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Two highly-related regulatory subunits of PP2A exert opposite effects on TGF- β /Activin/Nodal signalling

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Summary

We identify B α (PPP2R2A) and B δ (PPP2R2D), two highly-related members of the B family of regulatory subunits of the protein phosphatase PP2A, as important modulators of TGF- β /Activin/Nodal signalling, which affect the pathway in opposite ways. Knockdown of B α in *Xenopus* embryos or mammalian tissue culture cells suppresses TGF- β /Activin/Nodal-dependent responses, whereas knockdown of B δ enhances these responses. Moreover, in *Drosophila*, overexpression of Smad2 rescues a severe wing phenotype caused by overexpression of the single *Drosophila* PP2A B subunit, Twins. We show that in vertebrates B α enhances TGF- β /Activin/Nodal signalling by stabilising the basal levels of type I receptor, whereas B δ negatively modulates these pathways by restricting receptor activity. Thus, these highly-related members of the same subfamily of PP2A regulatory subunits differentially regulate TGF- β /Activin/Nodal signalling to elicit opposing biological outcomes.

Introduction

TGF- β superfamily ligands signal through complexes of type I and type II receptors, both of which are serine/threonine kinases. Binding of a TGF- β superfamily ligand allows the type II receptor kinase to phosphorylate and activate the type I receptor kinase, which in turn phosphorylates receptor-regulated Smads or R-Smads. Phosphorylated R-Smads form complexes with Smad4, which accumulate in the nucleus and directly regulate target gene transcription (ten Dijke and Hill, 2004). The pathway consists of two distinct branches, which are defined by the type I receptors and the R-Smads that are activated. The type I receptors ALK4, ALK5 and ALK7 specifically phosphorylate Smad2 and Smad3, whereas the type I receptors ALK1, ALK2, ALK3 and ALK6 are specific for Smad1, Smad5, and

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Smad8 (Schmierer and Hill, 2007). Broadly speaking, the Activin, Nodal and TGF- β subfamilies of ligands induce phosphorylation and activation of ALK4/5/7 and thus Smad2/3, whereas ligands of the BMP subfamily activate ALK1/2/3/6 and consequently Smad1/5/8. These two branches are frequently referred to as the TGF- β /Activin/Nodal-branch and the BMP/GDF-branch, respectively (Feng and Derynck, 2005).

Receptor levels are an obvious determinant of a cell's responsiveness to TGF- β superfamily ligands and are extensively regulated. Irrespective of the absence or presence of a signal, endocytosis of receptors by a clathrin-dependent mechanism operates in parallel with a caveolin-dependent mechanism, the former recycling receptors to the membrane, the latter promoting receptor degradation through the proteasomal pathway (Di Guglielmo et al., 2003). A balance between the two is thought to determine the amount of receptors that are present at the plasma membrane and thus competent for signalling. Lysosomal degradation of ALK4 and ALK5 also occurs and is promoted by the protein Dapper2 in zebrafish (Zhang et al., 2004), and degradation of BMP receptors in *Xenopus* is promoted by a phosphatase, Dullard (Satow et al., 2006).

In addition to receptor levels, the phosphorylation status of both the receptors and the Smads is also tightly controlled. Thus, protein phosphatases have long been postulated to influence TGF- β superfamily signalling, but concrete roles have only recently started to emerge. PP1 is targeted to active receptors by signal-induced feedback to dephosphorylate and inactivate the type I receptor (Shi et al., 2004) in both mammalian cells and *Drosophila* (Bennett and Alphey, 2002). Downstream of the receptors, a number of different phosphatases have been implicated in the removal of activating phosphates from the R-Smads (Chen et al., 2006; Knockaert et al., 2006; Lin et al., 2006). Finally, PP2A, a regulatory subunit of which can be phosphorylated by ALK5, has been implicated in the TGF- β signalling pathway as a downstream effector (Griswold-Prenner et al., 1998; Petritsch et al., 2000).

Here, we report an entirely novel role for specific regulatory subunits of PP2A in modulating TGF- β /Activin/Nodal signalling at the receptor level. PP2A is a multimeric serine/threonine protein phosphatase consisting of a 36 kD catalytic subunit (PP2A_C) and a 65 kD scaffolding subunit (PR65 or A subunit) (Janssens et al., 2005). An additional regulatory subunit, of which four distinct classes exist (B, B', B'' and B'''), associates with this dimer. The B family (PR55) comprises four highly homologous, mammalian genes (*PPP2R2A*, *PPP2R2B*, *PPP2R2C* and *PPP2R2D*) with *PPP2R2A* and *PPP2R2D* being widely expressed, and *PPP2R2B* and *PPP2R2C* expression being restricted to neural tissues (Janssens and Goris, 2001; Strack et al., 1999). To date, only redundant functions of these family members have been described (Adams et al., 2005). Here we show that *PPP2R2A* (referred to throughout as B α) and *PPP2R2D* (referred to throughout as B δ) have distinct and opposing roles in the regulation of TGF- β /Activin/Nodal signalling.

Materials and Methods

Plasmids and recombinant proteins

Full length mouse B α , B δ , B' δ and *Xenopus* B δ were PCR-amplified from EST clones, sequence checked and cloned into pEF-Flag and pFTX9 (Howell et al., 2002), for N-

terminal Flag tagging. Anti *Xenopus* B α and B δ in situ hybridisation probes corresponding to nucleotides 1988-2176 and 9-194 of the full length mRNA, respectively, were generated by PCR from *Xenopus* cDNA libraries and cloned into pCR2.1 (Invitrogen). *Xbra* and *gsc* probes (Howell et al., 2002), pCS2-GFP, pFTX4K-EGFP-Smad2 and the plasmid expressing activated ALK4 (Batut et al., 2007), the plasmid expressing HA-ALK4 (Jullien and Gurdon, 2005), pCMV5-HA-ALK5 and pCMV-HA ALK5^{ca} (Nicolás and Hill, 2003) and EF-LacZ (Bardwell and Treisman, 1994) were as described. HA-Raf1 was a kind gift from Richard Marais. Recombinant full-length human Smad2 was purified from bacterial lysates as a GST fusion by affinity purification followed by thrombin cleavage. Concentration and quality of the recombinant Smad2 were checked by SDS-PAGE and Coomassie staining. *In vitro* translations using the TnT system (Promega) were performed according to manufacturer's instructions.

Morpholinos, RNA isolation, RT-PCR and q-PCR

Extraction of total mRNA from *Xenopus* embryos, reverse transcription and q-PCR were performed as described (Batut et al., 2007). RT-PCR was performed as described (Levy and Hill, 2005). For the sequences of the morpholino oligonucleotides (Gene Tools, LLC) and the oligonucleotides used for RT-PCR and q-PCR see Table S1.

