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Hyperactive BMP signaling induced by ALK2^{R206H} requires type II receptor function in a *Drosophila* model for classic Fibrodysplasia Ossificans Progressiva

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Abstract

Fibrodysplasia Ossificans Progressiva (FOP) is an autosomal dominant skeletal disorder characterized by widespread and debilitating bone formation in place of soft connective tissue. All mutations associated with FOP map to the BMP type I receptor, ALK2, with the vast majority of patients possessing the ALK2^{R206H} mutation which results in hyperactive signaling. Here, we show that human ALK2^{R206H} exhibits hyperactive signaling both in *Drosophila* cell culture and *in vivo*. As true for ALK2^{R206H}–induced signaling in vertebrates, we find that the increase in signaling is also ligand-independent in *Drosophila*. Using the *Drosophila* system to identify factors required for this hyperactivity, we identified the type II receptor as a key determinant for mutant ALK2^{R206H} receptor signaling. In addition, we found that the wild-type ALK2 receptor can antagonize, as well as promote, BMP signaling. Due to the heterozygosity typical of FOP, this dual function is of particular interest given that the interplay between the two disparate behaviors of wild-type ALK2 could be shifted by the presence of the hyperactive ALK2^{R206H} mutant receptors. We present our work as a compelling example for the use of *Drosophila* as a model organism to study the molecular underpinnings of a complex human syndrome such as FOP.

Keywords

BMP signaling; ALK2; ACVR1; FOP; receptor kinase activation; Sax; Gbb; Dpp; Drosophila; type I receptor; type II receptor

INTRODUCTION

Fibrodysplasia Ossificans Progressiva (FOP) is a rare genetic disorder marked by the episodic deposition of heterotopic bone in the place of muscle and connective tissues throughout the life of a patient. All individuals with FOP have been found to carry a point mutation in one copy of their *ALK2/ACVR1* genes that encodes a bone morphogenetic protein (BMP) type I receptor (Shore et al., 2006; Kaplan et al., 2009). FOP-associated mutations in ALK2/ACVR1 appear to produce hyperactive receptors, resulting in inappropriate BMP signaling (Billings et al., 2008; Fukuda et al., 2009; Kaplan et al., 2009; van Dinther et al., 2010).

Transforming Growth Factor-β (TGF-β)/BMP type I receptors are highly conserved, transmembrane receptor serine/threonine kinases that are an integral part of the TGF-β/BMP signal transduction pathway acting in a diverse array of cellular and developmental processes. Type I receptors mediate extracellular TGF-β/BMP signals as part of a receptor

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complex made up of two types of transmembrane serine/threonine kinase receptors, the type I receptors and the related type II receptors. This heteromeric receptor complex has been shown to assemble by two different mechanisms: 1) secreted ligands can induce receptor complex formation by binding the extracellular ligand-binding domains of the type I and type II receptors (Groppe et al., 2008; Nickel et al., 2009), or 2) the type I and type II receptors can interact independently of ligand to generate preformed complexes that then bind ligand to initiate signal transduction (Nohe et al., 2002; Hassel et al., 2003; Ehrlich et al., 2011; Marom et al., 2011). Upon formation of the ligand-bound receptor complex, activation of the type I receptor results from trans-phosphorylation by the constitutive kinase activity of the type II receptor. The cytoplasmic signal transducers, receptor-mediated Smads (R-Smads), are then phosphorylated by the activated type I receptors, enabling nuclear accumulation of co-Smad/R-Smad complexes that interact with other proteins to regulate transcription of specific target genes (Massagué, 1998; Wu and Hill, 2009).

The TGF-β/BMP type I receptors are characterized by a cysteine-rich, extracellular ligand-binding domain, a single-pass transmembrane domain, and a well-conserved, intracellular kinase domain (Massagué, 1998). The intracellular domain contains regulatory regions such as the L45 loop, which confers R-Smad specificity (Feng and Derynck, 1997; Persson et al., 1998), and a glycine-serine rich GS domain required for activation of the type I receptor kinase (Wrana et al., 1994; Franzén et al., 1995). The GS domain, once phosphorylated by the type II receptor, forms a secondary binding site for R-Smads in addition to the L45 loop (Huse et al., 2001).

Based largely on sequence homology and ligand specificity, two main groups emerge from the family of BMP type I receptors represented by the mammalian ALKs and *Drosophila* Tkv/Sax receptors: ALK3/ALK6/Tkv and ALK1/ALK2 (ACVR1)/Sax (Chen and Massagué, 1999; Newfeld et al., 1999). These two groups exhibit higher affinities for secreted BMP ligands from the two subfamilies, BMP2/4/Dpp and BMP5/6/7/Gbb, respectively. The differential affinity for ligands among type I receptors is thought to reside in the extracellular domain of the receptor, the least conserved domain of the polypeptide. The total number of ligands far outweighs the number of type I and type II receptors and while there is thought to be some functional redundancy, it is clear that the tissue context of various combination of ligands and receptors impacts signaling output. As such, mutations in just the type I receptors are associated with a number of unique diseases including, hereditary hemorrhagic telangiectasia type 2 (HHT2) (ALK1/ACVRL1), juvenile polyposis syndrome (ALK3/BMPR1A), brachydactyly type A2 (ALK6/BMPR1B) and the focus of this study fibrodysplasia ossificans progressiva (FOP) (ALK2/ACVR1) (Abdalla and Letarte, 2006; Bayrak-Toydemir et al., 2006; Wehner et al., 2006; Olivieri et al., 2007; Howe et al., 2001; Zhou et al., 2001; Kim et al., 2003; Lehmann et al., 2003; Lehmann et al., 2006).

Classic, as well as atypical FOP is characterized by progressive, heterotopic ossification that occurs through an endochondral process (Pignolo et al., 2005). Extraskeletal ossification is especially detrimental when it leads to immobilization of joints and restriction of organ function. Mortality associated with FOP often results from respiratory complications due to the fusion of rib bones interfering with the function of muscles, connective tissue and nerves in the intercostal space (Kaplan and Glaser, 2005). Interestingly, for the most part clinical features are not apparent at birth other than great toe malformations, a characteristic that is invariant in classic FOP (Shore et al., 2006). As a general rule the onset of FOP is delayed until early childhood, suggesting that the disease is developmental in nature and may require other triggers. Ossification is episodic and tends to occur in association with trauma or inflammation, thus rendering surgery an ineffective treatment (Kaplan et al., 2008).

Patients with the classic FOP mutation are defined by a 617G>A mutation in one copy of their *ALK2* gene resulting in a histidine substitution at arginine 206 (R206H) (Shore et al., 2006), however, a small but growing list of variant mutations in other domains of the receptor are being identified (Billings et al., 2008; Furuya et al., 2008; Bocciardi et al., 2009; Petrie et al., 2009; Ohte et al., 2011). The classic R206H FOP mutation, which lies C-terminal to the GS domain in ALK2, leads to high levels of BMP signaling in a variety of systems (Billings et al., 2008; Shen et al., 2009; Song et al., 2010; van Dinther et al., 2010). Interestingly, the classic FOP mutation alters the identity of the amino acid just N-terminal to the conserved Thr/Gln residue that when mutated to Asp confers constitutive activity to TGF-β and BMP type I receptors (see Fig. 2A) (Wieser et al., 1995; Attisano et al., 1996; Akiyama et al., 1997; Macías-Silva et al., 1998; Chen and Massagué, 1999). Both the classic FOP mutation and the Thr/Gln to Asp mutation emphasize the importance of this region upstream of the type I receptor kinase domain as critical in controlling kinase activity.

While the mutations responsible for FOP have been identified, the molecular details that result in the hyperactive behavior of mutated ALK2/ACVR1 type I receptors are not yet fully understood. Given the multiple roles for BMP signaling in early development, high levels of sustained signaling produced by a constitutively active receptor would certainly affect embryogenesis and result in lethality. Instead, the episodic nature of FOP, as well as the long latency or quiescent period prior to heterotopic bone formation in patients, indicates that the hyperactivity of the mutant receptor must be unleashed. This could occur through a conformational change in the $ALK2^{FOP}$ protein or through a change in its interaction with *trans*-acting factors that then enable an increase in overall signaling. As such, studies to elucidate the mechanism of ALK2/ACVR1 receptor activation and its function within an organismal context will most certainly advance our understanding of FOP. Finally, identifying the molecular events that are responsible for FOP-induced hyperactive BMP signaling will open up avenues for potential therapeutic approaches.

