The finger 2 tip loop of Activin A is required for the formation of its non-signaling complex with ACVR1 and type II Bone Morphogenetic Protein receptors

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Running title: Activin A F2TL binds Acvr1 in a non-signaling complex

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Abstract

Activin A, a ligand that belongs to the BMP/TGFß family, functions in BMP signaling in two distinctly different ways: it binds to its cognate type II receptors – ACVR2A, ACVR2B, and BMPR2 – and the resulting complex either engages the type I receptor ACVR1B to activate Smad2/3 signaling or binds with the type I receptor ACVR1 to form a non-signaling complex. In order to set the stage for exploring potential biological roles of the non-signaling complex, we engineered Activin A variants that retain their ability to activate ACVR1B but are unable to generate the Activin A•type II receptor•ACVR1 non-signaling complex. This was accomplished by designing Activin A muteins wherein type I-binding regions were replaced with those of Nodal, a BMP/TGFß family member that utilizes ACVR1B but not ACVR1 as its type I receptor. Of the resulting muteins, an Activin A utilizing the finger 2 tip loop of Nodal (Activin A.Nod.F2TL) fulfilled our specifications; it failed to generate the non-signaling complex, yet activated ACVR1B akin to wild type Activin A. Furthermore, a single amino acid, D406, appears to be a main driver of this interaction, as its deletion results in a mutein that is similar in activity to Activin A.Nod.F2TL. These results define a region of Activin A that is important for the formation of the non-signaling complex and set the stage for engineering knock-in mice where the role of the non-signaling complex can be explored.

Introduction

Activin A is a protein that belongs to the TGFß/BMP family of ligands (1). These ligands initiate signaling by driving the formation of heterotetrameric complexes of their cognate type I and type II receptors, and where a dimeric ligand brings together two type I and two type II receptors (2). There are seven type I and five type II receptors that mediate signaling for this family of ligands. The choice of type I receptor in the signaling complex is the main determinant of which one of two signaling pathways is activated: Smad1/5/8, when the type I receptors are ACVRL1, ACVR1, BMPR1A or BMPR1B; and Smad2/3, when the type I receptors are ACVR1B, TGFBR1, or ACVR1C. Activin A utilizes ACVR1B as its type I receptor in conjunction with the type II receptors ACVR2A, ACVR2B, and to a lesser extent

BMPR2, and primarily signals through Smad2/3 (3). The biological functions of Activin A have been investigated widely using multiple approaches (4,5) including reverse genetics experiments wherein the gene encoding for Activin A – *Inhba* – has been 'knocked out' (6-8). The results of those experiments have been largely interpreted in the context of Activin A acting as a agonist to induce Smad2/3 or other signaling pathways (4,5,7,8).

However, recent studies pinpoint to a previously unrecognized property of Activin A: that it can engage the type I receptor, ACVR1, to form an ACVR1•Actvin A•type II receptor non-signaling complex. This ties down the type II receptors and ACVR1 and renders them unavailable for engagement with BMPs, hence resulting in an apparent inhibition of ACVR1-mediated BMP signaling. This property was discovered independently, in multiple myeloma-derived cell lines (9) and in the context of the rare genetic disorder Fibrodysplasia Ossificans Progressiva (10). *In vitro*, formation of this non-signaling complex results in inhibition of signaling by BMPs, primarily those BMPs that utilize ACVR1 as their preferred type I receptor. Therefore, in cells where ACVR1 is the main type I receptor, Activin A inhibits BMP6- (10) or BMP7-induced signaling (this work). This property is independent of ACVR1B, as neither stimulation of Smad2/3 signaling from ACVR1B nor inhibiting that signal affect either the formation of the ACVR1•Actvin A•type II receptor non-signaling complex or the resulting inhibition of ACVR1-mediated BMP signaling (9,10).

In contrast to the fairly detailed picture that has emerged from these *in vitro* studies, the physiological role of the non-signaling ACVR1•Activin A•type II receptor complex is less clear. In myeloma cells in culture, Activin A protects from BMP9-induced apoptosis, but whether a similar phenomenon takes place *in vivo* is currently unknown (9). Hence, thus far, the physiological role of ability of Activin A to form a non-signaling complex with ACVR1 has only been explored in the context of FOP. FOP is an autosomal-dominant genetic disorder that arises from missense mutations in the sequence encoding the intracellular domain of ACVR1. The resulting mutated variants of ACVR1 display the neomorphic property of recognizing Activin A (as well as other Activins) as an agonistic ligand, much like a BMP (10,11). Therefore, in FOP, Activin A has been converted from an antagonist to an agonist of signaling via ACVR1. In mouse FOP, this property is physiologically important as we have shown that inhibition of Activin A using highly specific monoclonal antibodies halt both the occurrence and the progression of heterotopic ossification, i.e. the phenotype that is the main hallmark (and medically most important aspect) of this disease (10). However, given that FOP is autosomal-dominant, one wild type copy of ACVR1 remains intact. In mouse FOP, removal of the wild type copy of *Acvr1* exacerbates the degree of heterotopic ossification observed (12). These data indicate that the non-signaling complex is indeed operant *in vivo*, at least in FOP. These results, however, do not explore whether there is a role for the non-signaling complex outside of ACVR1-centric disease settings such as FOP. We report here a set of Activin A muteins specifically engineered to address this question.