Cell culture and transfection of siRNA and plasmids

HeLa TK- and HaCaT cell lines expressing EGFP-Smad2 were generated and cultured as previously described (Nicolas et al., 2004; Schmierer and Hill, 2005). TGF- β (PeproTech) was used at 2 ng/ml. Bafilomycin A1 (Calbiochem) at 10 nM, MG132 and lactacystin (Sigma) at 25 μ M. Treatment of extracts with PNGase F was as described (Dorey and Hill, 2006). Cells were transfected with plasmids using Lipofectamine 2000 (Invitrogen) or Eugene HD (Roche), and with siRNAs using Dharmafect II (Dharmacon). Pools of 4 siRNA oligos (*SMART* pools, Dharmacon) targeting B α , B δ , Dapper-2 or T β R-II were transfected at a final concentration of 75 nM. Experiments were performed 72 hours after the transfection. As controls, a *SMART* pool of non-targeting siRNAs were used. Knockdowns using the individual oligonucleotides of a *SMART* pool were performed as above at a final concentration of 75 nM. For siRNA sequences see Table S1. Knockdown efficiency was assessed by RT-PCR and/or immunoblotting.

IP phosphatase assay

As substrates, endogenous and EGFP-tagged phospho-Smad2 were immunoprecipitated from TGF- β -treated HaCaT EGFP-Smad2 cells (Batut et al., 2007; Schmierer and Hill, 2005) with an anti-Smad2/3 antibody in lysis buffer (50 mM Tris-Cl pH7.5, 100 mM NaCl, 10% glycerol, 0.1% NP40, 2 mM DTT, protease inhibitors). HA-Raf-1 was expressed in HeLa TK- cells and immunoprecipitated with anti-HA antibody. Flag-B α , Flag-B δ or Flag-B' δ were expressed in HeLa TK- cells. PP2A holocomplexes containing these subunits were purified by Flag pulldown and eluted with Flag peptide. Alternatively, complexes were purified from stable HEK T-Rex cell lines harbouring Flag-B α or FLAG-B δ in the tetracycline-inducible pcDNA5/TO construct (Adams et al., 2005) induced with 1 μ g/ml tetracycline for 48 h, and treated or not for 1 hour with TGF- β . Phosphatase activity in the eluates was routinely assessed by phosphate release from a synthetic phosphopeptide using a

colorimetric assay (Upstate). Phospho-Smad2 or HA-Raf-1 bound to protein G beads were incubated with equal phosphatase activities for 1 hr at 37°C in lysis buffer and analysed for dephosphorylation by immunoblotting.

IP kinase assay

Active, endogenous ALK5 receptor kinase was immunoprecipitated from TGF- β treated HaCaT cells lysed in lysis buffer using anti-ALK5 antibodies that had been chemically crosslinked (Harlow and Lane, 1988) to protein A beads. Kinase activity was assessed by in vitro phosphorylation of 800 ng recombinant human Smad2 protein in the presence of 5 mM ATP, 2 mM MgCl₂ and 2 mM MnCl₂ at 37°C for 90 minutes. For IP kinase phosphatase assays, purified PP2A holocomplexes containing Flag-B α , Flag-B δ or Flag-B' δ were included in the reaction mixture.

Xenopus embryo injections, in situ hybridisation and immunofluorescence

Fertilisation, culture, staging, preparation of synthetic mRNAs and microinjection of *Xenopus* embryos were performed as described (Howell et al., 2002). Activin A (R&D Systems) was used at 20 ng/ml. Okadaic acid (Calbiochem) was used at 25 nM. In vitro transcribed mRNAs were injected into *Xenopus* at 500 pg for EGFP-Smad2, 250 pg for HA-ALK4, 100 pg for activated ALK4 and 200-500 pg for B α and B δ . GFP mRNA was used as a tracer at 50 pg. Total concentrations of morpholinos were 20 ng. In all cases the injection volume was 4-5 nl. Animal caps were dissected at stage 8-9 and harvested at the indicated stages. Whole-mount in situ hybridisation and immunofluorescence were performed as described (Batut et al., 2007). Antisense probes for in situ hybridisation were labelled with digoxigenin-UTP (Roche).

Genetic Interactions in the *Drosophila* wing

The following fly strains were used:

1. *w P{GAL4}A9* (Brand and Perrimon, 1993)
2. *w; P{UAS-*tw*s.B}23* (II) (Bajpai et al., 2004)
3. *w; +/-CyO; P{UAS-*dSmad2.Z}8D3** (III) (Zheng et al., 2003)
4. *y[1] w[1118]; Sp; +/-TSTL, CyO, TM6B, Tb[1]*
5. *tw*s[60]/*TM6b*, *Tb Hu* (Uemura et al., 1993).
6. *w[67c23] P{w[+mC]=lacW}Smox[G0348]/FM7c* (Peter et al., 2002).

Flies were reared on cornmeal-agar-dextrose. Loss of function interactions were tested at 25°. *smox/FM7a* females were mated to a loss of function *tw*s strain or to *y[1] w[c67c23]*. Progeny that were *smox/Y; tw*s/+ had no difference in viability or visible phenotypes compared to control *smox/Y*. Gain of function interaction tests were performed at 22° and 25° in males, which had stronger *tw*s overexpression phenotypes. Wings were mounted in Euparal and imaged digitally using a 4 $\times$ objective. It was previously reported that larger wings result from *A9-GAL4*-driven expression of *P{UAS-Smox.A}* (Marquez et al., 2001). The genotype used here gave only a subtle increase in the average wing dimensions compared to the control genotype. Control matings to produce *w P{GAL4}A9/Y; P{UAS-*

twi.Bj23/+ were performed at 23–24°C to permit recovery of mature wings. Tests for genetic interactions between loss-of-function mutations in each gene yielded no phenotypic evidence for a trans-heterozygous interaction (data not shown).

Western blotting, immunoprecipitation, antibodies and confocal microscopy

Whole cell extracts from *Xenopus* embryos and tissue culture cells, Western blotting and immunoprecipitation procedures were as described (Batut et al., 2007; Howell et al., 1999). Confocal microscopy was performed as described (Batut et al., 2007; Schmierer and Hill, 2005). The following commercial antibodies were used: anti-phospho-Smad2 (S465/S467), anti-phospho-Smad2 (S245/S250/S255), anti-phospho-Raf-1 (S259), anti-phospho-ERK (all Cell Signaling Technology); anti-Smad2/3 (BD Biosciences Pharmingen); anti-ALK5 (v-22, Santa Cruz Biotechnology); anti-T β RII, anti-pan B subunit and anti-PP2A catalytic subunit (Upstate); anti- β -Catenin, anti-Flag, anti-Flag-HRP and anti-Flag beads (Sigma); anti-HA, anti-HA-HRP and anti-GFP (Roche); anti-tubulin (YL1/2, Abcam).

Results

We initially identified the PP2A B δ subunit as a protein whose overexpression in early *Xenopus* embryos causes loss of anterior structures. Embryos overexpressing B δ exhibited a delayed gastrulation as judged by the time of blastopore closure, and showed greatly reduced anterior structures at the tailbud stage (Fig. 1A and Table S2). In contrast, knockdown of B δ using a specific morpholino resulted in embryos with a shortened axis compared with wild type embryos and slightly larger anterior structures relative to the trunk (Fig. 1A and Table S2). The morphant phenotype was identical when two distinct morpholino oligonucleotides were used (data not shown), and was rescued by co-expression of a mouse B δ mRNA (Fig. 1B).