Drosophila has proven to be an outstanding model organism to study an increasing number of human diseases based on the high degree of molecular and functional conservation observed for genes known to be involved in both the signaling pathways and regulatory mechanisms governing development and homeostasis (Veraksa et al., 2000; Reiter and Bier, 2002; O'Kane, 2003; Bier, 2005; Botas, 2007; Chintapalli et al., 2007; Pandey and Nichols, 2011). *Drosophila* signaling components are largely non-redundant which circumvents the potential difficulty in interpreting pathway manipulations made in vertebrate systems where two or more closely related proteins may exhibit functional redundancy. As such, the initial identification of the core TGF-β/BMP signaling components benefited from the genetically tractable *Drosophila* system (Sekelsky et al., 1995; Newfeld et al., 1996; Zhang et al., 1996; Botas, 2007) as have many subsequent studies investigating their developmental roles and mode of action. For example, the role for BMP signaling in wing development has been particularly well-characterized from its importance in wing disc growth and establishing the overall pattern of longitudinal provein and intervein tissue, to its role in the actual differentiation of vein and intervein tissues during pupal development (Rogulja and Irvine, 2005; Bangi and Wharton, 2006a; O'Connor et al., 2006; Affolter and Basler, 2007; Blair, 2007; Rogulja et al., 2008; Oh and Irvine, 2011; Schwank et al., 2011; Wartlick et al., 2011a; Wartlick et al., 2011b). In the larva, a gradient of BMP signaling activity is generated across the wing primordia of the wing imaginal disc, through the action of two *Drosophila* BMP ligands, Dpp and Gbb, and their interactions with the type I receptors, Sax and Tkv, and the type II receptor, Punt. The resulting phospho-Mad (pMad) gradient reflects the output of BMP signaling, critical for regulating growth, cell survival, and cell fate specification through its transcriptional targets.

The high degree of functional conservation between the *Drosophila* and vertebrate BMP signaling pathway components is underscored by the interchangeability of their respective signaling components at each level of the pathway. For instance, *dpp* mutant phenotypes in *Drosophila* can be rescued by human BMP2 and BMP4, while BMP5, 6 & 7 can apparently rescue *gbb* lethality (Padgett et al., 1993; Fritsch et al., 2010). In mammalian cell culture, both Dpp and Gbb are capable of inducing bone formation (Sampath et al., 1993). At the receptor level, both of the *Drosophila* type I receptors Tkv and Sax can bind human BMP2 in combination with exogenous DAF-4, a *C. elegans* BMP type II receptor (Brummel et al., 1994; Penton et al., 1994). Furthermore, Smads can function in heterologous systems, as *Drosophila* Mad is able to direct the induction of ventral mesoderm in response to *Xenopus* BMP4 in *Xenopus* animal caps (Newfeld et al., 1996).

Previous work from our lab has demonstrated that Sax, the *Drosophila* ALK2 orthologue, has a dual function in its ability to both promote and to antagonize signaling (Bangi and Wharton, 2006b). Based on the ability of Sax to suppress wing phenotypes associated with *gbb* or *dpp* overexpression and the reduction of endogenous *sax* function to enhance these phenotypes, we hypothesized that Sax antagonizes BMP signaling by directly binding, in particular, its high-affinity ligand Gbb in receptor complexes that are not competent to transduce a signal. Consistent with this proposal, we find that Sax can block Gbb-induced signaling in a quantitative, cell-based signaling assay (Bangi and Wharton, 2006b). The ability of Sax to antagonize BMP signaling alone is borne out by the fact that the other type I receptor Tkv enhances rather than inhibits signaling induced by either Gbb or its high-affinity ligand Dpp. The ability of type I receptors from other organisms to antagonize BMP signaling has not been investigated, although recent studies suggest that ALK2 is able to inhibit activin signaling in MA-10 cells and inhibit BMP6-induced signaling in COS cells (Renlund et al., 2007; van Dinther et al., 2010). These reports coupled with the evolutionary relatedness of ALK2 and Sax raises the possibility that ALK2, like Sax, may have the ability to inhibit BMP signaling.

Here we report on a series of studies that investigated the use of *Drosophila* as a model to assess the consequences of, as well as the molecular factors required for hyperactive kinase activity associated with FOP mutant receptors. Our findings reveal that the ALK2^{R206H} mutant receptor functions as a ligand-independent hyperactive type I receptor both *in vivo* and in *Drosophila* cell culture. *Drosophila* components are able to mediate the hyperactive signaling of the mutant human receptor and importantly, our findings have contributed to the mechanistic understanding of how defective FOP receptors signal by revealing that the type II receptor is a critical, molecular determinant required for ALK2^{R206H} mutant receptor signaling. Additionally, we investigated the functional similarities between Sax and ALK2 and found that wild-type ALK2 is also able to block BMP signaling but it achieves this inhibition in a manner different from that employed by Sax. These results provide an important advance in our understanding of both the molecular events required for hyperactive signaling by a FOP mutant receptor and the wild-type behavior of the ALK2/ACVR1 receptor. Moreover, these studies provide a new tool for future investigations of the mechanistic attributes and the triggers responsible for activating FOP mutant receptors.

Results

FOP mutant receptor ALK2^{R206H} stimulates increased BMP signaling in *Drosophila*

In order to test the ability of the human ALK2^{R206H} classical FOP mutant receptor to signal in *Drosophila,* we generated transgenic lines that allowed us to control the expression of ALK2^{R206H} in a tissue-specific manner (Brand and Perrimon, 1993) and assayed for the ability of ALK2^{R206H} to induce BMP signaling in the developing wing. When *UAS-ALK2^{R206H}* was expressed under the control of *ap-GAL4* or *A9-GAL4*, lines that primarily

express the transcriptional activator Gal4 in the dorsal compartment of the wing imaginal disc, adults were obtained, albeit unable to fully emerge from the pupal case. The wings from these individuals were misshapen and marked by ectopic veins (Fig. 1B; data not shown). In larvae of the same genotype we found a high level of pMad throughout the dorsal compartment of the wing imaginal disc compared to endogenous levels of pMad (Fig. 1C ′,D). The ectopic pMad observed in these discs indicate that ALK2^{R206H} is able to stimulate BMP signaling in *Drosophila* imaginal disc tissues, presumably through the direct phosphorylation of the *Drosophila* Smad1/5/8 orthologue Mad by its own kinase activity (Fig. 1D). As expected from the known role of BMP signaling in tissue growth (Capdevila and Guerrero, 1994; Haerry et al., 1998; Rogulja and Irvine, 2005; Affolter and Basler, 2007), we observed an increase in the size of the dorsal compartment of *ap-GAL4>ALK2^{R206H}* wing discs (Fig. 1D) also evident in the adult as downwardly curved wings resulting from the enlargement of the dorsal surface. In a separate, quantitative cell-based BMP signaling assay, we obtained similar results as our *in vivo* studies indicating that ALK2^{R206H} can induce an increase in BMP signaling (Fig. 1F). This cell-based assay makes use of a *lacZ* reporter construct under the control of the *brinker* silencer element (*brkS*) which is known to quantitatively repress transcription in response to Mad-mediated signaling (Müller et al., 2003; Bangi and Wharton, 2006b; Twombly et al., 2009). S2 cells transfected with a plasmid construct encoding the *Drosophila* ligand Gbb, exhibited a reduction in β-gal activity reflecting the repression of *lacZ* transcription as a result of an increase in BMP signaling (Fig. 1F). Cells transfected with a construct encoding the FOP mutant receptor ALK2^{R206H} showed very high levels of BMP signaling (Fig. 1F).

Extracellular ligand binding domain is not required for hyperactivity of ALK2^{R206H}

The wild-type ALK2 receptor has been shown to promote Müllerian-inhibiting substance (MIS)-dependent signaling in mammalian systems (Clarke et al., 2001; Visser et al., 2001) and to bind the vertebrate ligands, Activin and BMP7 (Attisano et al., 1993; ten Dijke et al., 1994). However, ALK2^{R206H} has been reported to signal independently of BMP ligands in zebrafish embryos and mammalian cells (Billings et al., 2008; Fukuda et al., 2009; Shen et al., 2009). In *Drosophila,* orthologues are evident for both BMP ligand subfamilies (BMP2/4 = Dpp and BMP5/6/7 = Gbb) and for the TGF-β/Activin subfamily (Daw, Actβ, Myo, Mav) (Kutty et al., 1998; Lo and Frasch, 1999; Nguyen et al., 2000; Parker et al., 2004; Parker et al., 2006; Moustakas and Heldin, 2009). Given ALK2's promiscuity in binding ligands from different TGF-β families, we generated an ALK2^{R206H} receptor that lacked the cysteine-rich (C38-C99), ligand-binding domain (LBD) to definitively test for the ability of ALK2^{R206H} to signal independently of a specific ligand in the *Drosophila* system. By deleting the LBD of ALK2^{R206H}, the mutated receptorwould be precluded from binding any TGF-β/BMP ligands (Fig. 1E). Cells transfected with the ALK2$^{\Delta LBD\text{-}R206H}$ construct were able to induce BMP signaling at comparable levels to that achieved by the full-length ALK2^{R206H} receptor (Fig. 1F), indicating that the signaling activity of the FOP mutant receptor is not only ligand-independent but that its ability to promote BMP signaling does not even require the presence a large portion of its extracellular domain (ECD) (Fig. 1E).