Results

A conceptual framework for engineering 'agonist-only' Activin A muteins

In order to set the stage for exploring the role of the non-signaling complex formed by Activin A when it engages ACVR1, we attempted to engineer Activin A muteins with the following properties (Fig. 1):

1. Ability to activate ACVR1B in a manner indistinguishable to wild type Activin A.
2. Preserving type I receptor usage and avoiding the generation of chimeric ligands that utilize other type I receptors such as TGFBR1 or ACVR1C.
3. Preservation of interaction with Activin A's natural antagonists, Follistatin and Follistatin-like 3.
4. Inability to form a non-signaling complex with ACVR1 as evidenced by a reduction of inhibition of BMP7-induced activation down to the level of inhibition that is driven by binding of Activin A to its type II receptors. (Since binding of Activin A to type II receptors is a required property for signaling through ACVR1B (13), it follows that it is not possible to engineer an Activin A that

will not compete with BMPs for type II occupancy; therefore, any Activin mutein that retains the ability to activate ACVR1B, will also, at high levels, antagonize signaling initiated by any BMP that utilizes ACVR2A, ACVR2B, or BMPR2, irrespective of its interaction with ACVR1.)

As a first step to engineer Activin A muteins with such properties, we determined which ligands of the BMP-TGFß family members utilize ACVR1B as their type I receptor, but do not engage ACVR1. Based on type I and type II receptor utilization, three ligands fit this requirement: Nodal, MSTN, and GDF11 (2). Given that of these three, MSTN and GDF11 can also utilize TGFBR1 (14,15), we focused on Nodal as the main donor of type I receptor-binding regions to substitute into the corresponding regions of Activin A, while also exploring the outcomes of substituting the same regions from other ligands. To define the specific regions of Activin to replace with their Nodal-derived counterparts, we initially relied on biochemical experiments where one region of Activin A had been replaced by the corresponding region from another BMP (16-18), and based on the premise that the regions that are most likely to define receptor utilization preferences are also those that are more divergent, we chose regions where the sequence diversity of TGF□ ligands was significant.

More specifically, several studies have demonstrated that the pre-helix and post-helix regions define the choice of type I receptors. Substitution of the region encompassed by the pre-helix and post-helix region (amino acids 355 to 391 of mature Activin A) with the corresponding region from BMP2, switched type I (but not type II) receptor utilization (17), whereas swapping the pre-helix region of Activin A with that of MSTN switched the preference of Activin A from ACVR1B to TGFBR1 (18). Moreover, the recently solved structure of GDF11 (an Activin family ligand) in complex with TGFBR1 and ACVR2B (PDB:6MAC), shows that the Finger 2 region is particularly important for determining type I receptor specificity for the Activin class (Goebel et al, under review). Superimposition of Activin A into this ternary complex predicts that Activin A's finger 2 tip loop (F2TL; amino acids 406-409) also participates in binding to Type I receptors (Fig. S1). In more detail, we generated predictions of specific molecular interactions through structural modeling. We started with the structure of Activin A bound to Fs288 (PDB:2B0U) (19), as Fs288 engages Activin A using the same interface as the type I receptor. This fixed the structure of Activin A as would be expected when receptor-bound. We then generated several models of ACVR1 based on closely related receptors and found BMPR1A to provide the best structural prediction. Superimposition of these models to the complex structure of TGFBR1•GDF11•ACVR2B followed by energy minimalization of amino acid side chains suggested a key interaction at the fingertip. Specifically, D406 extends into the type I binding site (seen in several Activin A structures – PDB: 1S4Y,2B0U, 2ARV, 3B4V) and is flanked by two lysine residues from ACVR1 (Fig. S2) (13,19-21). This observation supports a key role for D406 in binding ACVR1. Our model is further supported by the fact that there is a conserved hydrophobic residue (M79 in ACVR1) that rests in a hydrophobic pocket formed by the Activin A dimer, forming a "knob-in-hole" motif, which is also in the BMP2:BMPR1A (22) and GDF11•TGFBR1 complexes (PDB:2GOO, 6MAC) (Goebel et al in review}.

In addition, along with the F2TL region, the pre- and post-helix regions exhibit significant sequence diversity between ligands, hence making them attractive candidates for Type I receptor selectivity. In contrast, other regions that are known to be involved in engaging the type I receptors, such as finger 1 and H3 helix were excluded as donors for substitution analysis; finger 1 was excluded because it displays a high level of sequence conservation between ligands (Fig. S2), whereas the H3 helix was not utilized because it appears to play a structural role and not to be a major determinant of type I receptor choice (23). Hence, we chose to focus on these three regions – F2TL, pre-helix, and post-helix – as sources of sequences to transfer from Nodal (and other Activin class ligands) into Activin A.

Activin A with Nodal F2TL activates ACVR1B

Based on the design criteria and biochemical and structural information summarized above, we engineered Activin A muteins in which their pre-helix, post-helix, and F2TL regions were replaced with the corresponding regions of Nodal, generating **Activin A.Nod.pre**, **Activin A.Nod.post**, and **Activin A.Nod.F2TL**, respectively (Fig. 2). After expression and purification of their mature forms (Fig. S3), we first tested their ability to activate Smad2/3 signaling, a measure for utilization of ACVR1B as the type I receptor. Of these three muteins, the Activin A.Nod.F2TL exhibited activity very close to that of wild type Activin A (Fig. 3), whereas the other two were less active (Fig. S4).

