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Functional interaction between BMPR-II and Tctex-1, a light chain of Dynein, is isoform-specific and disrupted by mutations underlying primary pulmonary hypertension

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Diverse heterozygous mutations of bone morphogenetic receptor type II (BMPR-II) underlie the inherited form of the vascular disorder primary pulmonary hypertension (PPH). As yet, the molecular detail of how such defects contribute to the pathogenesis of PPH remains unclear. BMPR-II is a member of the transforming growth factor-β cell signalling superfamily. Ligand binding induces cell surface receptor complex formation and activates a cascade of phosphorylation events of intracellular intermediaries termed Smads, which initiate transcriptional regulation. Some 30% of PPH-causing mutations localize to exon 12, which may be spliced out forming an isoform depleted of the unusually long BMPR-II cytoplasmic tail. To further elucidate the consequences of *BMPR2* mutation, we sought to characterize aspects of the cytoplasmic domain function by seeking intracellular binding partners. We now report that Tctex-1, a light chain of the motor complex dynein, interacts with the cytoplasmic domain of BMPR-II and demonstrate that Tctex-1 is phosphorylated by BMPR-II, a function disrupted by PPH disease causing mutations within exon 12. Finally we show that BMPR-II and Tctex-1 co-localize to endothelium and smooth muscle within the media of pulmonary arterioles, key sites of vascular remodelling in PPH. Taken together, these data demonstrate a discrete function for the cytoplasmic domain of BMPR-II and justify further investigation of whether the interaction with and phosphorylation of Tctex-1 contributes to the pathogenesis of PPH.

INTRODUCTION

The transforming growth factor beta (TGF-β) superfamily of cytokines includes bone morphogenetic proteins (BMPs), activins and transforming growth factors that each play a complex and multifunctional role in the regulation of cell proliferation, differentiation and apoptosis during embryogenesis and throughout adult life (1).

The TGF-β cytokines signal through a cooperative ligand-binding mechanism, which initiates signalling by binding to a type I (e.g. BMPRIA/IB) and type II (e.g. BMPR-II) serine/threonine kinase receptor on the cell surface (2,3). Phosphorylated receptor kinases then propagate intracellular signalling via intermediary Smad molecules. Activated Smad complexes translocate to the nucleus and in conjunction with nuclear cofactors, result in transcriptional regulation of target genes. Although the Smad family of signalling molecules has been extensively characterized, BMP-activated Smad-independent pathways have also been identified, including members of the mitogen-activated protein kinase (MAPK) pathways, notably $p38^{MAPK}$ (1,4).

Heterozygous mutations identified in *BMPR2*, encoding a type II BMP receptor, have been associated with the human disease primary pulmonary hypertension (PPH) (MIM 178600) (5–7). This lethal vascular disorder is characterized by the obstruction and occlusion of pulmonary arterioles through proliferation and migration of endothelial and smooth muscle cells, leading to a sustained elevation of pulmonary arterial pressure. PPH is usually fatal, typically as a consequence of right heart failure (8).

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The gene *BMPR2* is located on chromosome 2q33 (9,10) and encodes a polypeptide of 1038 amino acids. The mature protein comprises an extracellular ligand binding domain and an intracellular serine/threonine kinase domain, both linked through a single transmembrane domain. BMPR-II has a long carboxy terminal tail (508 amino acids), a feature unique amongst TGF-β receptors. This cytoplasmic region comprises almost 50% of the protein, encoded by exon 11 (amino acids 509–529), exon 12 and exon 13 (amino acids 955–1038), yet is of unknown function (11,12). A short isoform of only 530 amino acids, the result of a splice variant which lacks exon 12, is also capable of phosphorylating both the Smads and $p38^{MAPK}$ (12,13). Northern blot and RT–PCR of human tissue demonstrate both short and long transcripts to be ubiquitously expressed (J.A. Flanagan *et al.*, unpublished data).

