C2C12$^{TVA+}$ cells overexpressing

$ACVR1^{R206H}$ showed an increased sensitivity towards all ligands, resulting in enhanced ALP production and decreased myosin staining in comparison to $ACVR1^{WT}$. Of note, overexpression of $ACVR1^{WT}$, even led to inhibition of ALP production in response to BMP2 stimulation, when compared to the empty vector control. A: Staining for Myosin (upper rows) and ALP (lower rows). B-D: ALP quantification. To test for statistical significance three independent experiments with three replicates each were analyzed using 1way ANOVA (Scale bar = 50 μm).

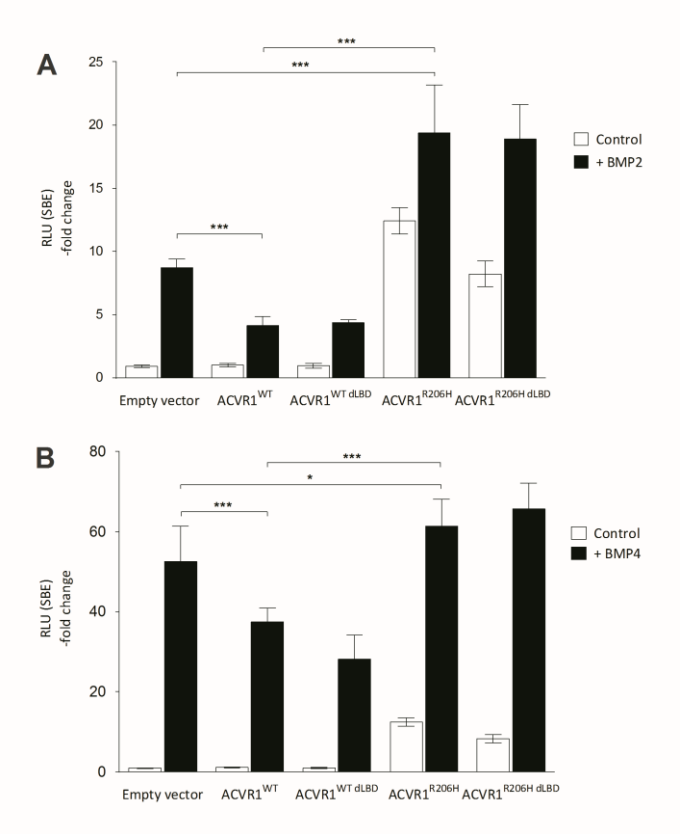


**Figure 2: The inhibitory effect of ACVR1 on SMAD signalling after BMP2 or BMP4 stimulation vanishes in FOP in a LBD independent manner.**

NIH/3T3 cells were transfected with SBE and pRL-TK for normalization and either an empty vector or Acvr1$^{WT}$, Acvr1$^{WT\ dLBD}$, Acvr1$^{R206H}$ or Acvr1$^{R206H\ dLBD}$ and stimulated with BMP2 or BMP4 on the next day for 24 h. A: Stimulation with BMP2 led to an inhibition of SMAD signalling by ACVR1$^{WT}$, but to an activation by ACVR1$^{R206H}$. B: Stimulation with BMP4 also led to an inhibition of SMAD signalling by ACVR1$^{WT}$ and to an activation by ACVR1$^{R206H}$. For both BMPs the LBD was not required for this response. To test for statistical significance a representative experiment with 9 replicates was analyzed using 1way ANOVA (RLU = relative luciferase units).