The realization that the tip loop variant performed according to our specifications for activation of ACVR1B prompted closer examination of the finger 2 tip loop region sequences between Activin A and Nodal. This comparison revealed an intriguing difference between the fingertips of these ligands: that Nodal lacks an aspartic acid at position 406 (Fig. 2*B*). Moreover, an alignment of Activin A from different species reveals that this residue is highly conserved (Fig. S5), whereas it varies when the different Activins are compared (Supplemental Fig. S2). These observations propelled us to question whether a deletion of D406 would generate an Activin A mutein with similar properties compared to the Nodal tip loop variant. We therefore compared the ability of Activin A.ΔD406, with that of Activin A.Nod.F2TL and wild type Activin A to activate ACVR1B using a CAGA-luciferase assay as a readout for Smad2/3 activation. As shown on Figure 3A, the Nodal tip loop variant is slightly less active that Activin A, whereas Activin A.ΔD406 exhibits identical activity to wild type Activin A, making Activin A.ΔD406 a second viable mutein to study further.

Activin A.Nod.F2TL and Activin A.ΔD406 retain their type I receptor preferences

In order to exclude the possibility that by substituting the sequence of finger 2 we have inadvertently altered type I receptor preferences (precluding the deliberate reduction of binding to ACVR1), we employed an assay that measures dimerization of type I with type II and investigated whether Activin A.Nod.F2TL and ΔD406 muteins displayed altered type I preferences. In line with our bioassay results, both Activin A.Nod.F2TL and Activin A.ΔD406 are identical to Activin A in their ability to dimerize ACVR1B and TGFBR1 along with the corresponding type II receptors. In addition, as would be expected for any Activin A variant that has reduced ability to form the non-signaling complex, both Activin A.Nod.F2TL (Fig. 3B) and Activin A.ΔD406 (Fig. S6) display greatly reduced ability to induce dimerization of ACVR1 with ACVR2A.

Activin A.Nod.F2TL and Activin A.ΔD406 cannot form a non-signaling complex with ACVR1

In order to determine whether Activin A.Nod.F2TL and Activin A.ΔD406 have lost their ability to form the non-signaling complex with ACVR1 and type II receptors, we tested their ability to inhibit BMP7-mediated activation of ACVR1 using a Smad1/5/8-responsive luciferase-based assay. Consistent with previous observations utilizing BMP6 and BMP9 (9,10), Activin A was able to inhibit activation of ACVR1 by BMP7 in dose-dependent manner. In contrast, both Activin A.Nod.F2TL and Activin A.ΔD406 were approximately 60-fold and 15-fold less effective at inhibiting BMP7, respectively (Fig. 4; Fig. S7)).

The remaining apparent inhibition can be attributed to binding of these muteins to the type II receptors, as would be expected for any Activin variant that can activate ACVR1B. To demonstrate this, we utilized an anti-Activin A monoclonal antibody, REGN2476, that neutralizes the activity of Activin A by blocking its binding to its type II receptors (Fig. S8). In addition, we also tested another neutralizing anti-Activin A antibody, H4H10442, that inhibits biding of Activin A to type I receptors by binding to the F2TL. Both antibodies can completely inhibit Activin A's ability to activate signaling through ACVR1B (Fig. S9) as well as FOP-mutant ACVR1 (10). When complexed with the F2TL mutants, REGN2476 completely abolished the remaining inhibition of BMP7-initiated signaling by Activin A.Nod.F2TL and

Activin A.ΔD406, (Fig. 4A; Fig. S9). In addition, we tested Activin A.Nod.F2TL and Activin A.ΔD406 for their ability to engage ACVR2B, one of the main type 2 receptors, using Biacore. As expected, in this binding assay, Activin A.Nod.F2TL and ActivinA.ΔD406 bound with high affinity to type II receptor like wild-type Activin confirming that this substitution does not affect type II receptor binding site (Fig. S10). These results clearly indicate that the remaining ability of Activin A.Nod.F2TL and Activin A.ΔD406 to antagonize BMP7-initiated signaling is indeed through engagement of the type II receptors.

Also in line with the results described above, we observed that H4H10442 shifts the dose response of the inhibition brought about by wild type Activin A on BMP7-induced signaling to that corresponding to Activin A.Nod.F2TL (Fig. 4B). This is consistent with the idea that the greater degree of inhibition seen with wild type Activin A is indeed driven by formation of the non-signaling complex. Using the same criteria, our data shows that Activin A.ΔD406 has partially retained the ability to antagonize BMP7 signaling via ACVR1, making it a less desirable 'agonist only' Activin A mutein (Fig. S7). Incidentally, in contrast to what is seen with Activin A.Nod.F2TL and Activin A.ΔD406, inhibition of binding of wild type Activin A to the type II receptor (via REGN2476) does not 'flat-line' the ability of wild type Activin A to inhibit BMP7-mediated signaling (Fig. 4, and Fig. S7). In fact, the response to the two antibodies - H4H10442, which blocks binding to ACVR1 by occupying the Activin A F2TL (Fig. S9), and REGN2476, which blocks binding to the type II receptors – is nearly identical when Activin A is the ligand outcompeting BMP7. This indicates that Activin A can engage ACVR1 even in the absence of binding to type II, at least under conditions where ACVR1 is expressed at high levels. This finding further highlights the fact that Activin A.Nod.F2TL and Activin A.ΔD406 cannot form a non-signaling complex with ACVR1.