To understand how specific these effects were for this particular B subunit, we investigated the effects of manipulating levels of the highly homologous B α , which we found also to be expressed in early *Xenopus* embryos in a similar pattern to B δ (Fig. S1). Surprisingly, morpholino knockdown of B α resulted in a very different phenotype to the B δ knockdown. The embryos exhibited a short anterior-posterior axis, but in this case anterior structures were much reduced (Fig. 1A and Table S2). In fact, the phenotype was similar to that caused by overexpression of B δ . The effect of B α knockdown was rescued by overexpression of mouse B α (Fig. 1B). Embryos overexpressing B α were phenotypically normal (Fig. 1B), perhaps because B α levels are not limiting in the early embryo. Consistent with the distinct phenotypes resulting from knockdown of B α and B δ , overexpression of B α could not rescue B δ morphant embryos and overexpression of B δ could not rescue B α morphant embryos (Fig. S2). We thus conclude that these two regulatory B subunits have distinct functions in the early *Xenopus* embryo.

Both B α and B δ affect Activin/Nodal-dependent processes

The phenotype of B α morphant and B δ overexpressing embryos was similar to the phenotypes of various Nodal signalling mutants in fish (Schier, 2001), and also to phenotypes of fish and frog embryos in which Nodal signalling had been inhibited by the

pharmacological type I receptor inhibitor, SB-431542 (Batut et al., 2007; Ho et al., 2006; Sun et al., 2006). Conversely, the phenotype of B δ morphant embryos was consistent with increased Activin/Nodal and/or Wnt signalling (Tada et al., 2002; Whitman, 2001). To investigate this in more detail, we examined the effect of manipulating the levels of B α and B δ on the expression of the Activin/Nodal target genes *gsc* and *Xbra* (Howell et al., 2002). Both *in situ* hybridisation and quantitative PCR revealed that either overexpression of B δ or knockdown of B α greatly inhibited the expression of *gsc* and *Xbra* in early gastrula embryos (Fig. 2). In contrast, knockdown of B δ increased the expression of these genes (Fig. 2). We also analysed β -catenin localisation as a readout for Wnt activity, but found no evidence for enhanced Wnt activity in B δ morphant embryos or reduced Wnt activity in B α morphant and B δ -overexpressing embryos (see Fig. 5A). Thus, the observed phenotypes are most likely due to a modulation in the intensity of Nodal signalling, with knockdown of B δ promoting Nodal signalling and knockdown of B α or overexpression of B δ , inhibiting Nodal signalling.

To corroborate our data from whole embryos we investigated the effects of B α and B δ on Activin-dependent animal cap elongation, which is a functional readout for Activin/Nodal activity (Smith, 1993). Animal cap explants cultured in buffer alone heal into balls of ciliated epidermis, whilst those treated with Activin elongate and differentiate to form mesoderm. Activin-induced elongation of animal cap explants was completely abrogated by overexpression of B δ , but not B α (Fig. 3A). Conversely, morpholino knockdown of B δ enhanced cap elongation, whereas knockdown of B α completely abolished elongation (Fig. 3B). Importantly, the effects of B α knockdown could be rescued by overexpression of an EGFP-tagged version of Smad2, which is the intracellular mediator of the Activin/Nodal pathway (Fig. 3C). As a control, overexpression of EGFP:Smad2 had no effect on Activin-induced animal cap elongation by itself (Fig. 3C).

Altogether, these results indicate that altering the expression levels of B α or B δ in the early embryo modulates the Activin/Nodal signalling pathway. Importantly, B α and B δ affect the strength of Activin/Nodal signalling in *Xenopus* in opposite directions, with B α normally acting positively, and B δ acting negatively.

Wing phenotypes caused by overexpression of the *Drosophila* B subunit Twins can be rescued by overexpression of Smad2 and vice versa

We next extended our analysis of the role of the B family of PP2A regulatory subunits in Activin/Nodal signalling by analysing the genetic interaction between *twins* and *smox* in *Drosophila*. *Twins* is the only *Drosophila* B family member (Mayer-Jaekel et al., 1993), and *Smox* is *Drosophila* Smad2 (Henderson and Andrew, 1998), which transduces signals from the Activin type I receptor Baboon (Brummel et al., 1999; Parker et al., 2006; Serpe and O'Connor, 2006). Overexpression tests yielded a genetic interaction between *smox* and *twins*. Overexpression of *Twins* alone throughout the developing wing primordium with the UAS-Gal4 binary expression system (Brand and Perrimon, 1993) yielded very small wings with minimal venation in males, which had reduced survival (Fig. 4B); control wings that lacked the A9-Gal4 expression driver were wild type in phenotype (Fig. 4A). Overexpression of *Smox* alone yielded wings with abnormal venation ($n > 40$, Fig. 4C). When the two transgenes were co-expressed, however, 24 out of 30 wings were completely

suppressed to wild type shape and venation; 6 wings had partially suppressed phenotypes (Fig. S3). Co-expression of the Baboon A isoform with Twins also suppressed, but not as well as Smox (Fig. S3). In summary, the effects of Twins overexpression on wing size and vein structure was strongly or completely suppressed by increased levels of Smox/dSmad2 in 93% of cases, suggesting that Twins antagonises Activin/Smad2 signalling in the wing primordium. In *Drosophila* therefore, the only B subunit Twins acts similarly to B δ in *Xenopus*.

B α and B δ exert opposite effects on the levels of active Smad2

We next analysed in detail at what point in the TGF- β /Activin/Nodal pathway these phosphatase subunits acted. TGF- β /Nodal/Activin stimulation leads to C-terminal phosphorylation of Smad2 (and Smad3), which then accumulate in the nucleus (Massagué et al., 2005). At early gastrula stages endogenous Nodal signalling in *Xenopus* embryos is stronger dorsally (Lee et al., 2001) as seen by nuclear localisation of Smad2 on the dorsal, but not on the ventral side (Fig. 5A panels a and c). Knockdown of B α or overexpression of B δ suppressed this Smad2 nuclear accumulation on the dorsal side, whilst knockdown of B δ permitted Smad2 nuclear accumulation even on the ventral side (Fig. 5A, top panels). The effects were specific to Nodal signalling as levels of nuclear β -catenin, which reflect active Wnt signalling, were not similarly affected (Fig. 5A, middle panels). In animal cap explants expressing a constitutively active version of the Activin/Nodal type I receptor ALK4 to mimick Nodal signalling (Wieser et al., 1995), morpholino knockdown of B α prevented nuclear accumulation of EGFP-Smad2 (Fig. S4A). Moreover, Activin-induced nuclear accumulation of EGFP-Smad2 in animal cap explants was also abrogated by overexpression of B δ , but not B α (Fig. S4B). Taken together, these results indicate that B α and B δ act on Activin/Nodal signalling downstream of the ligands.