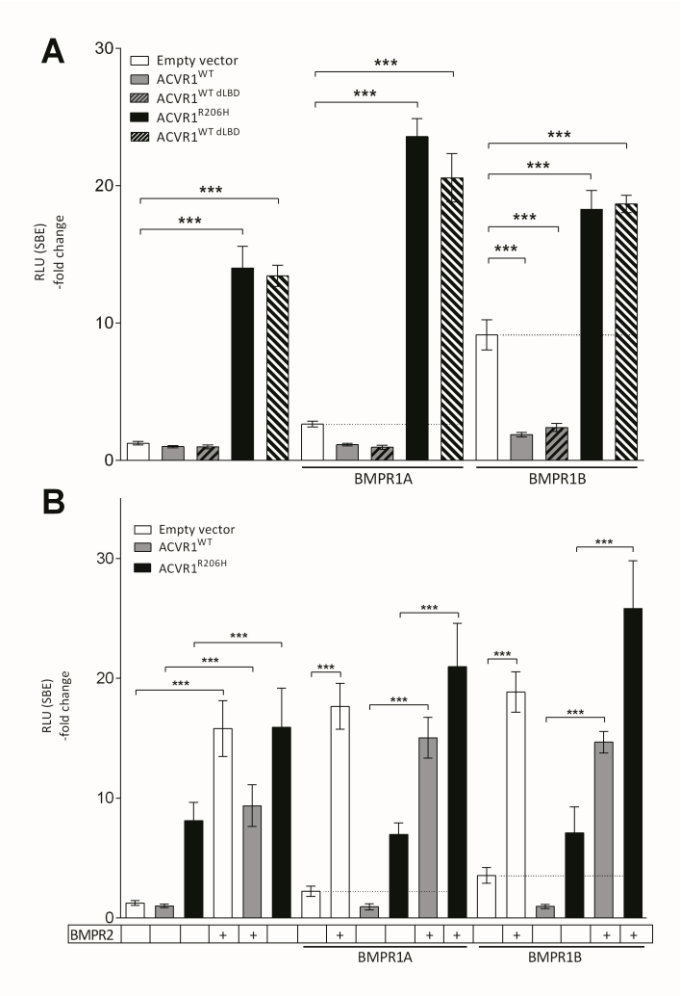


**Figure 3: The inhibitory effect of ACVR1$^{WT}$ on BMPR1A or BMPR1B function is not observed for ACVR1$^{R206H}$.**

NIH/3T3 cells expressing SBE and pRL-TK for normalization and either an empty vector, ACVR1$^{WT}$, ACVR1$^{WT\ dLBD}$, ACVR1$^{R206H}$ or ACVR1$^{R206H\ dLBD}$. Additionally, co-expression of BMPR1A, BMPR1B or BMPR2 was performed. A: In the presence of ACVR1$^{WT}$ and ACVR1$^{WT\ dLBD}$ the activating function of BMPR1A or BMPR1B on SMAD signalling was reduced. For ACVR1$^{R206H}$ and ACVR1$^{R206H\ dLBD}$ this reduction was turned into an activation of SMAD signalling. B: Overexpression of BMPR2 led to a rescue of the inhibitory effect of ACVR1$^{WT}$ on BMPR1A and BMPR1B induced SMAD signalling. For ACVR1$^{R206H}$, BMPR2 overexpression

resulted in an even higher activation of SMAD signalling. To test for statistical significance a representative experiment with 8 replicates was analyzed using 1way ANOVA (RLU = relative luciferase units).

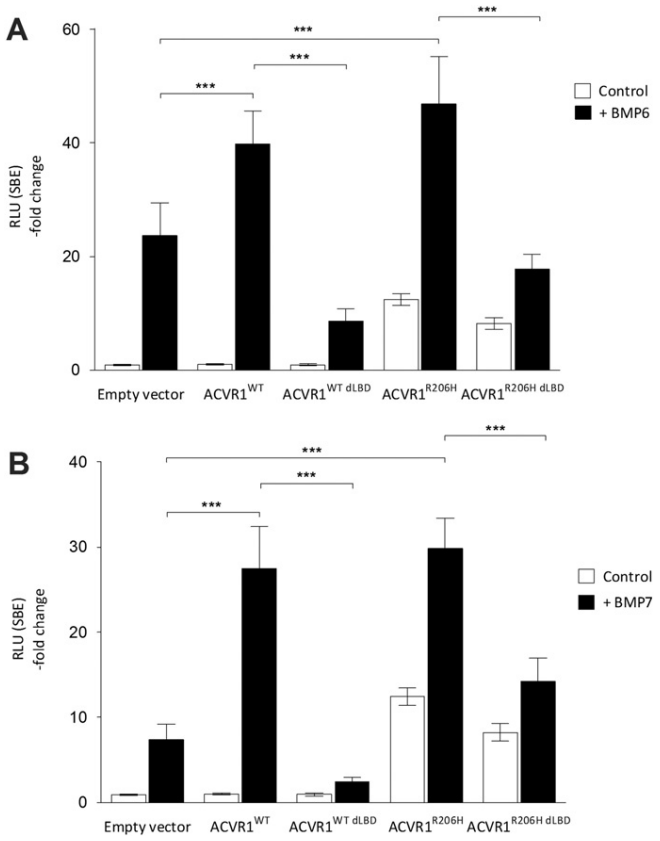


**Figure 4: The activating effect of ACVR1 on SMAD signalling after BMP6 or BMP7 stimulation is further enhanced in FOP in a LBD dependent manner.**

NIH/3T3 cells were transfected with SBE and pRL-TK for normalization and an empty vector or Acvr1$^{WT}$, Acvr1$^{WT\ dLBD}$, Acvr1$^{R206H}$ or Acvr1$^{R206H\ dLBD}$ and stimulated with BMP6 or BMP7 on the next day for 24 h. A: Stimulation with BMP6 led to an activation of SMAD signalling by ACVR1$^{WT}$ and to a hyperactivation by ACVR1$^{R206H}$. B: A comparable activation by ACVR1$^{WT}$ and hyperactivation of ACVR1$^{R206H}$ was seen for BMP7. Without the LBD this activation by BMP6 or BMP7 was neither seen for ACVR1$^{WT}$, nor for ACVR1$^{R206H}$. To test for statistical significance a representative experiment with 9 replicates was analyzed using 1way ANOVA (RLU = relative luciferase units).