Type II receptor is required for hyperactivity of ALK2^{R206H}

Given the proximity of the R206H FOP mutation to the GS domain, we questioned whether the hyperactivity of ALK2^{R206H} was in fact dependent on the phosphorylation of its GS domain by the type II receptor as is typical during type I receptor activation, or if the R206H mutant receptor could induce signaling independently of the type II receptor as had been observed for the constitutively activating mutation at the adjacent residue in the TGF-β type I receptor (TβR1^{T204D}) (Wieser et al., 1995; Macías-Silva et al., 1998). The importance of trans-phosphorylation of the ALK2^{R206H} GS domain by the type II receptor kinase was tested by mutating GS domain Ser/Thr residues to Ala and assaying the mutated constructs

for signaling activity (Fig. 2A,B). Both ALK2$^{GS1\text{-}R206H}$ (three Ser mutated to Ala) and ALK2$^{GS2\text{-}R206H}$ (all three Ser and the Thr mutated to Ala) resulted in the abrogation of signaling activity (Fig. 2B) indicating that the identity of these residues as Ser or Thr is critical for signaling, thus suggesting that their phosphorylation is required for the hyperactive signaling of the ALK2^{R206H} receptor.

To test more rigorously the importance of type II receptor function for ALK2^{R206H} hyperactivity, we made use of a UAS-*put* RNAi construct to knock down the expression of the *Drosophila* type II receptor, Punt, *in vivo*. Directed expression of *put* RNAi to the dorsal compartment of the wing imaginal disc using *ap-GAL4* resulted in a dramatic loss of pMad (Fig. 2C) consistent with the requirement for *put* in BMP signaling (Letsou et al., 1995; Ruberte et al., 1995). The elevated levels of pMad induced by expression of *ALK2^{R206H}* in the dorsal wing compartment (Fig. 2D) was largely suppressed when *put RNAi* was coexpressed (Fig. 2E) demonstrating that the activity of ALK2^{R206H} is dependent on the presence of type II receptor function *in vivo*.

We next tested if the activity of ALK2^{R206H} depends on a specific type II receptor. Given that knocking down endogenous Punt completely suppressed the elevated pMad levels in wing discs induced by ALK2^{R206H}, we tested for the ability of the other *Drosophila* type II receptor, Wit, to restore hyperactive signaling in this experimental context. Indeed, we found that the expression of *wit-HA* with *ALK2^{R206H}* and *put* RNAi led to elevated pMad indicating that the ability of ALK2^{R206H} to signal is not dependent on a specific type II receptor (Fig. S1). The ability to restore signaling by ALK2^{R206H} with the expression of Wit is consistent with our finding that ALK2^{R206H} signaling requires the presence of a type II receptor.

The QD activating mutation in BMP type I receptors is dependent on type II receptor function

Previous reports have shown that in all members of the TGF-β/BMP family of type I receptors, mutation of the conserved Thr/Gln residue neighboring R206 (in ALK2) to Asp results in constitutive signaling (Wieser et al., 1995; Attisano et al., 1996; Akiyama et al., 1997; Macías-Silva et al., 1998; Chen and Massagué, 1999). The constitutive activity associated with TβR1^{T204D} has been described as TβRII-independent (Wieser et al., 1995). However, as shown above, we found that the presence of a type II receptor is absolutely required for the signaling hyperactivity associated with ALK2^{R206H}. These somewhat conflicting observations led us to question whether activating mutations in other type I receptors have a requirement for type II receptors. Indeed, we found that unlike TβR1^{T204D}, constitutive signaling produced by the *Drosophila* BMP type I receptor, Tkv, carrying the equivalent mutation (Gln to Asp; Tkv^{QD}) is type II receptor-dependent. The high levels of pMad induced by Tkv^{QD} are suppressed by knocking down Punt with *put* RNAi (Fig. 2F,G). These data indicate an inherent difference in how TβR1 responds to an activating mutation compared to how the BMP type I receptor, Tkv, does. It is not yet clear whether this reflects a fundamental difference in the mechanism used by TGF-β and BMP type I receptors as it remains a possibility that TβR1 in the previous studies could have interacted with BMP or Activin type II receptors (Wieser et al., 1995; Chen et al., 1997).

ALK2 can inhibit BMP signaling

Clearly the FOP mutant receptor, ALK2^{R206H}, exhibits high levels of signaling in *Drosophila* when expressed *in vivo* as well as in cell culture (Fig. 1, 2). We have previously shown that the *Drosophila* ALK2 orthologue, Sax, exhibits a dual function in its transduction of BMP signals (Bangi and Wharton, 2006b). Given this ability of Sax to both antagonize and mediate signaling, we considered the possibility that the hyperactivating

ALK2 mutations associated with FOP may actually mask a normal dual function for ALK2. To test for the ability of wild-type ALK2 to inhibit BMP signaling we first compared the *in vivo* overexpression of wild-type ALK2 to that of Sax, under conditions known to reveal the inhibitory function of Sax (Fig. 3A,B). In both cases, we observed a loss or thinning of longitudinal vein 5 (L5), a phenotype associated with a loss of *gbb* function, as well as a reduction in the overall size of the wing, an indication of reduced BMP signaling (Wharton et al., 1999). In general, the ectopic expression of *ALK2* produced more severe phenotypes than *sax* including a reduction in wing size and the additional loss of L4 (Fig. 3A, B). Similarly, a more dramatic loss of pMad is observed in the dorsal compartment of the wing pouch in wing imaginal discs when ALK2 is overexpressed compared to the narrowing of pMad distribution in discs from overexpressing Sax (Fig. 3C–D′). Taken together these results indicate that like overexpression of Sax, the overexpression of ALK2 leads to an effective reduction in BMP signaling.

We and others have observed that Sax binds Gbb more effectively than Dpp, and as such we found that Sax can suppress the wing phenotypes produced by overexpression of Gbb better than those produced by the overexpression of Dpp (Haerry et al., 1998; Haerry, 2010). ALK2 has been shown to bind BMP7 but not BMP4 (ten Dijke et al., 1994; Macías-Silva et al., 1998; Greenwald et al., 2003) and given the evolutionary relatedness of Gbb and Dpp to BMP7 and BMP4, respectively (Sampath et al., 1993; Fritsch et al., 2010), we hypothesized that ALK2 would be able to effectively inhibit Gbb-induced BMP signaling. As observed previously, *A9-GAL4>UAS-gbb* resulted in an array of wing phenotypes marked by ectopic vein material, indicative of an increase in BMP signaling. The distribution of wing phenotypes can be shifted toward the less severe phenotypic classes when *sax* is coexpressed (Bangi and Wharton, 2006b) (Fig. 4A), consistent with the ability of Sax to antagonize BMP signaling and suppress the ectopic Gbb signaling. In a second set of experiments we made use of this phenotypic assay to test for the ability of *ALK2* to antagonize signaling. We found that not only did coexpressing *ALK2* with *gbb* suppress wing defects associated with ectopic Gbb signaling but that all *A9-GAL4>UAS-ALK2; UAS-gbb* wings exhibited phenotypes consistent with a reduction in endogenous BMP signaling, such as a reduction in wing size and a loss of longitudinal vein material (class 6) (Fig. 4A). An examination of pMad distribution in the wing disc confirmed this conclusion in that not only was the ectopic expression of pMad induced by *gbb* overexpression eliminated, but pMad associated with endogenous BMP signaling was also been dramatically reduced (Fig. 4B).

The ability of ALK2 to inhibit BMP signaling was also tested in the quantitative, cell-based BMP signaling assay. Cotransfection of either *sax* or *ALK2* with *gbb* resulted in a suppression of Gbb-induced signaling, indicating that both receptors were capable of inhibiting signaling (Fig. 4C). Interestingly, we found that signaling induced by transfections with either *dpp* or human *BMP4* was also inhibited by ALK2 (Fig. 4D) while signaling induced by transfection of mouse *BMP7* (see Fig. S2) was enhanced by ALK2, indicating that the ability of ALK2 to inhibit or promote BMP signaling is ligand-specific (Fig. 4D,E).

Inhibition by ALK2 is ligand-independent

The ability of ALK2 to inhibit signaling induced by BMP4, a ligand it does not bind, coupled with its ability to enhance signaling induced by BMP7, a ligand that it does bind, raises the possibility that ALK2 may only inhibit signaling induced by BMP ligands that ALK2 itself does not bind. We investigated this possibility by testing the ability of ALK2 to bind Gbb and Dpp by co-immunoprecipitation. We were not able to detect an interaction between ALK2 and either Gbb or Dpp (Fig. 5A, lane 4 & Fig. 5B, lane 13, respectively) while the expected association between Gbb and Sax (Fig. 5A, lane 2) was apparent, as was

a strong interaction between Dpp and Tkv (Fig. 5B, lane 11) with no to little interaction between Dpp and Sax (lane 12).

It is possible that the affinity of ALK2 for the *Drosophila* BMP ligands was below the detectable limit of co-immunoprecipitations. Therefore, to more rigorously test for the importance of ALK2-ligand interactions, we deleted the cysteine-rich region of the extracellular (C38-C99) ligand-binding domain of ALK2 ($ALK2^{\Delta LBD}$) (Fig. 5C) and tested for the ability of this mutated receptor to block signaling. Interestingly, we found that $ALK2^{\Delta LBD}$ was able to effectively block Gbb induced signaling in S2 cells (Fig. 5D), indicating that the ability of ALK2 to block BMP signaling is independent of a direct interaction with ligand and for that matter independent of a large portion of its extracellular domain. Thus, ALK2 must inhibit BMP signaling by some mechanism other than ligand sequestration.