Given that these two muteins have a greatly reduced ability to engage ACVR1, we tested whether they can still activate ACVR1[R206H] (i.e. the FOP-causing variant that utilizes Activins as agonists (10,11)). Both muteins were found to transduce a signal through ACVR1[R206H] (Fig. S11). This indicates that they retain enough binding to A to induce signaling, but not enough to form the non-signaling complex. This result is corroborated by the observation that both muteins can still dimerize ACVR1 with ACVR2A to some extent, though not to the degree that Activin A does (Fig. 3B, and S6). In line with these results, structural modeling of Activin A.Nod.F2TL suggests that D405 can shift into the type I interface to compensate for the loss of D406, albeit interacting with only one lysine residue (K78), and hence resulting a weakened interaction (Fig. S1).

Activin A.Nod.F2TL and Activin A.ΔD406 retain their interactions with their antagonists

As a last step in examining potential differences between Activin A and these two muteins, we tested their interaction with Activin's natural inhibitors, Follistatin (FST) and Follistatin-like 3 (FSTL3) (Fig. 5). Both isoforms of FST, differing in the absence (Fs288, Fig. 5*A*) or presence (Fs315, Fig. 5*B*) of c-terminal acidic tail, inhibit Activin A signaling to baseline in Smad2/3 reporter assays. Activin A.Nod.F2TL displays nearly identical inhibition profile compared to wild type, whereas Activin A.ΔD406 shows a slightly decreased ability to be inhibited by both FST isoforms but still can be fully inhibited to baseline (Fig. S13). Contrasting this, both F2TL muteins display decreased ability to be inhibited by FSTL3 (Fig. 5*C*, Fig. S13). This is due to the difference in the way FST and FSTL3 engage Activin A. Both FST and FSTL3 engage the Activins and other TGF□ ligands, in a 2:1 stoichiometric ratio with 2 Follistatin molecules per one dimeric ligand. When FST engages Activin A the FSD3 domain reaches around the ligand to non-covalently contact the N-terminal domain of the other FST molecule, essentially fully wrapping around the ligand. There are relatively few specific contacts between FST and the ligands that it interacts with. This makes FST a quite promiscuous modulator of multiple BMP/TGFß family members. In contrast, FSTL3 lacks the FSD3 domain and engages Activin A using several amino acid-specific contacts, which includes direct contacts with the finger 2 tip loop. Specifically the ring nitrogen of His91 in FSTL3 makes a charge contact with D406 of Activin A, and

both Ser221 and Arg225 directly interact electrostatically with Activin A Q408 (24). Since both Activin A.Nod.F2TL and Activin A.ΔD406 lack D406, the reduced inhibition of these Activin A muteins by FSTL3 was expected.

Discussion

Activin A, the most widely studied of the Activin branch of BMP/TGFß family ligands, has been primarily studied as an agonistic ligand that activates signaling through the type I receptor, ACVR1B (4,5). However, we and others have uncovered an additional role for Activin A – the generation of a non-signaling complex with a different type I receptor, ACVR1 (9,10). Interestingly, ACVR1 was originally cloned as the type I receptor for Activin A (25,26), but the lack of induction of an ACVR1 signal from Activin A along with the ability of BMP7 to activate it, led to a 'relabeling' of ACVR1 as a BMP receptor (27). The discovery that Activin A has a dual role – (a) acting as an inducer of signaling through ACVR1B; and (b) tying down ACVR1 along with the corresponding type II receptors into a non-signaling complex which in turn inhibits signaling by BMPs that utilize those receptors – has propelled us to ask whether this non-signaling complex has physiological roles. Evidence that this is the case already exists in the context of (mouse) FOP, as removal of the remaining wild type copy of *Acvr1* greatly enhances HO (the main disease phenotype in FOP), whereas overexpression of wild type *Acvr1* largely blocks HO. However, these results only speak to the role of the non-signaling complex within FOP and leave open the question of the biological functions of that complex in normal physiology or disease settings where Activin may play a role. To set the stage for experiments that can explore these questions at the organismal level and specifically in genetically modified animal models, we engineered Activin A muteins that preserve the ability to act as agonists of ACVR1B but fail to form the non-signaling complex with ACVR1 and the corresponding type II receptors.

To design such muteins we made use of biochemical information regarding receptor utilization by Activin family ligands, prior mutagenesis studies, and structural modeling. We chose Nodal as the main donor for replacements of type I-binding regions of Activin A and focused on three regions for engineering the different chimeric ligands: the pre-helix, the post-helix, and the finger 2 tip loop (F2TL) (see Results section). Of the resulting Activin A-Nodal chimeras, the one that displays properties very close to our specifications was Activin A.Nod.F2TL, where the finger 2 tip loop was substituted with that of Nodal. In addition, based on sequence homology, we engineered a single amino acid deletion variant – Activin A.ΔD406 – that displays properties close to that of its parent, Activin A.Nod.F2TL, but retains more binding to ACVR1, hence rendering it less desirable for modeling *in vivo*. Nonetheless, Activin A.ΔD406 demonstrates the importance of D406 as a major determinant of the interaction of Activin A with ACVR1, an idea that is supported by our structural modeling (Fig. S1).

In line with the findings we describe here, the importance of the finger 2 tip loop as a major determinant of type I receptor choice has been concurrently demonstrated for GDF11's interaction with TGFBR1 (Goebel et al, under review). Both findings are further supported by an additional chimeric ligand that we have engineered using GDF8 as the 'donor' to generate the corresponding F2TL variant – Activin A.GDF8.F2TL. This variant utilizes TGFBR1 as the type I receptor to a greater degree than Activin A, while retaining its ability to engage ACVR1B in a manner identical to Activin A (Fig. S12). Therefore, these results lend further credence to the concept that the finger 2 tip loop is a key determinant of type I receptor choice in the Activin A class.