TGF- β /Activin/Nodal-induced nuclear accumulation of Smad2 is driven by Smad2 C-terminal phosphorylation (Massagué et al., 2005). In accordance with the observed changes in Smad2 nuclear accumulation, we found that overexpression of B δ caused a decrease in Smad2 phosphorylation in whole embryos in response to endogenous Nodal signalling, whereas overexpression of B α had no effect (Fig. 5B). The same was true in animal caps in response to exogenous Activin (Fig. S5 and data not shown). In knockdown experiments, Smad2 phosphorylation was increased by B δ knockdown and decreased by B α knockdown (Fig. 5C). The effect of B δ knockdown could be mimicked by a 1-hour treatment of animal caps with the PP2A catalytic subunit inhibitor, okadaic acid (Fig. 5D), suggesting that B δ 's action on TGF- β /Activin/Nodal signalling requires the phosphatase activity of PP2A (see also Discussion). Previous work has suggested that B family members have cell type specific effects on the ERK MAPK pathway (Adams et al., 2005; Strack, 2002; Van Kanegan et al., 2005). However in *Xenopus* embryos, knockdown of B δ or B α or their overexpression had no effect on ERK phosphorylation in response to endogenous receptor tyrosine kinase signalling (Fig. 5B and C).

The observed effects on Smad2 phosphorylation and nuclear accumulation are not confined to *Xenopus* embryos but are conserved in mammalian systems. We used siRNAs to knock down B α and B δ in a HeLa cell line stably expressing EGFP-Smad2. As in *Xenopus*,

knockdown of B α decreased the nuclear accumulation of Smad2 in response to TGF- β in these cells (Fig. 5E) and inhibited Smad2 phosphorylation (Fig. 5F). In contrast, knockdown of B δ enhanced Smad2 nuclear accumulation relative to control cells (Fig. 5E) and increased Smad2 phosphorylation (Fig. 5F). These effects were observable in a number of different human and mouse cell lines (data not shown) and were specific, as several different individual siRNA oligonucleotides which were proven to specifically knock down B α or B δ (Fig. S6A, B), elicited the same effect (Fig. S6B, C). Consistent with the decrease in TGF- β -induced Smad2 phosphorylation caused by B α knockdown, TGF- β was also less effective at mediating growth arrest in these conditions (Fig. S7). Overexpression of either B α or B δ had little effect on the level of phosphorylated Smad2 in tissue culture cells (data not shown), perhaps because their levels are not limiting or because free subunits which are not incorporated into holocomplexes, are unstable (Strack et al., 2002).

In conclusion, B α and B δ modulate the level of active phosphorylated Smad2 and hence its nuclear accumulation in both *Xenopus* embryos and tissue culture cells, with B α normally promoting Smad2 phosphorylation and B δ inhibiting Smad2 phosphorylation.

B α - and B δ -containing PP2A holocomplexes do not dephosphorylate pSmad2

The simplest hypothesis to explain the effects on Smad2 phosphorylation was that B δ acted directly on the C-terminal phosphates of activated Smad2 and that B α negated this action. We therefore immunopurified heterotrimeric active PP2A complexes containing either Flag-tagged B α or B δ or a distinct regulatory subunit, B' δ (Janssens and Goris, 2001) together with the catalytic and scaffolding subunits (Fig. 6A-C and data not shown). These complexes were all active since they dephosphorylated a control phospho-peptide (Fig. 6C) and the PP2A complexes containing either B α or B δ , but not complexes containing B' δ , efficiently dephosphorylated an immunopurified Raf substrate (Fig. 6D), as previously reported (Adams et al., 2005). However, we could not detect any dephosphorylation of an immunopurified, C-terminally phosphorylated Smad2 substrate by any of the phosphatase complexes (Fig. 6D). This was also true for PP2A holocomplexes purified from TGF- β -induced cells (Fig. 6E and F). Furthermore, we could not detect significant dephosphorylation of a number of phosphorylated residues within the Smad2 linker region (Kretzschmar et al., 1999) (Fig. 6G). Thus, neither B α nor B δ seem to act directly on phosphorylated Smad2.

B α and B δ do not affect receptor kinase activity in vitro

Since B α and B δ do not act on Smad2 directly, but do affect Smad2 phosphorylation in vivo we asked whether they could regulate the activity of the TGF- β receptor complex. The TGF- β receptor complex comprises two type II receptors (T β R-II) and two type I receptors (ALK5). ALK5 activity requires its phosphorylation by the constitutively active type II receptor, and thus we reasoned that dephosphorylation of ALK5 by a PP2A complex in vitro would reduce ALK5 activity. In an in vitro kinase assay (see Fig. 7A for the experimental scheme) immunopurified active receptor complexes from TGF- β -induced cells phosphorylated recombinant Smad2 at its C-terminus (Fig. 7B, lane C). However, incubation of receptor and substrate with either phosphatase complex had no significant effect on the

ability of receptors to phosphorylate Smad2 (Fig. 7B, lanes B α , B δ , B' δ). Thus in vitro, neither phosphatase subunit affected receptor kinase activity.

Knockdown of B δ enhances ALK4 activity

We therefore investigated whether B α or B δ affected receptor activity in vivo. We have recently shown that ALK4 receptor clustering provides a convenient readout of receptor activity in vivo (Batut et al., 2007) as it is induced in response to ligand and requires ALK4 kinase activity (Fig. S8). In untreated animal caps, which exhibit very low levels of Activin/Nodal signalling, morpholino knockdown of B δ , or inhibition of PP2A catalytic activity by okadaic acid, was sufficient to induce ALK4 clustering (Fig. 7C, compare untreated animal caps). Thus a reduction of B δ activity lowers the threshold of ligand required for ALK4 signalling, indicating that B δ normally restricts receptor activity and might act to suppress signalling at very low ligand concentrations.

Knockdown of B α promotes degradation of ALK4 and ALK5

In the course of investigating whether knockdown of B α would inhibit Activin/Nodal-induced ALK4 clustering, we noticed that levels of HA-ALK4 were extremely low when B α was depleted (Fig. 7D). Moreover, overexpression of B δ , which has the same functional effects as B α knockdown, similarly reduced HA-ALK4 levels (Fig. 7D).

These data were corroborated in HaCaT cells, where knockdown of B α led to a strong decrease in levels of endogenous ALK5 protein (Fig. 7E). This effect was specific as knockdown of B α or B δ did not affect endogenous T β R-II levels (Fig. 7F). We conclude that the effect on receptor levels is post-transcriptional in both model systems. In tissue culture cells, knockdown of B α had no effect on endogenous ALK5 mRNA levels (Fig. S9A) and also substantially reduced protein levels of exogenously expressed HA-tagged ALK5 (Fig. S9B). Similarly in *Xenopus* embryos, the effects of B α knockdown or B δ overexpression on HA-ALK4 are not at the level of transcription since the receptor is expressed from an injected synthetic mRNA. In principle, B α could affect translation of the type I receptors, or their stability. We favour the latter possibility since the mRNAs used in both systems have no 3' or 5' UTRs, which are usually required for translational regulation. Furthermore, we could detect a weak interaction between HA-ALK4 and Flag-tagged B α in a co-immunoprecipitation (Fig. S10) as was reported previously for ALK5 (Griswold-Prenner et al., 1998), suggesting that B α might affect ALK4 and ALK5 at the protein level.