DISCUSSION

The BMP signaling pathway exhibits a high degree of conservation throughout the metazoans (Newfeld et al., 1999). Consistent with this, we found that when the mutant form of the human ALK2 type I receptor ($ALK2^{R206H}$), which is associated with the vast majority of fibrodysplasia ossificans progressiva (FOP) cases, (Shore et al., 2006; Billings et al., 2008) is expressed in *Drosophila* we mimic the misregulation of BMP signaling previously described in vertebrate systems (Billings et al., 2008; Fukuda et al., 2009; Shen et al., 2009; Song et al., 2010; van Dinther et al., 2010). Our results provide clear evidence that the *Drosophila* BMP signaling components are compatible with the human ALK2 type I receptor, such that the classic FOP mutation $ALK2^{R206H}$ manifests as hyperactive BMP signaling in *Drosophila* tissues as well. This finding bodes well for the use of the *Drosophila* system as a future tool to elucidate the molecular details responsible for misregulated BMP signaling associated with FOP despite the obvious differences in the ultimate consequence of this hyperactive signaling in *Drosophila* versus the formation of heterotopic bone in mammals.

Hyperactive BMP signaling requires type II receptor function

As in mammalian cells, we found that in *Drosophila,* $ALK2^{R206H}$ is able to induce high levels of phosphorylated Mad, the *Drosophila* Smad1/5/8 orthologue, in the absence of ligand. The ability of $ALK2^{R206H}$ to induce high levels of pMad is abrogated when the activation domain (GS domain) of the ALK2 receptor is mutated, suggesting that the kinase of $ALK2^{R206H}$ is directly responsible for phosphorylating Mad. While it remains formally possible in the various *Drosophila* assay systems we have tested that the endogenous *Drosophila* type I receptors Sax or Tkv are instead responsible for Mad phosphorylation in response to $ALK2^{R206H}$ expression, the level of these receptors should be far lower than $ALK2^{R206H}$ and the dependency of the pMad increase on an intact ALK2 GS domain makes this possibility less likely. Importantly, we found that the hyperactivity of $ALK2^{R206H}$ to signal is completely dependent on the function of a type II receptor kinase, which is responsible for activating type I receptors at their GS domain (Fig. 6A). The dependency of hyperactive signaling by $ALK2^{R206H}$ on the function of a type II receptor had not previously been appreciated in studies of FOP, and the fact that $ALK2^{R206H}$ can signal independently of ligand but requires type II receptor function indicates that $ALK2^{R206H}$ must be able to associate with type II receptors independently of ligand. Taken together, we envision a model in which the classic FOP mutation exposes the serine/threonine residues in the GS domain to phosphorylation by the type II receptor, thus circumventing the requirement for ligand to activate signaling (Fig. 6A).

Interestingly, our results show that the constitutively active BMP type I receptor Tkv^{QD} also shows a dependency for type II receptor function (Fig. 2G). This result is in contrast to that previously shown for the constitutively active $T\beta R1^{T204D}$ which signals in the absence of the TGF-β type II receptor, TβR-II (Wieser et al., 1995; Chen et al., 1997). While it has not yet been definitively shown that $T\beta R1^{T204D}$ signals independently of BMP or Activin type II receptors, these apparently conflicting data could reflect a fundamental difference in either the requirement for type II receptors or for the interaction of type I and type II receptors in BMP versus TGF-β signaling. Other key distinctions between TGF-β and BMP receptor signaling have been previously noted. For example, structural studies have shown that the assembly of BMP and TGF-β receptor complexes differ in that the extracellular domains of the BMP type I and II receptors do not contact one another, while an N-terminal extension in the extracellular domain of TGF-β type II receptors has been shown to directly interact with the type I receptor (Kirsch et al., 2000; Allendorph et al., 2006; Groppe et al., 2008). In addition, the minimal receptor complex required for BMP versus TGF-β signaling appears to differ. While a heterotrimeric (type I:type II:type II) BMP receptor complex is minimally required to transduce BMP signals (Isaacs et al., 2010), autonomously functioning TβRI:TβRII (type I:type II) heterodimers have been shown to be sufficient for the transduction of TGF-β signals (Huang et al., 2011).

In addition to divergent type II receptor requirements, mutations that confer hyperactivity or constitutive activity to TGF-β/BMP type I receptors differ in their respective effect on binding of the intracellular inhibitor FKBP12 to the type I receptor. FKBP12 has been proposed to prevent “leaky” ligand-independent signaling by masking the GS domain, until which time ligand binding results in its dissociation and signaling ensues (Chen et al., 1997; Huse et al., 1999; Huse et al., 2001; Wang and Donahoe, 2004). In a number of experiments, it has been shown that the R206H mutation reduces binding of FKBP12 making this an attractive molecular explanation for the hyperactivity displayed by $ALK2^{R206H}$ (Groppe et al., 2007; Shen et al., 2009; Song et al., 2010; Groppe et al., 2011). Curiously, the constitutively active Q207D mutation in ALK2 does not disrupt binding of FKBP12, whereas the equivalent constitutively active mutation in TβR1(T204D) does (Chen et al., 1997). In the case of the *Drosophila* FKBP12 orthologue FKBP2, our preliminary studies indicate that the loss of FKBP2 function *in vivo* did not produce phenotypes consistent with a substantial increase in BMP signaling (V. Le, S. Ballard, data not shown). Taken together, there does not appear to be a clear correlation between a loss or disruption of FKBP12 binding and the hyperactivity of mutant type I receptors. While we do not yet understand the mechanisms underlying the differential association of FKBP12 to $ALK2^{R026H}$ versus $ALK2^{Q207D}$, such differences raise the possibility that *in vivo,* the constitutively active $ALK2^{Q207D}$ receptor behaves differently from the $ALK2^{R206H}$ FOP mutant receptor.

The finding that $ALK2^{R206H}$ hyperactive signaling depends on type II receptor function provides a new angle in the search for FOP therapeutics. Current strategies for drug development have focused on identifying small molecule inhibitors of type I receptor kinase activity (Yu et al., 2008a; Yu et al., 2008b; Hao et al., 2010). One such inhibitor, dorsomorphin, has been shown to effectively inhibit $ALK2^{R206H}$ kinase activity (Yu et al., 2008a; Fukuda et al., 2009; Shen et al., 2009; van Dinther et al., 2010), but unfortunately dorsomorphin non-specifically inhibits the kinase activity of other BMP type I receptors and appears to exhibit “off-target” effects on VEGF signaling (Yu et al., 2008b; Hao et al., 2010). In addition to future efforts to improve the selectivity of dorsomorphin analogs (Hao et al., 2010), alternative approaches that disrupt FOP-induced signaling are needed. An exciting new prospect for drug development could exploit our recently identified type II receptor requirement for $ALK2^{R206H}$ hyperactivity by focusing on the identification of molecules or factors that specifically block the interaction between $ALK2^{R206H}$ and type II receptors in FOP cells.

The wild-type ALK2 receptor can inhibit BMP signaling

In addition to our studies of $ALK2^{R206H}$ in the *Drosophila* system, we also analyzed the ability of wild-type ALK2 to mediate signaling. Since FOP is a dominant autosomal disease and all mutations isolated thus far are protein coding point mutations, the FOP mutant receptors must always be expressed in the presence of wild-type ALK2 receptor. Therefore, in order to understand the mechanistic underpinnings of FOP it is critical that we have a full understanding of wild-type ALK2 receptor function in addition to elucidating the consequences of the R206H mutation. Thus, we investigated the possibility that ALK2 can both promote and antagonize signaling, a behavior we discovered is exhibited by the *Drosophila* ALK2 orthologue, Sax (Bangi and Wharton, 2006b). Our results revealed that wild-type ALK2 receptor is indeed able to inhibit BMP signaling *in vivo* as well as in *Drosophila* cell culture. Interestingly, we found that the mechanism by which ALK2 accomplishes signaling inhibition likely differs from that employed by Sax. Whereas Sax likely inhibits signaling via the incorporation of its high-affinity ligand, Gbb, into inactive complexes, ALK2 appears to inhibit signaling induced by ligands that ALK2 itself does not actually bind. We propose that ALK2 accomplishes this inhibition by interacting with a type II receptor, such as Punt, and dominantly prevents Punt's participation in a signaling complex that is activated by the binding of ligands such as Gbb, Dpp or BMP4 (Fig. 6B). A similar mechanism has been proposed to explain the negative effect of ALK2/ACVR1 on signaling induced by Activin which acts through a different set of core signaling components (Renlund et al., 2007). While specific binding of BMP6 to ALK2 has not been reported, ALK2 has also been observed to inhibit BMP6 induced signaling (van Dinther et al., 2010). However, in the case of BMP7 ligand, which has been shown to bind ALK2 (ten Dijke et al., 1994; Greenwald et al., 2003), Punt would not be excluded from signaling complexes, but rather ALK2 would facilitate BMP7 binding and enhance BMP7-induced signaling (Fig. 6A). In our model, ALK2 would then act as a modifier of receptor complex activity by dictating which BMP ligand the complex can bind. In other words, we propose that ALK2 determines whether the receptor complex that a type II receptor has participated in will be an active or inactive signaling complex depending on which BMP ligand is present (Fig. 6B). Therefore, the ability of ALK2 to regulate signaling based on BMP ligand type may have a profound impact on ligand-specific responses and warrants further investigation to determine if this dual behavior of ALK2 is observed endogenously.