Although neither one of the two muteins that we present here is absolutely perfect in its activity profile in that they display a small increase in resistance to inhibition by FSTL3 (Fig. 5), a property that is unavoidable for Activin A variants that bear changes in the finger 2 tip loop. Whether this deviation from our intended design will have physiological consequences, remains to be seen. It should be noted however, that FSTL3 knockout mice have a relatively benign phenotype that has been well characterized and that has been attributed to an increase in Activin A activity (28). Given the relatively small reduction in the ability of Activin A.Nod.F2TL to be inhibited by FSTL3 (compared to what would be expected from a complete loss of FSTL3), it is unlikely that this difference will result in a phenotype. The availability of phenotypic data on FSTL3 knockout mice should facilitate the assessment of any

differences that might arise in the physiology of mice bearing Activin A.Nod.F2TL as a knock-in into *Inhba*.

Irrespective of these considerations, the finding that substitution of finger 2 tip loop with that of Nodal without significantly changing Activin A's interaction with ACVR1B is a significant advance in understanding the molecular mechanisms of signaling through this receptor system. Our findings provide additional evidence that the non-signaling complex is a stable entity mediated through very specific contacts between Activin A and ACVR1, and particularly those that are involved in determining type I receptor choice by Activin family ligands. The amino acids of Activin A that are involved in interacting with ACVR1 to form the non-signaling complex are at least in part different that those required for the interaction of Activin A with ACVR1B (16,29). Nonetheless, we note that we did not attempt an exhaustive search to define all possible Activin A muteins with the desired properties, and hence it is possible that other such muteins can be engineered. Along these lines, since Activin B and Activin AB are also capable to forming non-signaling complexes with ACVR1 and the corresponding type II receptors, our findings should enable exploration of the formation of the respective complexes by following similar methodology. Lastly, given the level of evolutionary conservation in this receptor-ligand system, our data opens the field of investigating whether the ability of forming the non-signaling complex was an early or a late event during evolution.

Experimental Procedures

Antibodies and protein reagents

Activin A antibodies H4H10442 and REGN2476 are human monoclonal antibodies specific to Activin A and utilizing an IgG4 constant region. They were generated using Regeneron's VelocImmune® mice (30,31).

Cloning, expression and purification of Activin A muteins

The mature regions of Activin A or Activin A muteins were cloned downstream of mouse *Inhba* pro-peptide using isothermic Gibson assembly (32) of synthetic gene blocks (Integrated DNA Technologies) into a CMV-based mammalian expression vector. The corresponding expression vectors were introduced into CHO-K1 cells expressing human Furin (CHO-KI.Furin). The resulting conditioned media was concentrated approximately ten-fold using a filtration column with a 3K molecular weight cut off (Pierce) before biochemical characterization of the Activin A muteins in cell-based assays. Following this primary analysis, Activin A and the Activin A F2TL mutants were stably expressed in CHO-K1.Furin cells. The Activin A pro-peptide complex was first purified to homogeneity by heparin chromatography followed by size exclusion chromatography on a preparative scale S200 column. To isolate the mature Activin A mutein dimers, reverse phase chromatography on C4 column was used, and mature Activin A was eluted with an acetonitrile gradient.

Structural modeling and alignments

ClustalW alignment in MacVector was used for amino acid sequence alignments. Structural alignments using known crystal structures as a reference were carried out using STRAP, a web-based alignment program (33). All figures with structural models were generated in PyMol (The PyMol Molecular Graphics System, Schrödinger, LLC). The model of ACVR1 was built using Swiss-model (34). After manual adjustment that resulted in no clashes and contained realistic contacts, energy minimization of amino acid side chains was performed utilizing YASARA (35).

Binding kinetics measurements

Kinetic binding parameters for the interaction of human ACVR2B.hFc fusion protein with recombinant Activin A and Activin A muteins were determined under neutral pH on Biacore 3000 using dextran-coated (CM5) chips at 25°C. The running buffer was prepared using filtered HBS-ET (10 mM Hepes, 150 mM NaCl, 3.4mM EDTA, 0.05% polysorbate 20, pH 7.4). The capture sensor surface was prepared by covalently immobilizing Protein A (Sigma-Aldrich, St. Louis, MO) to the chip surface using (1¬Ethyl-3-[3-dimethylaminopropyl]carbodiimide-hydrochloride)/N-hydroxysuccinimide (EDC/NHS) coupling chemistry. Binding kinetics of Activin A and Activin A muteins were measured by flowing 100nM- 0.14nM of ligand, serially diluted three-fold, at 100uL/minute for two minutes and monitored for dissociation for 15 minutes. All capture surfaces were regenerated with one 30-s pulse of 10 mM glycine–HCl (pH 1.5, (GE Healthcare, Marlborough, MA). Kinetic parameters were obtained by globally fitting the data to a 1:1 binding model with mass transport limitation using Scrubber 2.0c Evaluation Software. The equilibrium dissociation constant (KD) was calculated by dividing the dissociation rate constant (kd) by the association rate constant (ka).

Cell culture and cell-based assays

Unless otherwise noted, cells were cultured in Dulbecco's modified Eagle's medium containing 10% (v/v) fetal bovine serum (FBS), penicillin/streptomycin (50 U/ml), and 2 mM L-glutamine. Generation of ACVR1 and ACVR1.R206H expressing HEK293 BRE-luciferase reporter cells has been previously described (10). For this study, a reporter construct with twelve tandem repeats of the $(CAGACagc)_{12}$ response element was introduced into HEK293 cells to engineer the HEK293 CAGA-luciferase reporter line (36).