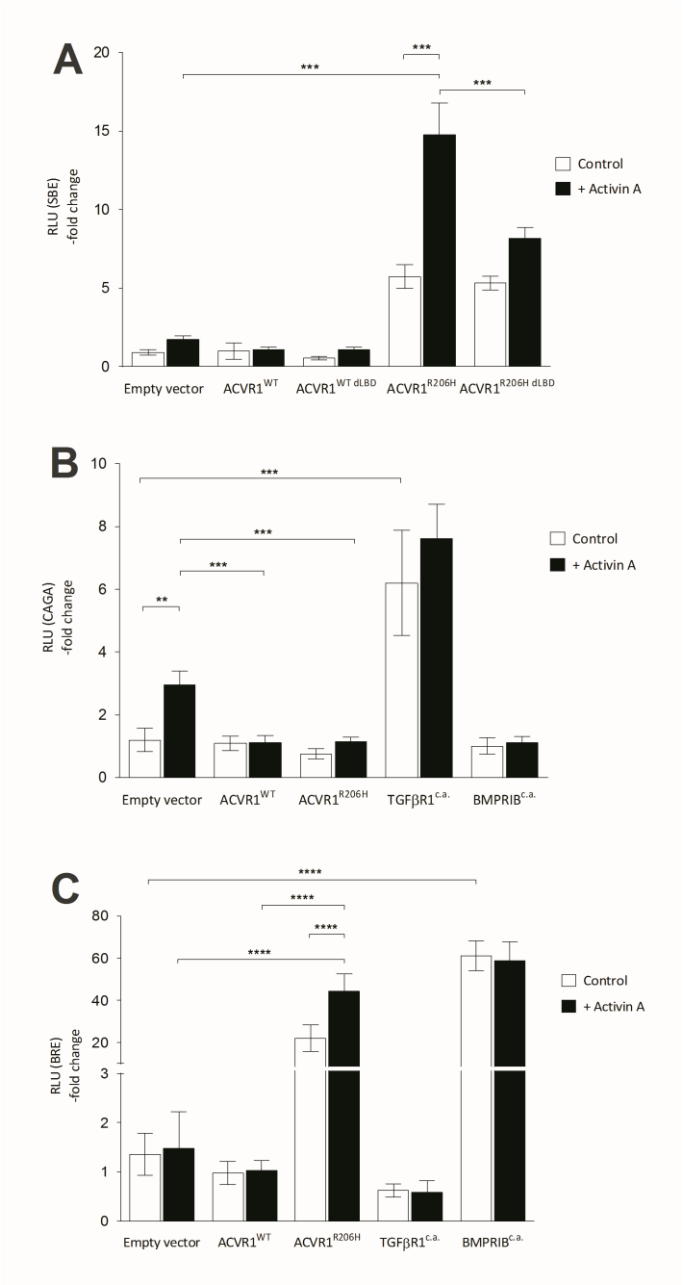


**Figure 5: ACVR1$^{R206H}$ shows an activating effect on BMP signalling after stimulation with Activin A in a LBD dependent manner.**

NIH/3T3 cells were transfected with SBE, CAGA or BRE and pRL-TK for normalization and an empty vector or Acvr1$^{WT}$, Acvr1$^{WT\ dLBD}$, Acvr1$^{R206H}$ or Acvr1$^{R206H\ dLBD}$ and stimulated with Activin A. TGFβR1$^{c.a.}$ served as positive control for SMAD2/3 signalling and BMPR1B$^{c.a.}$ for BMP signalling. A: Stimulation with Activin A led to an activation of general SMAD activity in

the presence of $ACVR1^{R206H}$, which was significantly reduced in the absence of the ligand binding domain. B: There was no increase in SMAD2/3 signalling for either $ACVR1^{WT}$ or $ACVR1^{R206H}$ in response to Activin A, instead C: $ACVR1^{R206H}$ activates BMP signalling in the presence of Activin A. To test for statistical significance on SMAD signalling three independent experiments with 3 replicates each and on BMP/SMAD2/3 signalling a representative experiment with 9 replicates were analyzed using 1way ANOVA (RLU = relative luciferase units).