On a separate note, the inability of ALK2 to bind Gbb was unexpected based on the demonstrated ability of ALK2 to bind BMP7 and the evolutionary relatedness of Gbb to the BMP5/6/7 subgroup. While the conserved domains of BMP5, BMP6 and BMP7 can reportedly rescue *gbb* mutant phenotypes (Fritsch et al., 2010), our results suggest that it is unlikely that Gbb can fully substitute for BMP7 function in vertebrates, specifically for BMP7-induced signaling mediated through ALK2.

Impact of *Drosophila* models for the study of FOP

Perhaps the least well understood aspect of FOP and most difficult for patients, is the sporadic and progressive nature of the disease. One of the primary difficulties still facing the FOP field is reconciling the molecular events of hyperactive signaling induced by the FOP receptor in animal models with the clinical features that manifest in patients. The sporadic nature of the disease contrasts with the hyperactivity that the mutant receptor displays in experimental assays suggesting that under endogenous conditions the activity of the mutant receptor must be regulated or muted until an event triggers a flare-up.

To date, all FOP patients are heterozygous for mutations in ALK2 regardless of whether they harbor the classic R206H or an atypical mutation. It is possible that one copy of $ALK2^{WT}$ can compete with $ALK2^{FOP}$ receptors for type II availability thereby keeping final

output of BMP signaling below a threshold required for bone formation. Therefore, in an endogenous context the relative ratio between FOP type I receptors, wild-type type I receptors, and type II receptors may be a key determinant in whether or not activation of the pathway reaches a threshold necessary for bone formation. Alternatively, at physiological levels, the FOP mutant receptor activity might be inhibited by a different factor in *trans* and only when the mutant receptor is overexpressed under experimental conditions does it escape this negative regulation. Thus, in the future it will be important to study the behavior of the FOP mutant receptors when expressed at physiologically relevant levels achievable through homologous recombination. Making use of the *Drosophila* model system to express both mutant and wild-type receptors at endogenous levels will enable *in vivo* mutagenic screens to identify factors that suppress or enhance the effects of the $ALK2^{R206H}$ activity and in turn provide us with new targets for therapy and treatment. Furthermore, given the correlation between ossification and trauma, it has been suggested that inflammation associated with injury may in some way "trigger" heterotopic ossification. The specific molecule(s) associated with such triggers could act to increase the accessibility of the GS domain to the kinase activity of the type II receptor by disrupting the binding of a putative inhibitor, for example, or they could influence the ability of the $ALK2^{R206H}$ receptor to interact with available type II receptors (Fig. 6C). Although the precise mechanism(s) by which such putative modulators may influence the behavior of $ALK2^{R206H}$ remains unknown, the *Drosophila* system is a particularly attractive model organism in which to undertake such studies given the high conservation of pathways governing cellular physiology.

In closing, our work has demonstrated the value of using a *Drosophila* genetic system to study the molecular foundation of altered BMP signaling characteristic of individuals with FOP. Our experiments reveal a requirement for type II receptor function in the hyperactivity displayed by the $ALK2^{R206H}$ mutant receptor, a fact previously unappreciated. In addition, we have also observed the ability of the wild-type ALK2 receptor to inhibit BMP signaling in a ligand-specific manner. How these findings contribute to the sporadic nature of FOP as well as potentially impact our broad understanding of other diseases associated with misregulated type I receptor activity warrants further investigation. We intend to exploit the comprehensive genetic tools in *Drosophila* system to screen for potential modifiers of FOP mutant receptor activity as a means to bridge this gap.

EXPERIMENTAL PROCEDURES

Plasmid Constructs

Gateway cloning (Invitrogen) was used to clone all cDNAs into the following *Drosophila* Gateway Vectors: pTWF for GAL4-UAS driven expression in transgenic animals and the Actin5C vector pAWF (C-terminal 3xFLAG) for constitutive expression in cell culture. *ALK2 and* $ALK2^{R206H}$ cDNAs were a generous gift from Eileen Shore. *punt* cDNA was a gift from Michael O'Connor. *pAW gbb*, *pAW dppHA* and *pAW hBMP4* were constructed by Takuya Akiyama.

Ligand-binding domain deletions—Ligand-binding domain deletion mutants were generated by Quikchange Site-directed Mutagenesis (Stratagene). For $ALK2^{\Delta LBD}$, sequences corresponding to Cys35 to Cys 99 were removed using the following primers: fwd 5′-CAA CCC CAA ACT CTA CAT GAA CAG GAA CAT CAC GGC C-3′ and rev 5′-GGC CGT GAT GTT CCT GTT CAT GTA GAG TTT GGG GTT G-3′. For Sax, sequences corresponding to Cys67 to Cys148 were removed using the following primers: fwd 5′-CGC ATC CCA GAT ACA AAA ATG AGG GAG ACT TTC C-3′ and rev 5′-GGA AAG TCT CCC TCA TTT TTG TAT CTG GGA TGC G-3′.

GS domain mutants—Two sets of GS domain mutations were generated in ALK2^{R206H} (Quikchange Site-Directed Mutagenesis). ALK2 $^{GS1\text{-}R206H}$: all three serines were mutated to alanine (TSGSGSG > TAGAGAG) using the following primers: (ALK2 S190,192,194A fwd) 5′-CAG ATT TAT TGG ATC ATT CGT GTA CAG CAG GAG CTG GCG CTG GTC TTC CTT TTC TGG TAC -3′ and (ALK2 S190,192,194A rev) 5′-GTA CCA GAA AAG GAA GAC CAG CGC CAG CTC CTG CTG TAC ACG AAT GAT CCA ATA AAT CTG-3′. ALK2 $^{GS2\text{-}R206H}$: a threonine and all three serines were mutated to alanine (TSGSGSG > AAGAGAG) using the following primers: (ALK2 T189A S190,192,194A fwd) 5′-CAG ATT TAT TGG ATC ATT CGT GTG CAG CAG GAG CTG GCG CTG GTC TTC CTT TTC TGG TAC-3′ and (ALK2 T189A S190,192,194A rev) 5′-GTA CCA GAA AAG GAA GAC CAG CGC CAG CTC CTG CTG CAC ACG AAT GAT CCA ATA AAT CTG-3′.

Drosophila melanogaster strains and crosses

All fly strains were cultured using standard sucrose, yeast extract agar food at 25°C. All fly strains as described in Flybase and obtained from Bloomington Stock Center except where noted: *UAS-gbb9.1* (Khalsa et al., 1998), *A9-GAL4*, *UAS- tkv^{QD}* (Haerry et al., 1998), *UAS-wit-HA31* (Michael O'Connor). *punt RNAi* (from NIG-FLY, NIG 7904 R-2*D*). *UAS-sax-3xFLAG(1-1M-A), UAS-ALK2-3xFLAG(8-1-9M-1a),* and *UAS-ALK2^{R206H}-3xFlag(3-4F1-a)* were germline transformants derived from constructs described above.

Receptor and *gbb* overexpression

Receptors and *gbb* were overexpressed using the UAS-GAL4 system (Brand and Perrimon, 1993). *A9-GAL4* and *ap-GAL4* drivers express primarily in the dorsal compartment of the wing imaginal disc.

in vivo Gbb signaling assay

A previously described *in vivo* assay (Bangi and Wharton, 2006b) was used to test for the ability of BMP type I receptors to affect Gbb signaling. Adult wings from the following genotypes were mounted (DPX, EM Sciences) and scored: *w A9-GAL4/yw; +/+; UAS-gbb9.1/+* were compared to *w A9-GAL4/yw, UAS-sax(1-1M-A)/+; UAS-gbb9.1/+* and *w A9-GAL4/yw; UAS-ALK2(8-1-9M-1a)/+; UAS-gbb9.1.*

Immunohistochemistry

Everted third instar larvae were dissected and fixed in 4% paraformaldehyde/PBS (v/v) for 20 minutes at room temperature followed by 5 washes in PBST (0.3% Triton X-100). Fixed tissues were then incubated overnight in blocking solution (10% NGS in PBST) at 4°C. After blocking, the cuticles were incubated in primary antibody diluted in blocking solution at the following dilutions: 1:1000 anti-FLAG M2 (Sigma, F3165), 1:1000 anti-HA 3F10 (Roche) and 1:1000 PS3 (Epitomics). Tissues were then washed 5 times with PBST and incubated in secondary antibody in blocking solution at the following dilutions: 1:1000 GAM Alexa Fluor 633, 1:1000 GARt Alexa Fluor405 (in WitHA experiments), 1:1000 GARb Alexa Fluor568. Following 5 washes in PBST, wing discs were removed and mounted in 80% glycerol/0.5% N-propyl gallate. Confocal images were collected using a Zeiss LSM510 Meta confocal laser scanning microscope.