Cell based assays were conducted as previously described (10). Briefly, HEK293 reporter cells were plated in 96-well plates. After a 16 hours incubation with ligands alone, ligand mixtures or ligand/antagonist mixtures, luciferase expression was measured with Bright-Glo Luciferase Assay System (Promega).

Human U2OS cell lines for receptor dimerization assays were purchased from DiscoverX (Fremont, CA) and used following manufacturers protocols. Briefly, cells were treated with ligands for 16 hours. β-galactosidase enzyme fragment complementation was quantified by a luminescent signal following the addition of the manufacturer supplied substrate.

Conflict of interest

VI, HK, QZ, AR, RL, XW, JM, AJM, and ANE are employees of Regeneron Pharmaceuticals and own common stock as well as stock options of Regeneron shares. The remaining authors have no conflicts of interest to declare with respect to the contents of this article.

Author Contributions

VI and ANE conceived of the project. VI, ANE, EJG, AJM, and TBT designed experiments and were involved in the interpretation of results and wrote the manuscript. RAC, EJG, CJC, AD, HK, QZ, AR, RL, XW, and JM performed experiments and contributed data.

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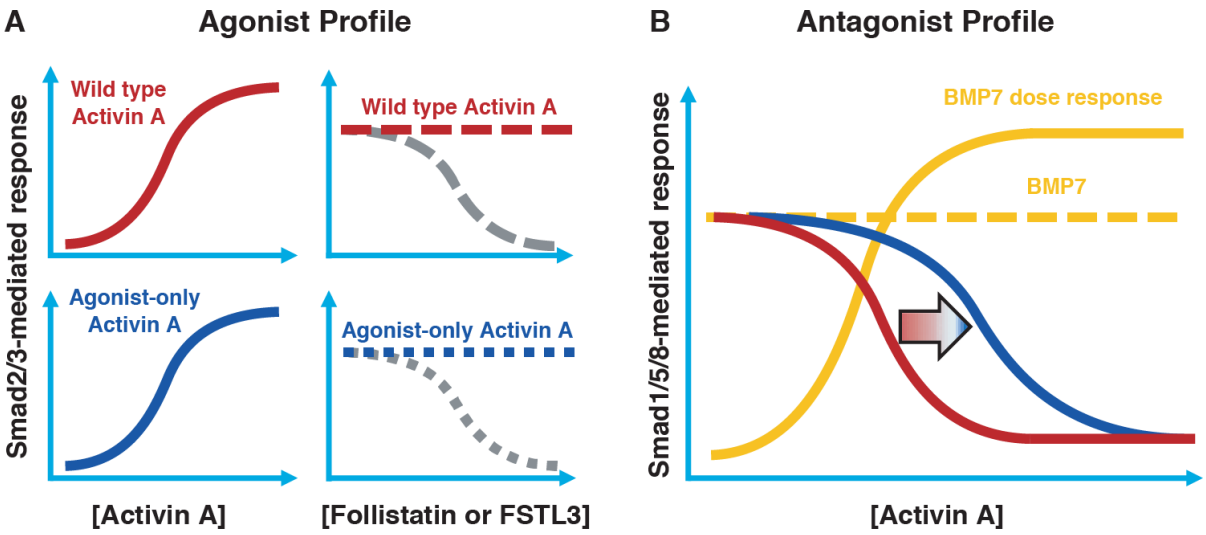

Figure 1: Conceptual framework for designing 'agonist-only' Activin A muteins
(A) In a luciferase reporter assay, agonist only Activin A muteins (blue) should retain activation of the Smad2/3 pathway like wild-type Activin A (red; left panels), and they should retain ability to be inhibited by the endogenous antagonists Follistatin and FSTL3 (grey; right panels). (B) As a result of a loss of ACVR1 binding, Activin A muteins should be less effective inhibitors of BMP mediated signaling to the Smad1/5/8 pathway. Therefore, we expect that the agonist only muteins should have reduced antagonism of Smad1/5/8 signaling compared to wild-type Activin A. However, antagonism will not be entirely lost, as the agonist-only mutein must still bind to Type II receptor for activation of Smad2/3 signaling.