To identify the pathway of degradation, we asked whether inhibitors of the lysosome (bafilomycin A1) or the proteasome (lactacystin or MG132) could rescue the effects of B α knockdown in tissue culture cells. We found that in HeLa cells overexpressing ALK5, the effects of B α knockdown was rescued by bafilomycin A1, but not MG132 (Fig. S11A). Similarly in HaCaT cells, a partial rescue of endogenous ALK5 levels was observed with bafilomycin A treatment, but not by treatment with lactacystin or MG132 (Fig. S11B).

Taken together, these data suggest that knockdown of B α (and in *Xenopus*, also overexpression of B δ) promotes degradation of the type I receptors ALK4 and ALK5 via a lysosomal pathway. We speculated that PP2A might regulate Dapper2, which is known to promote lysosomal degradation of ALK4 and ALK5 (Zhang et al., 2004). However,

knockdown of Dapper2 did not ameliorate the decrease in ALK5 levels resulting from B α knock-down, even though Dapper2 knockdown alone clearly raised levels of ALK5 (Fig. S11C). Thus, it is more likely that Dapper2 regulates PP2A or that the two act independently.

Discussion

It has long been speculated that the individual, yet highly homologous, members of each subfamily of PP2A regulatory subunits would have unique and specific roles in addition to their established redundant activities. We have demonstrated here for the first time such non-redundant functions of two members of the B subfamily of PP2A regulatory subunits. We have shown that B α and B δ perform important functions in modulating the intensity of TGF- β /Activin/Nodal signalling in different species by stabilising basal levels of the ALK4 and ALK5 receptors (B α) and by restricting receptor activity (B δ) (Fig. 7G). Perturbation of these mechanisms has dramatic functional consequences in vivo as demonstrated by altered gene expression and serious developmental defects.

We do not yet know the exact mechanism whereby B α and B δ regulate receptor levels and activity, respectively. Nevertheless it is clear from our knockdown experiments that each subunit affects a separate and distinct aspect of receptor biology, ruling out the possibility that B α and B δ merely compete with each other for catalytic and scaffolding subunits, so that knockdown of one B subunit increases the levels of complexes containing the other B subunit. The results of our loss of function experiments also exclude the possibility that B α and B δ have opposing activities on a single common substrate. However, it is likely that subunit competition within the PP2A holoenzyme does explain why B δ overexpression mimicks B α knockdown (both manipulations causing ALK4 destabilisation in *Xenopus* embryos). B δ has a higher affinity for the catalytic subunit than B α (Fig. 6C and E). Overexpressed B δ is thus more likely to compete out endogenous B α than vice versa, and B δ overexpression would mimick B α knockdown, whereas B α overexpression would not necessarily have an effect, as we observe.

We have shown that the PP2A catalytic inhibitor, okadaic acid, can mimick the effects of B δ knockdown suggesting that B δ functions as part of a PP2A holoenzyme complex. However, as okadaic acid is not absolutely specific for PP2A, a mechanism independent of the PP2A catalytic subunit cannot be definitively ruled out. We have shown that knockdown of B δ promotes receptor clustering at low endogenous ligand concentrations, suggesting that B δ normally inhibits receptor clustering and thus receptor activation at sub-threshold levels of ligand. Whether it does this by removing (in the context of a PP2A holoenzyme complex) an activating phosphate from serine/threonine residues in the receptors themselves, or via dephosphorylation of another component remains to be investigated.

In contrast to the role ascribed to B δ , our data demonstrate that B α regulates the levels of the type I receptors ALK4 and ALK5, most likely by stabilising the receptors and preventing their degradation via the lysosomal pathway. The involvement of phosphatases in protein turnover is not unprecedented. The protein phosphatase Dullard has recently been reported to promote the degradation of BMP type II receptors in *Xenopus*, thus repressing BMP-

dependent phosphorylation of the BMP type I receptor (Satow et al., 2006). More specifically to PP2A, the cycling of the protein Period in *Drosophila* is dependent upon the activity of Twins and loss of PP2A activity reduces Period expression (Sathyanarayanan et al., 2004). In this case, Twins acts in the context of a PP2A holoenzyme to dephosphorylate Period directly. Moreover, Twins also affects the levels of Armadillo, the *Drosophila* β -Catenin homologue, since it is required for stabilisation of Armadillo in response to Wingless signalling (Bajpai et al., 2004). Consistent with our demonstration that *Drosophila* Twins acts like vertebrate B δ , we have observed a reduction in levels of nuclear β -Catenin in B δ morphant embryos (Fig. 5A). Importantly however, this effect of B δ on the Wnt signalling pathway cannot explain the phenotypes we observe in B δ morphant embryos. In our present study it is not yet clear whether B α stabilises ALK4 and ALK5 by acting to dephosphorylate the receptors themselves, or whether it acts indirectly. Consistent with a previous report (Griswold-Prenner et al., 1998) we could detect a weak interaction between B α and ALK4, suggesting that it may act on the receptor directly, or possibly on another component of the receptor complex. We find that the effect of B α on type I receptor levels occurs in the absence of signalling, indicating that B α is required for regulating the Basal levels of receptor and not responsible for downregulating receptor levels after signal transduction.

In summary, we conclude that B α - and B δ -containing phosphatase complexes have distinct substrates whose net effects on the TGF- β /Activin/Nodal pathway are opposite. This strongly suggests that the ratio of B α to B δ in a particular cell will influence the threshold response to TGF- β /Activin/Nodal ligands, which will in turn determine the levels of target gene transcription and thus developmental programmes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

- Adams DG, Coffee RL Jr, Zhang H, Pelech S, Strack S, Wadzinski BE. Positive regulation of Raf1-MEK1/2-ERK1/2 signaling by protein serine/threonine phosphatase 2A holoenzymes. *J Biol Chem.* 2005; 280:42644–54. [PubMed: 16239230]
- Bajpai R, Makhijani K, Rao PR, Shashidhara LS. *Drosophila* Twins regulates Armadillo levels in response to Wg/Wnt signal. *Development.* 2004; 131:1007–16. [PubMed: 14973271]
- Bardwell VJ, Treisman R. The POZ domain: a conserved protein-protein interaction motif. *Genes Dev.* 1994; 8:1664–77. [PubMed: 7958847]
- Batut J, Howell M, Hill CS. Kinesin-mediated transport of Smad2 is required for signaling in response to TGF- β ligands. *Developmental Cell.* 2007; 12:261–274. [PubMed: 17276343]