Drosophila Schneider 2 (S2) cell maintenance and Transfections

S2 cells were cultured in Shields and Sang M3 Insect Medium (Sigma S8398) pH 6.5 containing 10% Insect Medium Supplement (Sigma I7267) and 2% Fetal Bovine Serum

(F3018). Transient transfections were carried out using Effectene Transfection Reagent (Qiagen 301427).

Quantitative Cell-based BMP Signaling Assay

An adapted protocol based on a previously described assay was used to measure BMP signaling activity (Bangi and Wharton, 2006b; Müller et al., 2003; Twombly et al., 2009). This assay makes use of a reporter construct expressing *lacZ* under the control of a *Su(H)* transcriptional activation response element as well as a *brk* transcriptional silencer element (*Su(H)/brkS-lacZ*). Cotransfection of the reporter construct with plasmids encoding Su(H) and an activated form of Notch (N*) lead to *lacZ* transcription while the activation of BMP signaling leads to a repression of *lacZ* expression by virtue of the BMP-responsive *brk* silencer element. BMP signaling can thus be measured as a loss of β-galactosidase activity. 2.8×10^6 cells were cotransfected with *Su(H), N*, Su(H)/brkS-lacZ*, and *luciferase* plasmids, all under the control of the actin 5C promoter. Constructs and their concentrations used in this assay are indicated in the figure legends. Cells were harvested and lysed 3 days post-transfection and β-galactosidase activity of cleared lysate was measured using the dual luciferase assay system (Dual-Light, Applied Biosystems) and normalized to luciferase activity which served as a transfection control for each sample. The normalized value obtained from the cleared lysate of cells cotransfected with only *Su(H), N*, Su(H)/brkS-lacZ* and luciferase was set to 100%. Statistical significance was determined using two-tailed T-Test with significance value of 0.05. Epitope tagged and untagged versions of the type I receptors investigated in this study were compared for signaling activity and showed no significant difference (data not shown).

Co-immunoprecipitation

8×10^6 S2 cells were transiently transfected with 1ug total DNA at the following ratios:

Receptor-Ligand interaction—300ng pAWF type I receptor constructs and 700ng of either pAW *dppHA* or pAW *gbb*; cells were incubated at 25°C for 4 days for protein production. Cells were solubilized in 1% Triton X-100 at 4°C for 1 hour. Cleared lysate was incubated with 1ug anti-FLAG M2 (Sigma F3165) bound to 20uL of Dynabeads Protein G Dynabead (Invitrogen 100-04D) per sample at 4° for 1 hour. An aliquot of cleared lysate was saved as soluble input for western blot analysis. The beads were then washed once with one volume of Wash Buffer 1 (20mM Tris-HCl pH 7.4, 150mM NaCl, 0.2% Triton X-100), twice with one volume of Wash Buffer 2 (20mM Tris-HCl pH 7.4, 150mM NaCl), and boiled for 5 minutes in 50uL 2xSDS buffer. IP and soluble input fractions were run on 12% SDS-PAGE gels and analyzed by western blot using standard protocols. Anti-HA 3F10 (Roche) was used at 0.1ng/uL. Anti-Flag M2 (Sigma) was used at 4ng/uL, mouse anti-Gbb (gift from Guillermo Marquez) was used at a 1:1000 dilution. Secondary antibodies GAM IgG-HRP light-chain specific (Jackson) and GARat HRP (Jackson, preabsorbed) were used at a 1:10,000 dilution.

Image analysis

pMad Intensity Profiles—Intensity profiles of pMad distribution were measured by the Fiji Image Processing Package (http://fiji.sc/wiki/index.php/Fiji). The profiles shown are the average intensity plots measured in the dorsal and ventral compartments of five wing discs and aligned by the posterior and anterior peaks of pMad distribution in the ventral compartment.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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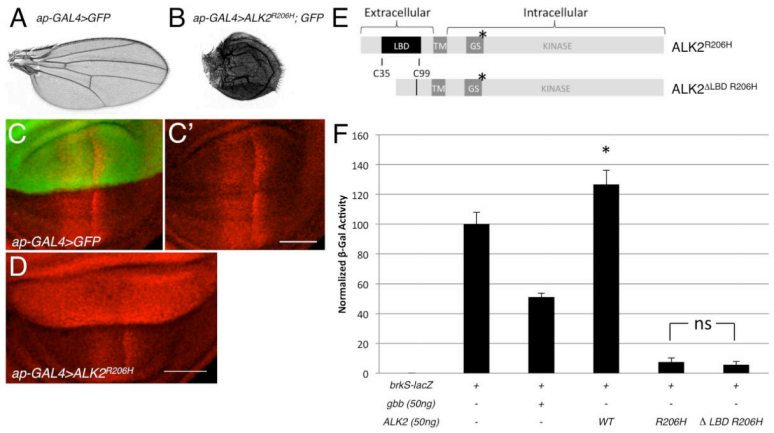

Figure 1.

In the Drosophila system the ALK2^{R206H} FOP mutant receptor stimulates BMP signaling even in the absence of its ligand binding domain. **A:** Wild-type wing from control *ap-GAL4, UAS-GFP/+* adult. **B:** Wing from *ap-GAL4, UAS-GFP/UAS-ALK2^{R206H}* adult. **C–D**: Confocal images of pMad distribution (red) in the wing pouch of third instar larval wing discs. Scale bar= 50μm. **C,C′:** A representative *ap-GAL4, UAS-GFP/+* control wing disc. The dorsal expression domain of *ap-GAL4* is marked by GFP expression (green). The ventral compartment lacks expression of GFP. **D:** A representative *ap-GAL4,UAS-GFP/ UAS-ALK2^{R206H} wing disc.* **E:** Diagram of full length ALK2^{R206H} and ligand-binding domain deletion mutant ALK2$^{\Delta LBD\ R206H}$ drawn to scale. Amino acids from Cys35 to Cys99 were removed by site-directed mutagenesis. * indicates position of the R206H mutation. **F:** Quantitative *brkS-lacZ* assay measuring BMP signaling activity of ALK2^{R206H} and ALK2$^{\Delta LBD\ R206H}$ in S2 cell culture. Data represent mean +/− standard deviation (n=4). ns = not significant, p>0.05 *brkS-lacZ +ALK2^{R206H}* versus *brkS-lacZ +ALK2$^{\Delta LBD\ R206H}$*(p=0.38). * p<0.05 versus *brkS-lacZ* transfection alone (p=0.005). We interpret this statistical significance to reflect the ability of ALK2 to inhibit endogenous BMP signaling in S2 cells. LBD = ligand binding domain. TM= transmembrane domain. GS = glycine-serine rich domain/box.

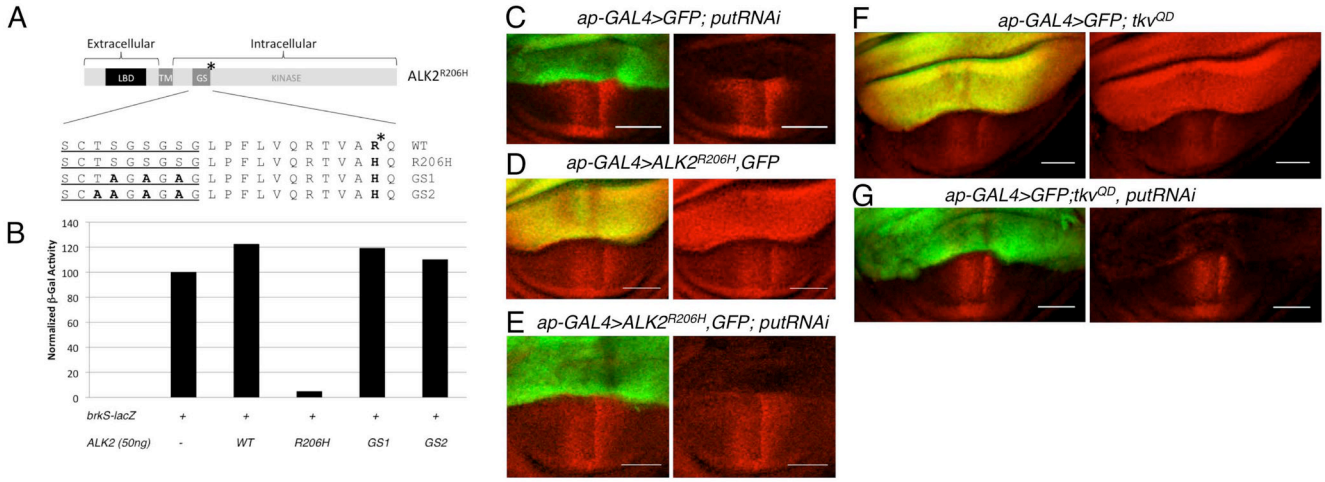

Figure 2.