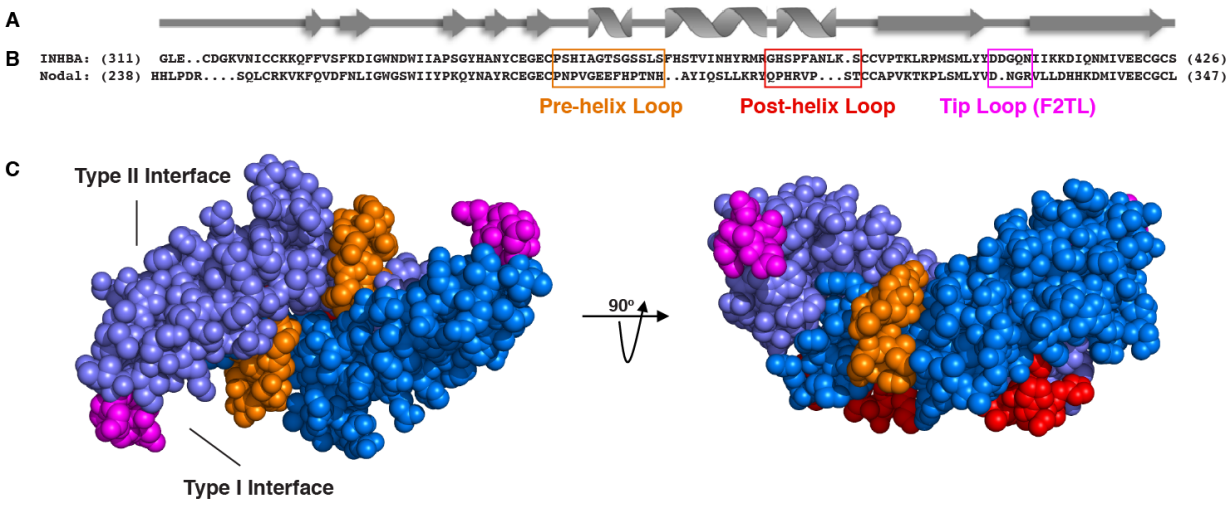

Figure 2: Activin A muteins were engineered with the Activin A pre-helix, post-helix, and F2TL regions replaced with the corresponding regions of Nodal

(A) Structural alignment of human Activin A and Nodal that highlights the Activin A pre-helix, post-helix and finger 2 tip loop (F2TL) sequences used to generate agonist-only Activin A muteins. Nodal sequences highlighted in the boxed areas were substituted for the corresponding sequences of Activin A. (B) Crystal structure of FSTL3-bound Activin A (space filled model, PDB 3B4V) with substituted areas colored as follows; pre-helix loop (orange), post-helix loop (red) and F2TL (magenta). Each Activin A monomer is depicted in either light or dark blue. Notice that these substitutions occupy the type I receptor binding site.

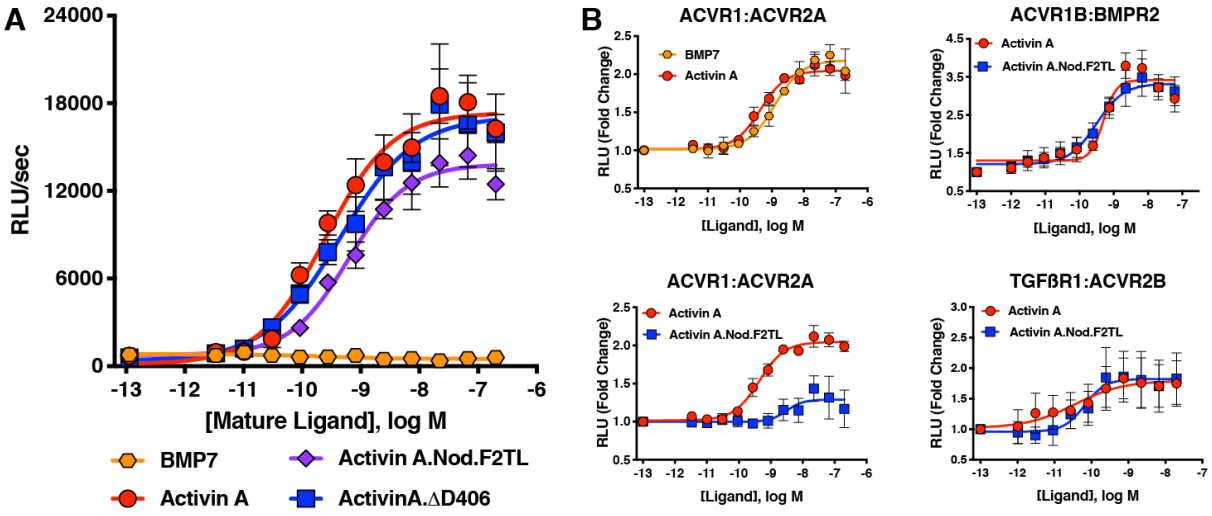

Figure 3: Activin A F2TL mutants signal normally through the Smad2/3 pathway, but have lost binding to Acvr1

(A) Activin A F2TL muteins activate Smad2/3 signaling to similar levels as wild type Activin A in HEK293 cells harboring a Smad2/3 reporter construct driving firefly luciferase. (B) U20S cells expressing split beta-galactosidase fusions of corresponding type I and type II receptors were treated with a dose response of BMP7, Activin A, or Activin A.Nod.F2TL. Type 1 receptor binding was measured by luminescence in these receptor dimerization assays. Activin A.Nod.F2TL has reduced ability to dimerize ACVR1:ACVR2A receptors, while retaining wild type capacity to dimerize the ACVR1B:BMPR2 and TGFBR1:ACVR2B receptor pairs.