- Bennett D, Alphey L. PP1 binds Sara and negatively regulates Dpp signaling in *Drosophila melanogaster*. *Nat Genet.* 2002; 31:419–23. [PubMed: 12134149]
- Brand AH, Perrimon N. Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development.* 1993; 118:401–15. [PubMed: 8223268]
- Brummel T, Abdollah S, Haerry TE, Shimell MJ, Merriam J, Raftery L, Wrana JL, O'Connor MB. The *Drosophila* activin receptor baboon signals through dSmad2 and controls cell proliferation but not patterning during larval development. *Genes Dev.* 1999; 13:98–111. [PubMed: 9887103]
- Chen HB, Shen J, Ip YT, Xu L. Identification of phosphatases for Smad in the BMP/DPP pathway. *Genes Dev.* 2006; 20:648–53. [PubMed: 16510868]
- Di Guglielmo GM, Le Roy C, Goodfellow AF, Wrana JL. Distinct endocytic pathways regulate TGF-beta receptor signalling and turnover. *Nat Cell Biol.* 2003; 5:410–21. [PubMed: 12717440]
- Dorey K, Hill CS. A novel Cripto-related protein reveals an essential role for EGF-CFCs in Nodal signalling in *Xenopus* embryos. *Dev Biol.* 2006; 292:303–16. [PubMed: 16497290]
- Feng XH, Derynck R. Specificity and versatility in TGF- β signaling through Smads. *Annu Rev Cell Dev Biol.* 2005; 21:659–93. [PubMed: 16212511]
- Griswold-Prenner I, Kamibayashi C, Maruoka EM, Mumby MC, Derynck R. Physical and functional interactions between type I transforming growth factor β receptors and B α , a WD-40 repeat subunit of phosphatase 2A. *Mol Cell Biol.* 1998; 18:6595–604. [PubMed: 9774674]
- Harlow, E.; Lane, D. *Antibodies, a Laboratory Manual.* Cold Spring Harbour Laboratory Press; 1988.
- Henderson KD, Andrew DJ. Identification of a novel *Drosophila* SMAD on the X chromosome. *Biochem Biophys Res Commun.* 1998; 252:195–201. [PubMed: 9813169]
- Ho DM, Chan J, Bayliss P, Whitman M. Inhibitor-resistant type I receptors reveal specific requirements for TGF- β signaling in vivo. *Dev Biol.* 2006; 295:730–742. [PubMed: 16684517]
- Howell M, Inman GJ, Hill CS. A novel *Xenopus* Smad-interacting forkhead transcription factor (XFast-3) cooperates with XFast-1 in regulating gastrulation movements. *Development.* 2002; 129:2823–34. [PubMed: 12050132]
- Howell M, Itoh F, Pierreux CE, Valgeirsdottir S, Itoh S, ten Dijke P, Hill CS. *Xenopus* Smad4 β is the co-Smad component of developmentally regulated transcription factor complexes responsible for induction of early mesodermal genes. *Dev Biol.* 1999; 214:354–69. [PubMed: 10525340]
- Janssens V, Goris J. Protein phosphatase 2A: a highly regulated family of serine/threonine phosphatases implicated in cell growth and signalling. *Biochem J.* 2001; 353:417–39. [PubMed: 11171037]
- Janssens V, Goris J, Van Hoof C. PP2A: the expected tumor suppressor. *Curr Opin Genet Dev.* 2005; 15:34–41. [PubMed: 15661531]
- Jullien J, Gurdon J. Morphogen gradient interpretation by a regulated trafficking step during ligand-receptor transduction. *Genes Dev.* 2005; 19:2682–94. [PubMed: 16260495]
- Knockaert M, Sapkota G, Alarcon C, Massague J, Brivanlou AH. Unique players in the BMP pathway: small C-terminal domain phosphatases dephosphorylate Smad1 to attenuate BMP signaling. *Proc Natl Acad Sci U S A.* 2006; 103:11940–5. [PubMed: 16882717]
- Kretschmar M, Doody J, Timokhina I, Massague J. A mechanism of repression of TGF β / Smad signaling by oncogenic Ras. *Genes Dev.* 1999; 13:804–16. [PubMed: 10197981]
- Lee MA, Heasman J, Whitman M. Timing of endogenous activin-like signals and regional specification of the *Xenopus* embryo. *Development.* 2001; 128:2939–52. [PubMed: 11532917]
- Levy L, Hill CS. Smad4 dependency defines two classes of transforming growth factor β (TGF- β) target genes and distinguishes TGF- β -induced epithelial-mesenchymal transition from its antiproliferative and migratory responses. *Mol Cell Biol.* 2005; 25:8108–25. [PubMed: 16135802]
- Lin X, Duan X, Liang YY, Su Y, Wrighton KH, Long J, Hu M, Davis CM, Wang J, Brunicaudi FC, et al. PPM1A functions as a Smad phosphatase to terminate TGF β signaling. *Cell.* 2006; 125:915–28. [PubMed: 16751101]
- Marquez RM, Singer MA, Takaesu NT, Waldrip WR, Kraytsberg Y, Newfeld SJ. Transgenic analysis of the Smad family of TGF- β signal transducers in *Drosophila melanogaster* suggests new roles and new interactions between family members. *Genetics.* 2001; 157:1639–48. [PubMed: 11290719]