Hyperactive signaling induced by ALK2^{R206H} requires BMP type II receptor function. **A:** (Top) Diagram of the full length ALK2^{R206H} receptor drawn to scale. LBD = ligand binding domain. TM= transmembrane domain. GS = glycine-serine rich domain/box. * indicates position of R206H mutation. (Below) Amino acid alignment of GS domains (from ALK2, ALK2^{R206H}, ALK2$^{GS1\text{-}R206H}$ (GS1)and ALK2$^{GS2\text{-}R206H}$ (GS2). Glycine-serine rich sequence containing serine and threonine targets of type II receptor phosphorylation is underlined. **B:** *brkS-lacZ* signaling assay indicates ALK2$^{GS1\text{-}R206H}$ (GS1)and ALK2$^{GS2\text{-}R206H}$ (GS2) lack BMP signaling activity. Data plotted are mean of two experiments performed in duplicate. **C–G:** Stimulation of BMP signaling by ALK2^{R206H} and Tkv^{QD} in the wing disc requires the type II receptor Punt. Confocal images of pMad distribution (red) in wing pouch of third larval instar wing discs, *ap-GAL4* expression domain marked by *UAS-GFP* (green). **C:** Expression of *putRNAi* in dorsal compartment leads to dramatic reduction in pMad. *ap-GAL4, UAS-GFP/+; UAS-put RNAi/+* **D:** Expression of *ALK2^{R206H}* in dorsal compartment leads to an increase in pMad (red) levels, *ap-GAL4, UAS-GFP/UAS-ALK2^{R206H}*. **E:** Coexpression of of *putRNAi* eliminates pMad increase associated with *ALK2^{R206H}* as well as endogenous pMad, *ap-GAL4,UAS-GFP/ UAS-ALK2^{R206H}; UAS-putRNAi/+*. **F:** High levels of pMad are associated with expression of *tkv^{QD}* in dorsal wing compartment, *ap-GAL4, UAS-GFP/+; UAS-tkv^{QD}/+*. **G:** Coexpression of putRNAi eliminates BMP signaling induced by *tkv^{QD}*as indicated by the loss of pMad, *ap-GAL4, UAS-GFP/+; UAS-tkv^{QD}/UAS-putRNAi*. Scale bar = 50μm.

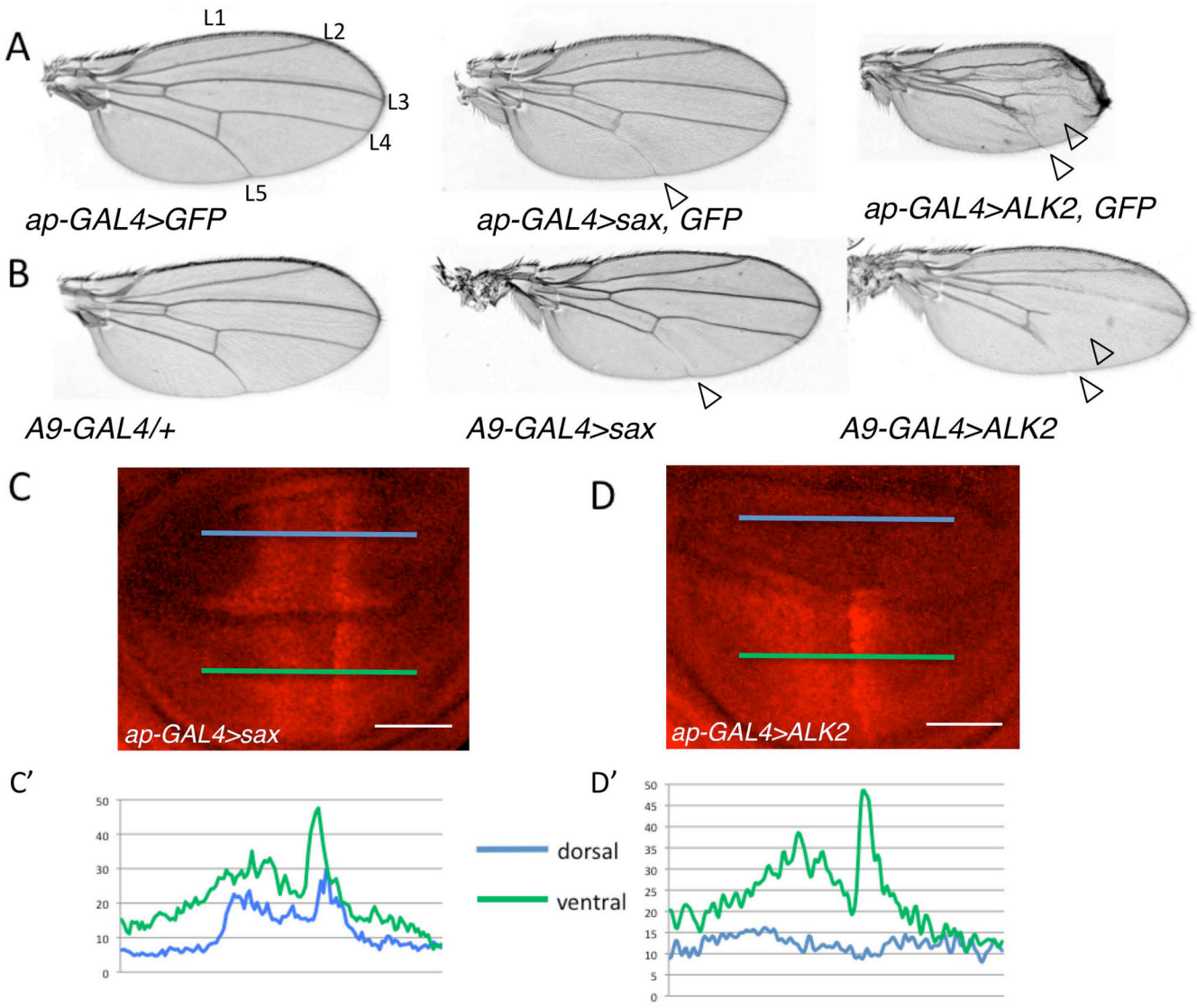

Figure 3.

ALK2 can inhibit endogenous BMP signaling. **A–B:** Expression of *ALK2*, like *sax*, leads to loss of vein tissue (open arrowheads). **A:** Adult wings from *ap-GAL4, UAS-GFP/+* (left), *ap-GAL4, UAS-GFP/UAS-sax* (middle), and *ap-GAL4, UAS-GFP/UAS-ALK2* (right). **B:** Adult wings from *A9-GAL4/+* (left), *A9-GAL4/+; UAS-sax/+* (middle) and *A9-GAL4/+; UAS-ALK2/+* (right). **C–D′:** ALK2 reduces pMad levels. **C,D:** Representative confocal images of pMad distribution (red) in wing pouch of third instar larval wing discs (C) *ap-GAL4, UAS-GFP/UAS-sax,* (D) *ap-GAL4, UAS-GFP/UAS-ALK2.* Scale bar = 50μm **C′:** Average pMad intensity profiles of the dorsal (blue line) and ventral (green line) compartments of *ap-GAL4, UAS-GFP/UAS-sax* wing discs (n=5). **D′:** Average pMad intensity profiles of the dorsal (blue line) and ventral (green line) compartments of *ap-GAL4, UAS-GFP/UAS-ALK2* wing discs (n=5).