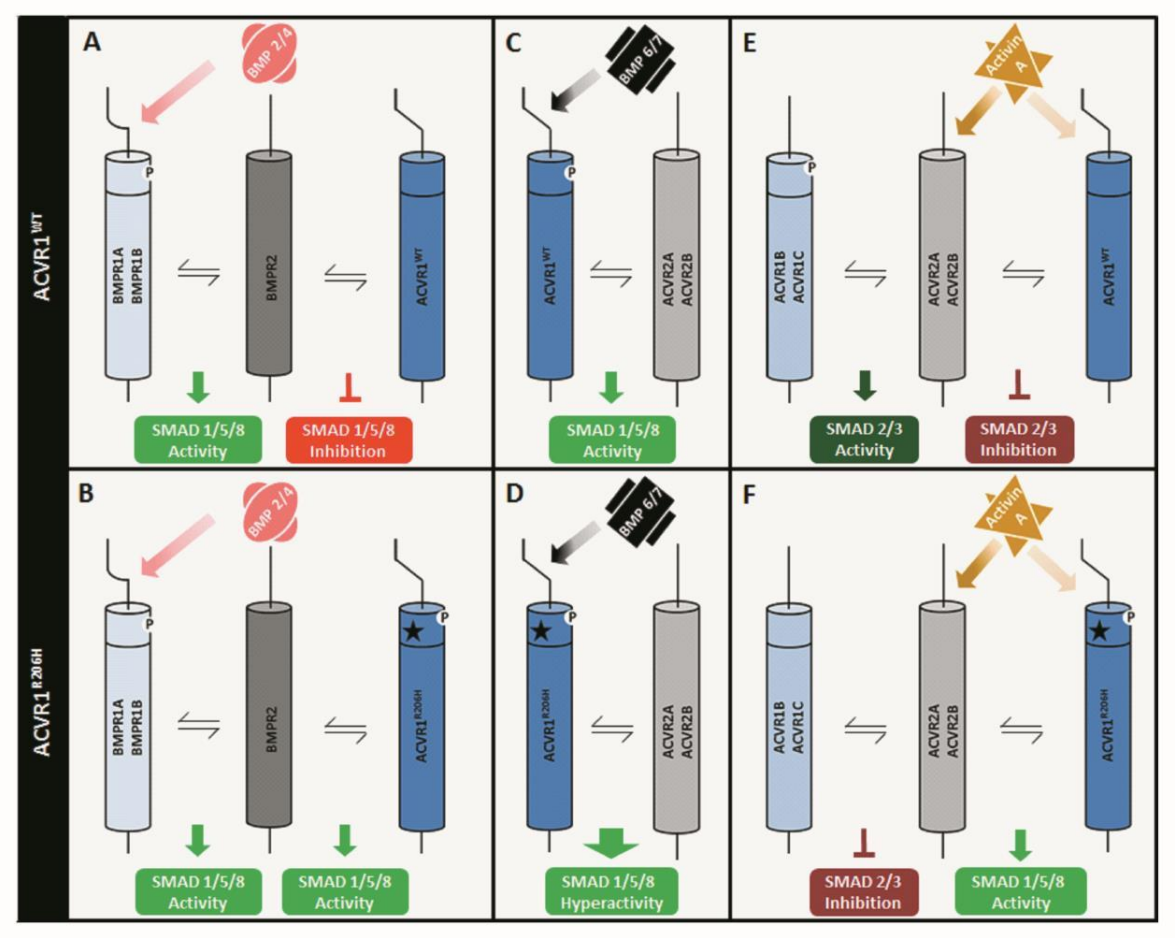


**Figure 6: Scheme of differential activation mechanisms of ACVR1$^{WT}$ and ACVR1$^{R206H}$ in response to BMP2 or BMP4, BMP6 or BMP7 and Activin A.**

A: BMP2 or BMP4 bind to the type I receptors BMPR1A or BMPR1B and induce BMP activity together with BMPR2. In the presence of ACVR1$^{WT}$ this activity is inhibited, due to competition for BMPR2. B: This inhibition in response to BMP2 and BMP4 is not present for ACVR1$^{R206H}$, which contrary to ACVR$^{WT}$ further activates BMP activity. C: BMP6 or BMP7 bind directly to ACVR1$^{WT}$ and activate BMP activity together with type II receptors ACVR2A/ACVR2B. D: This activation by BMP6 and BMP7 is even further enhanced with ACVR1$^{R206H}$ leading to BMP hyperactivity. E: Activin A binds to ACVR2A/ACVR2B and usually induces SMAD2/3 activity by interacting with the type I receptors ACVR1B (also known as ALK4) and ACVR1C (also

known as ALK7). This is inhibited in the presence of $ACVR1^{WT}$. F: In contrast, in response to Activin A, $ACVR1^{R206H}$ induces BMP activity, but still inhibits SMAD2/3 activity. The suggested scheme is a simplification of events, as crossover uses of the type II receptors may exist.

Graphical abstract