- Massagué J, Seoane J, Wotton D. Smad transcription factors. *Genes Dev.* 2005; 19:2783–810. [PubMed: 16322555]
- Mayer-Jaekel RE, Ohkura H, Gomes R, Sunkel CE, Baumgartner S, Hemmings BA, Glover DM. The 55 kd regulatory subunit of *Drosophila* protein phosphatase 2A is required for anaphase. *Cell.* 1993; 72:621–33. [PubMed: 8382567]
- Nicolas FJ, De Bosscher K, Schmierer B, Hill CS. Analysis of Smad nucleocytoplasmic shuttling in living cells. *J Cell Sci.* 2004; 117:4113–25. [PubMed: 15280432]
- Nicolás FJ, Hill CS. Attenuation of the TGF β -Smad signaling pathway in pancreatic tumor cells confers resistance to TGF β -induced growth arrest. *Oncogene.* 2003; 22:3698–3711. [PubMed: 12802277]
- Parker L, Ellis JE, Nguyen MQ, Arora K. The divergent TGF- β ligand Dawdle utilizes an activin pathway to influence axon guidance in *Drosophila*. *Development.* 2006; 133:4981–91. [PubMed: 17119022]
- Peter A, Schottler P, Werner M, Beinert N, Dowe G, Burkert P, Mourkioti F, Dentzer L, He Y, Deak P, et al. Mapping and identification of essential gene functions on the X chromosome of *Drosophila*. *EMBO Rep.* 2002; 3:34–8. [PubMed: 11751581]
- Petritsch C, Beug H, Balmain A, Oft M. TGF- β inhibits p70 S6 kinase via protein phosphatase 2A to induce G(1) arrest. *Genes Dev.* 2000; 14:3093–101. [PubMed: 11124802]
- Sathyanarayanan S, Zheng X, Xiao R, Sehgal A. Posttranslational regulation of *Drosophila* PERIOD protein by protein phosphatase 2A. *Cell.* 2004; 116:603–15. [PubMed: 14980226]
- Satow R, Kurisaki A, Chan TC, Hamazaki TS, Asashima M. Dullard Promotes Degradation and Dephosphorylation of BMP Receptors and Is Required for Neural Induction. *Dev Cell.* 2006; 11:763–74. [PubMed: 17141153]
- Schier AF. Axis formation and patterning in zebrafish. *Curr Opin Genet Dev.* 2001; 11:393–404. [PubMed: 11448625]
- Schmierer B, Hill CS. Kinetic analysis of Smad nucleocytoplasmic shuttling reveals a mechanism for transforming growth factor β -dependent nuclear accumulation of Smads. *Mol Cell Biol.* 2005; 25:9845–58. [PubMed: 16260601]
- Schmierer B, Hill CS. TGF β -SMAD signal transduction: molecular specificity and functional flexibility. *Nat Rev Mol Cell Biol.* 2007; 8:970–82. [PubMed: 18000526]
- Serpe M, O'Connor MB. The metalloprotease tolloid-related and its TGF- β -like substrate Dawdle regulate *Drosophila* motoneuron axon guidance. *Development.* 2006; 133:4969–79. [PubMed: 17119021]
- Shi W, Sun C, He B, Xiong W, Shi X, Yao D, Cao X. GADD34-PP1c recruited by Smad7 dephosphorylates TGF β type I receptor. *J Cell Biol.* 2004; 164:291–300. [PubMed: 14718519]
- Smith JC. Mesoderm-inducing factors in early vertebrate development. *EMBO J.* 1993; 12:4463–4470. [PubMed: 8223456]
- Strack S. Overexpression of the protein phosphatase 2A regulatory subunit Bgamma promotes neuronal differentiation by activating the MAP kinase (MAPK) cascade. *J Biol Chem.* 2002; 277:41525–32. [PubMed: 12191994]
- Strack S, Chang D, Zaucha JA, Colbran RJ, Wadzinski BE. Cloning and characterization of B δ , a novel regulatory subunit of protein phosphatase 2A. *FEBS Lett.* 1999; 460:462–6. [PubMed: 10556517]
- Strack S, Ruediger R, Walter G, Dagda RK, Barwacz CA, Cribbs JT. Protein phosphatase 2A holoenzyme assembly: identification of contacts between B-family regulatory and scaffolding A subunits. *J Biol Chem.* 2002; 277:20750–5. [PubMed: 11929880]
- Sun Z, Jin P, Tian T, Gu Y, Chen YG, Meng A. Activation and roles of ALK4/ALK7-mediated maternal TGF β signals in zebrafish embryo. *Biochem Biophys Res Commun.* 2006; 345:694–703. [PubMed: 16696945]
- Tada M, Concha ML, Heisenberg CP. Non-canonical Wnt signalling and regulation of gastrulation movements. *Semin Cell Dev Biol.* 2002; 13:251–60. [PubMed: 12137734]
- ten Dijke P, Hill CS. New insights into TGF- β -Smad signalling. *Trends Biochem Sci.* 2004; 29:265–73. [PubMed: 15130563]

- Uemura T, Shiomi K, Togashi S, Takeichi M. Mutation of twins encoding a regulator of protein phosphatase 2A leads to pattern duplication in *Drosophila* imaginal discs. *Genes Dev.* 1993; 7:429–40. [PubMed: 8383623]
- Van Kanegan MJ, Adams DG, Wadzinski BE, Strack S. Distinct protein phosphatase 2A heterotrimers modulate growth factor signaling to extracellular signal-regulated kinases and Akt. *J Biol Chem.* 2005; 280:36029–36. [PubMed: 16129692]
- Whitman M. Nodal signaling in early vertebrate embryos. Themes and variations. *Dev Cell.* 2001; 1:605–17. [PubMed: 11709181]
- Wieser R, Wrana JL, Massagué J. GS domain mutations that constitutively activate T β R-I, the downstream signaling component in the TGF- β receptor complex. *EMBO J.* 1995; 14:2199–208. [PubMed: 7774578]
- Zhang L, Zhou H, Su Y, Sun Z, Zhang H, Zhang L, Zhang Y, Ning Y, Chen YG, Meng A. Zebrafish Dpr2 inhibits mesoderm induction by promoting degradation of nodal receptors. *Science.* 2004; 306:114–7. [PubMed: 15459392]
- Zheng X, Wang J, Haerry TE, Wu AY, Martin J, O'Connor MB, Lee CH, Lee T. TGF- β signaling activates steroid hormone receptor expression during neuronal remodeling in the *Drosophila* brain. *Cell.* 2003; 112:303–15. [PubMed: 12581521]

Fig. 1. Manipulating the expression of Bα and Bδ in *Xenopus* embryos produces distinct phenotypes

(A) *Xenopus* embryos were injected with either control GFP mRNA, Flag-tagged mouse Bδ mRNA (Bδ), a morpholino control (MoC) or a specific morpholino against *Xenopus* Bα (MoBα) or Bδ (MoBδ) at the one-cell stage, and fixed when control embryos had reached either early gastrula or tailbud (stage 25). Representative embryos are shown, arrows indicate anterior. The anterior regions of embryos are magnified below. Note the lack of head structures in Bδ-injected embryos and MoBα-injected embryos.

(B) Embryos were injected as in A with the indicated mRNAs and morpholinos. The effect of MoBδ or MoBα could be rescued by coinjection with the cognate mRNA (mouse Bδ or Bα). Percentages of embryos showing wild type phenotype when control-injected embryos had reached stage 22 are given. Arrows indicate anterior.

Fig. 2. Manipulating the expression of $B\alpha$ and $B\delta$ in *Xenopus* embryos has opposing effects on Activin/Nodal target gene expression

(A) In situ hybridisation of gastrula-stage embryos injected with either a morpholino control (MoC) or a specific morpholino against *Xenopus* $B\alpha$ (MoBa) or $B\delta$ (MoBδ) or with Flag-tagged mouse $B\delta$ mRNA (Bδ) at the one-cell stage. The probes used were against *gsc* or *Xbra*. Staining was visualised with BM purple. Note the increased staining for both *Xbra* and *Gsc* in $B\delta$ morphants, and decreased staining in $B\alpha$ morphants and embryos overexpressing $B\delta$.

(B) Analysis of gene expression by q-PCR. Total RNA was isolated from stage 10.5 embryos that had been injected at the one-cell stage with either morpholino control, or morpholinos against $B\alpha$ or $B\delta$. Expression levels are normalised to ornithine decarboxylase (ODC).

Fig. 3. B α and B δ act on the Activin/Nodal signalling pathway in *Xenopus*

(A-C) One-cell embryos were injected with the indicated mRNAs (B α , B δ or EGFP-Smad2) and morpholinos (MoC, MoB α or MoB δ). When control embryos had reached stage 8, the animal pole was excised and incubated with or without Activin for 16 hrs, and visualised for the degree of elongation.

Fig. 4. Overexpression of *Drosophila* Smad2 (Smox) can rescue the effects of overexpression of *Drosophila* B subunit, Twins in the wing

(A) Phenotypically wild type wing from $+/Y; UAS-tws23; UAS-Smox8D3$ male.