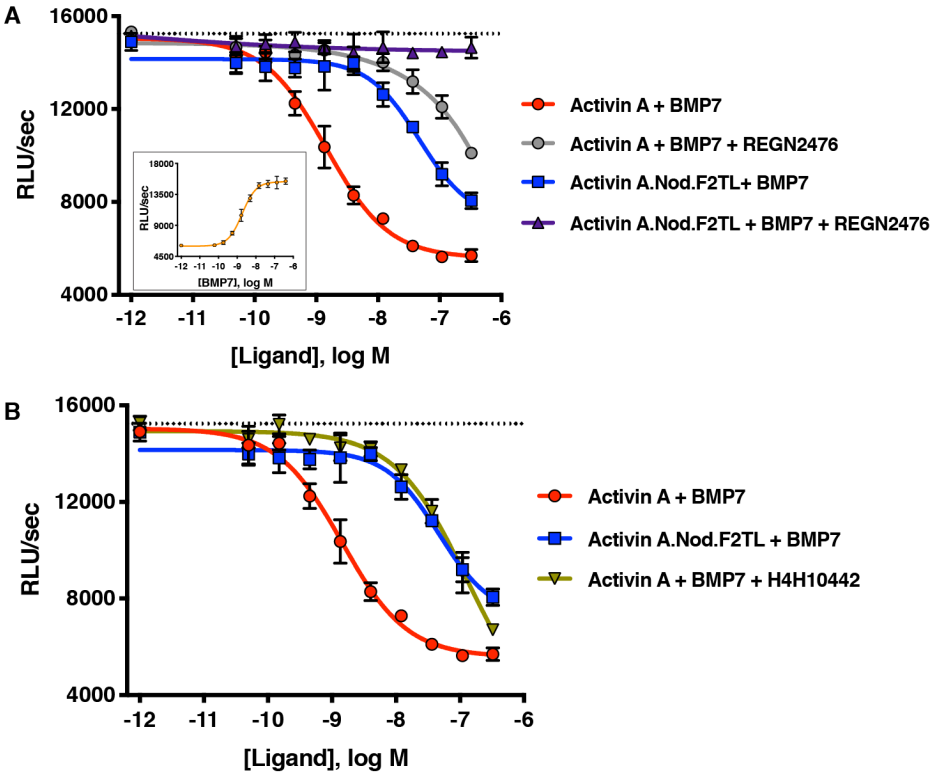

Figure 4: The remaining BMP antagonism of Activin A.Nod.F2TL is due to type II receptor binding A) Activin A.Nod.F2TL is a less effective inhibitor of BMP7 signaling to Smad 1/5/8 than wild-type Activin A. HEK293 cells harboring a Smad1/5/8 luciferase reporter construct were treated with varying concentrations of Activin A or Activin A.Nod.F2TL with a constant concentration of BMP7 (12nM) to stimulate Smad1/5/8 signaling. Inhibition of BMP7 is reduced ~ 60 fold with Activin A.Nod.F2TL compared to Activin A. Using an Activin A antibody that blocks interaction with type II receptor (REGN2476), the remaining inhibition of BMP7 by Activin A.Nod.F2TL is lost. B) Inhibition of type I receptor binding of Activin A with anti-Activin antibody H4H10442 shows a similar reduction in BMP inhibition to Activin A.Nod.F2TL. (The IC_{50}s of Acitivin A and Activin A.Nod.F2TL are 1.4×10^{-9} M and 9.7×10^{-8} M, respectively. Insert in Fig. 4A shows a dose response of BMP7 on the HEK293 reporter cells, and the dotted lines represents the Smad1/5/8 signal induced by 12nM BMP7 without inhibition by Activin A.)

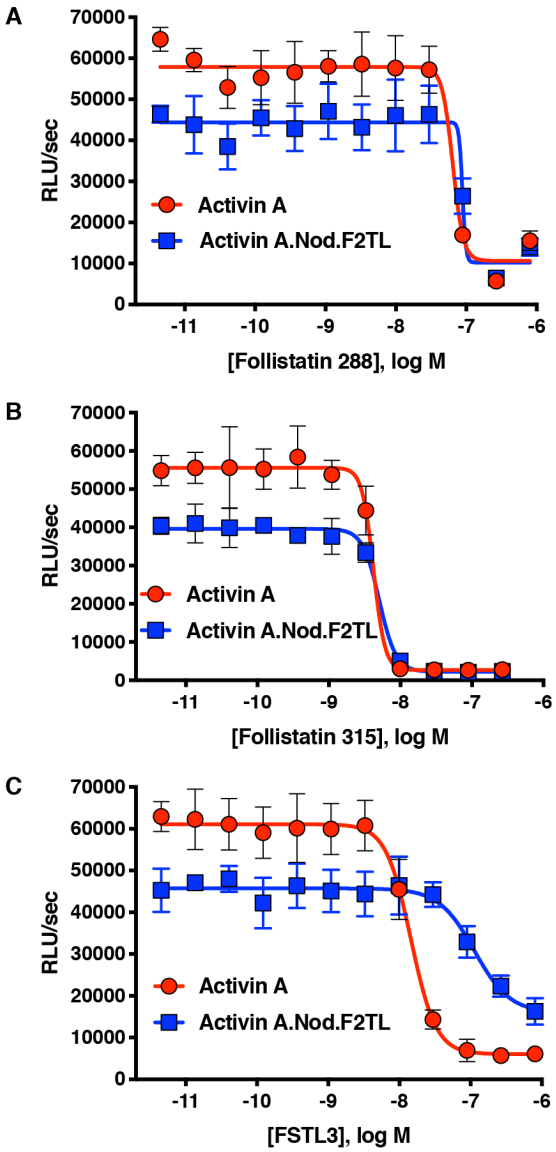

Figure 5: Activin A.Nod.F2TL mutein is inhibited by Follistatin but shows reduced inhibition by FSTL3

Varying concentrations of Follistatin and FSTL3 were preincubated with a constant concentration (10nM) of Activin A or Activin A.Nod.F2TL 'agonist only' mutein. Activity of both Activin A and Activin A.Nod.F2TL were tested in HEK293 cells harboring the Smad2/3 luciferase reporter. Activity of both Activin A and Activin A.Nod.F2TL was blocked by both follistatin-288 (A) and follistatin-315 (B). (C) FSTL3 is a less effective inhibitor of Activin A.Nod.F2TL.