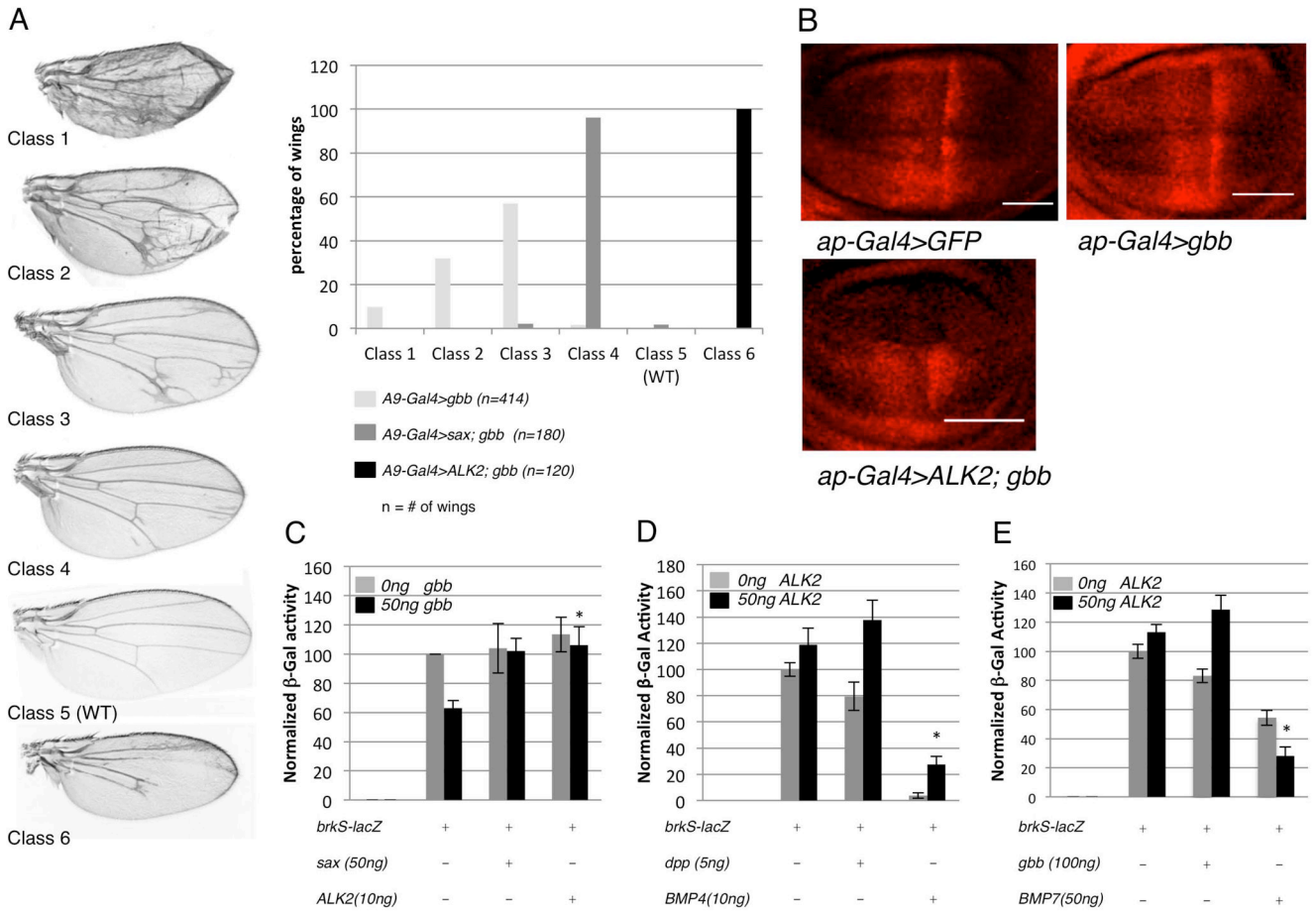

Figure 4.

ALK2 can inhibit exogenous, ligand-induced BMP signaling in a ligand-specific manner. **A:** (Left) Class 1 to Class 4: phenotypic distribution of adult wings from *A9-GAL4/+;UAS-gbb9.1/+*, Class 5: Wild-type, Class 6: Phenotype of *A9-GAL4/+; UAS-ALK2/+; UAS-gbb9.1/+* adult wings. (Right) The shift in the *gbb* overexpression phenotype associated with coexpression of either *sax* or *Alk2* suggests Gbb-induced signaling is antagonized. **B:** ALK2 can inhibit the increase in pMad (red) associated with Gbb expression in the dorsal compartment. (top left) *ap-GAL4, UAS-GFP/+* (top right) *ap-GAL4, UAS-GFP/+;UASgbb9.1*/+ (bottom left) *ap-GAL4, UAS-GFP/UAS ALK2; UASgbb9.1/+*. **C:** ALK2 can antagonize BMP signaling induced by Gbb as measured by the *brkS-lacZ* reporter assay in S2 cell culture. Data represent mean +/− standard deviation (n=4). *compared to *brkS-lacZ* + *50ng gbb* $p<0.05$ ($p=0.006$). **D:** ALK2 can antagonize BMP signaling induced by Dpp and human BMP4 (hBMP4) as measured by the *brkS-lacZ* reporter assay in S2 cell culture. Data represent mean +/− standard deviation (n=6). *compared to *brkS-lacZ* + *10ng BMP4* $p<0.05$ ($p=0.0006$). **E:** ALK2 enhances BMP signaling induced by mouse BMP7 (mBMP7) in the *brkS-lacZ* S2 cell culture assay. Data represent mean +/− standard deviation (n=3). *compared to *brkS-lacZ* + 5*0ng BMP7* $p<0.05$ ($p=0.005$).

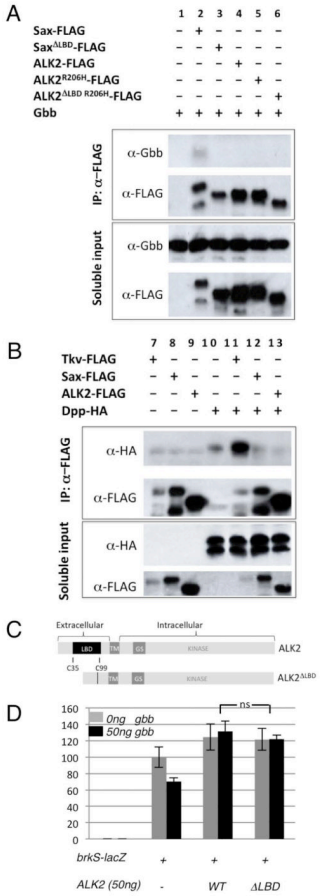

Figure 5.

ALK2 does not bind the *Drosophila* BMPs, Dpp or Gbb. **A,B:** Gbb co-immunoprecipitates with its high-affinity receptor Sax but not ALK2. Dpp-HA co-immunoprecipitates with its high-affinity receptor Tkv but not ALK2. **C:** Diagram of the full length ALK2 (LBD = ligand binding domain. TM= transmembrane domain. GS = glycine-serine rich domain/box) and ligand-binding domain deletion mutant $ALK2^{\Delta LBD}$ with amino acids from Cys35 to Cys99 removed by site-directed mutagenesis. **D:** $ALK2^{\Delta LBD}$ can inhibit Gbb-induced signaling in S2 cells. Data represent mean +/− standard deviation (n=3). ns (not significant) $p>0.05$ *brkS-lacZ* + $ALK2^{WT}$ + *50ng gbb* vs. *brkS-lacZ* + $ALK2^{\Delta LBD}$ + *50ng gbb* ($p=0.3$).

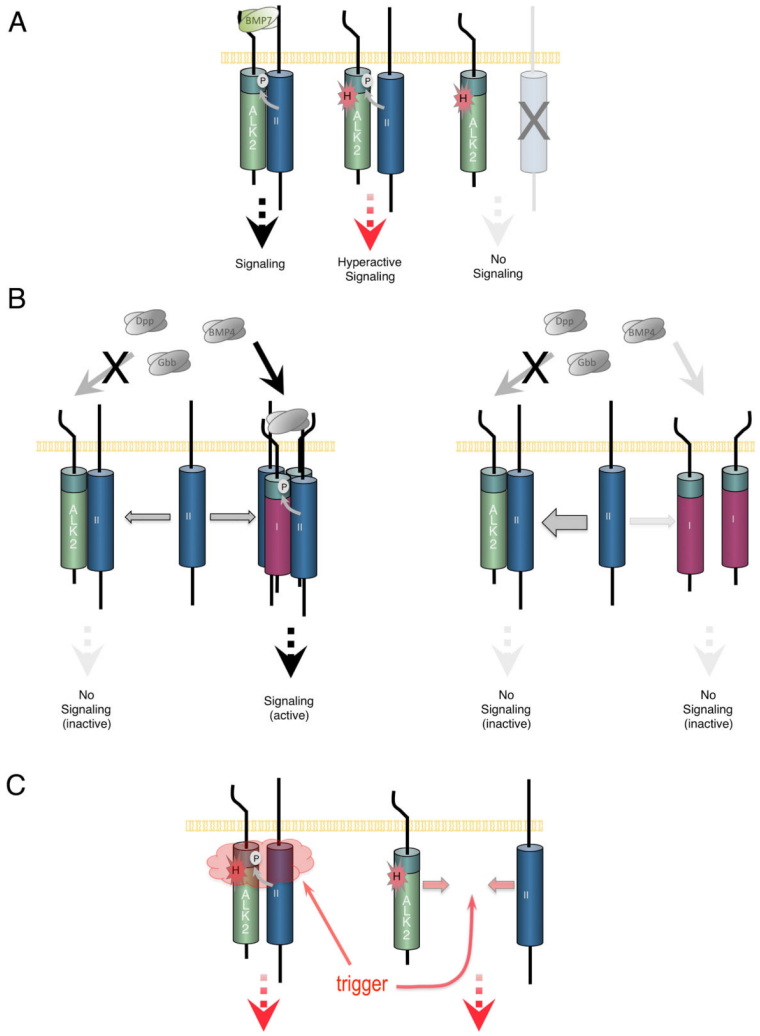

Figure 6.

Models for ALK2^{R206H} hyperactivity and ALK2 inhibition of BMP signaling. **A:** (Left) When bound by BMP7, the GS domain (green domain adjacent to membrane) of ALK2 is phosphorylated (P in white circle) by a type II receptor (blue receptor labeled “II”) leading to BMP signal transduction. (Middle) The classic R206H FOP mutation in ALK2 (H in red starburst) circumvents the ligand requirement for signaling by increasing the accessibility of ALK2’s GS domain to the type II receptor resulting in hyperactive signaling. (Right) In the absence of a functional type II receptor, ALK2^{R206H} is not activated and unable to signal. **B:** (left) While ALK2 is unable to mediate signaling by BMP4, Gbb, or Dpp, these ligands can signal through other type I receptors (purple receptor labeled “I”). (right) In the absence of BMP7 and under conditions when the type I receptor population at the cell surface is enriched for ALK2 (as is the case during experimental overexpression of ALK2), BMP signaling in general is suppressed as a result of the titration of type II receptors away from productive signaling complexes, into inactive complexes with ALK2. **C:** Various events (trigger) that may act to allow hyperactive signaling of ALK2^{R206H} could further increase GS domain accessibility by disrupting the interaction of a putative inhibitor, or could facilitate the interaction of ALK2^{R206H} with available type II receptors.