(B) Small, blistered wing from $A9-GAL4; UAS-tws23; +$ male. All wings from these males are smaller than wild type; approximately 80% were cupped and blistered with little or no evidence of veins.

(C) Wing from $A9-GAL4; +; UAS-Smox8D3$ male. In this genotype, wing veins formed a delta at the margin, and additional wing vein material was often observed (arrowheads).

(D) Phenotypically normal wing from $A9-GAL4; UAS-tws23; UAS-Smox8D3$ male. All veins terminated normally at the margin (arrows).

Fig. 5. Bα and Bδ exert differential effects on the level of phosphorylated Smad2

(A) Embryos were injected at the one-cell stage with morpholinos (Mo) against Bα or Bδ, or Bδ mRNA as indicated. Embryos were harvested at stage 10, fixed, dissected through the lip and analysed by immunofluorescence using anti-Smad2 and anti-β-Catenin antibodies. The nuclei were visualised with DAPI. Panels a and b show an area from the ventral vegetal region and panels c-e show an area from the dorsal vegetal region.

(B) Embryos remained uninjected (ui) or were injected with two doses of mouse Bα mRNA or mouse Bδ mRNA, cultured until control embryos reached stage 9 and analysed by immunoblotting with anti-phospho-Smad2, Smad2/3, or phospho-ERK (pERK) antibodies.

(C) Embryos were injected with distinct morpholinos (labelled 1 or 2) targeting Bδ, or Bα respectively, or with a control morpholino and analysed as in (B).

(D) Animal caps from stage 8 embryos were incubated with or without okadaic acid (OA, 25 nM) for 1 hr, treated with or without Activin for 20 min and processed for immunoblotting.

(E) HeLa EGFP-Smad2 cells were transfected with either an siRNA *SMARTpool* control or a human Bα- or Bδ-specific *SMARTpool*. Cells were incubated with TGF-β for the times indicated, fixed and visualised by confocal microscopy.

(F) HeLa EGFP-Smad2 cells were transfected as in E and incubated with TGF- β for the times indicated. Samples were analysed by Western blotting with anti-phospho-Smad2, anti-Smad2/3 and anti-pan B subunit antibodies.

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Fig. 6. Bα and Bδ do not act directly on phosphorylated Smad2

(A) Outline of the experimental procedure to isolate Bα- and Bδ-containing active PP2A holo complexes and to perform phosphatase assays.

(B) Silver-stained gel showing the composition of complexes isolated by Flag pulldown from HeLa cells transfected with the indicated Flag-tagged B subunits. The components of the complex are indicated including the catalytic subunit (PP2A_C) and the structural subunit (PP2A_A). * indicates that the Flag-Bδ overlies PP2A_A.

(C) Western blot analysis of immunopurified complexes showing the presence of appropriate B or B'δ (PPP2R5D) subunit (Flag blot) and co-purified catalytic subunit (anti-PP2A_C blot) for each complex. Phosphatase activity was assessed by a colorimetric assay using a phospho-peptide as substrate (bars).

(D) PP2A complexes as in (C) were incubated with phospho-Smad2 immunopurified from TGF-β-induced HaCaT EGFP-Smad2 cells. The reactions were then analysed by immunoblotting with anti-pSmad2 and anti-Smad2/3 antibodies. All PP2A complexes tested failed to dephosphorylate phospho-Smad2. Bα- and Bδ-containing complexes dephosphorylated pS259 of immunoprecipitated HA-tagged Raf-1 (lower panels)

(E) TGF-β treatment prior to immunopurification of the PP2A complexes does not affect the amount of co-purified catalytic subunit, nor the activity of the complexes in the colorimetric assay.

(F) As in (D), but PP2A complexes were purified from untreated (-) or TGF-β-induced cells (+), as shown in (E).

(G) Phosphorylated serines 245, 250 and 255 of Smad2 are not substrates for immunopurified Bα and Bδ complexes. Phosphatase complexes were immunopurified from either control cells (C) or cells expressing Flag-tagged Bα or Bδ as indicated, and incubated with either a Smad2/3 immunoprecipitate from TGF-β-induced HaCaT cells (upper panels) or, as a control, an immunopurified phosphorylated Raf substrate from HeLa cells

expressing HA-Raf (lower panel). Samples were analysed by Western blotting using antibodies recognising Smad2 phosphorylated at residues S245, S250, S255, as well as anti-Smad2/3, anti-phospho Raf and anti-HA as indicated.

Fig. 7. B α regulates the basal level of the type I receptor and B δ regulates its activity

(A) Outline of the experimental procedure to isolate B α - and B δ -containing active PP2A holocomplexes and to assay their ability to affect the kinase activity of ALK5.

(B) The presence of neither PP2A complex affects the kinase activity of ALK5 in vitro. Endogenous ALK5 complexes immunopurified from untreated or TGF- β -treated HaCaT cells were incubated with recombinant Smad2 substrate in the absence or presence of B-subunit-specific PP2A complexes purified as in Figure 6. C-terminal Smad2 phosphorylation was detected by immunoblotting. The activity of the PP2A complexes was confirmed by their ability to dephosphorylate pS259 of Raf-1 (lower panel).

(C) Knockdown of B δ promotes ALK4 clustering. Animal caps from embryos expressing either HA-ALK4 mRNA alone (upper panels) or in combination with morpholino against B δ (MoB δ , middle panels) were incubated for 1 hr in the presence or absence of Activin and stained with anti-HA antibody. HA-ALK4 clusters in response to Activin and in untreated embryos injected with MoB δ . Okadaic acid (OA) treatment (lower panels) also induces HA-ALK4 clustering and thus mimics B δ knockdown.

(D) B α knockdown strongly decreases basal protein levels of ALK5. HaCaT cells were transfected with siRNAs and treated with TGF- β as indicated. Extracts were immunoblotted with antibodies against ALK5, phospho-Smad2, pan B-subunits and Smad2/3.

(E) B α knockdown has no effect on T β R-II levels. HaCaT cells were transfected with the indicated siRNAs. Extracts were immunoblotted with antibodies against T β R-II, pan B-subunits and Smad2/3. Prior to electrophoresis, extracts were treated $\pm$ PNGase F to remove N-linked sugars from T β R-II and visualise it more clearly.

(F) B α knockdown or B δ overexpression decreases protein levels of HA-ALK4. *Xenopus* embryos were injected at the one-cell stage with HA-ALK4 and GFP mRNAs as well as with morpholinos or B δ mRNA as indicated, cultured until uninjected embryos (Ui) had reached stage 9 and analysed by immunoblotting.

(G) Model of the modulation of TGF- β /Activin/Nodal signalling by B α and B δ . B α normally stabilises the type I receptors ALK4 and ALK5, and B α knockdown promotes their basal degradation. B δ normally restricts ligand-dependent activation of ALK4 and ALK5, and B δ knockdown facilitates such activation. When overexpressed, B δ additionally inhibits endogenous B α by replacing it in the PP2A holoenzyme due to its higher affinity for the catalytic subunit.