Defects in *BMPR2* have been reported in at least 50% of probands with familial PPH, and are varied in form and predicted impact on *BMPR2* transcripts. The majority of PPH disease-causing mutations are nonsense or frameshift and many predict premature transcript truncation. The remainder (26%) alter highly conserved amino acids within BMPR-II domains of known function (14). At least *in vitro*, different mutations lead to heterogeneous functional defects, which include the intracellular retention of the mutant receptor and disruption of Smad-mediated signalling capacity (15). However, we have demonstrated that all deleterious *BMPR2* mutations lead to constitutive activation of $p38^{MAPK}$ (16). Whilst these *in vitro* observations identify aberrant cellular events, it remains unclear whether they are consequent upon BMPR-II haploinsufficiency or the result of a dominant negative effect and furthermore how such defects contribute to the pathogenesis of PPH.

At least a third of pathogenic mutations in *BMPR2* are located within the cytoplasmic domain (14) (www.pphgenes.net). These defects would be unlikely to impact on the splicing of exon 12, nor the generation of the short isoform, findings that imply a specific functional requirement for the long cytoplasmic tail of this type II receptor. In this study we sought to investigate the role and function of the large cytoplasmic domain of BMPR-II. Using a yeast two-hybrid screen and *in vitro* biochemical assays, we describe an interaction between the cytoplasmic tail of BMPR-II and Tctex-1, a 14 kDa light chain of the cytoplasmic dynein complex. Furthermore, we demonstrate that the ability to bind and phosphorylate dynein differs between the short and long isoforms of BMPR-II and is severely compromised by pathogenic mutations underlying PPH. Finally, we demonstrate co-localization of both BMPR-II and Tctex-1 within the pulmonary vascular endothelial and smooth muscle cells, both prominent sites of pathological remodelling in PPH.

RESULTS

Identification of Tctex-1 as an interacting protein with BMPR-II

The Gal4 yeast two-hybrid system was used to screen a human lung cDNA library for proteins that interacted with BMPR-II. A fragment of BMPR-II (amino acids 209–1038) was used as bait, with the inclusion of the kinase domain (amino acids 209–508) as a positive control to identify known binding partners. A total of 40 positive clones, as determined by their ability to grow in the absence of adenine and histidine and activate the *lacZ* gene were identified. Of these, three contained TGF-βRI, a known interactor with BMPR-II in yeast (3). Two positive clones contained inserts of unknown function and await characterization. The remainder represented inserts comprising the entire open reading frame, albeit with variable lengths of untranslated sequence, of a known gene encoding Tctex-1, a 14 kDa dynein light chain. Cytoplasmic dynein, a molecular motor protein structured around two heavy chains, two intermediate chains and a variable number of light–intermediate and light chains, is responsible for the retrograde transport of protein cargo along the microtubular network (17).

Refinement of the interaction between Tctex-1 and BMPR-II by the yeast two hybrid system

The interaction between BMPR-II and Tctex-1 was confirmed by its reconstitution in an *in vivo* yeast growth assay. Additionally, the inability of yeast expressing Tctex-1 and two randomly chosen transcripts, lamin A and SREBP1a, to grow on selective media suggested that the interaction with BMPR-II was both real and specific.

To determine the region of interaction with BMPR-II we designed four constructs, labelled B–E, encoding truncated and overlapping products of Tctex-1. Each of these constructs, and full-length Tctex-1 (construct A), was tested independently for interaction with BMPR-II. On selective media, growth of yeast containing BMPR-II was only observed when co-transformed with constructs A and E (Fig. 1). These data indicate that Tctex-1 interacts with BMPR-II at its carboxy terminal end. This 37 amino acid region of the light chain bears consensus sequences for casein kinase II and protein kinase C as well as clusters of serine residues reminiscent of the SSxS motif found in Smads phosphorylated by receptors of the TGF-β family (18).

An analysis of the interaction between BMP receptors and Tctex-1 by *in vitro* binding assays

To substantiate the interaction between BMPR-II and Tctex-1 *in vitro*, GST pull-down assays were performed. A fusion protein of GST and full-length Tctex-1 (GST-Tctex1) was tested against radiolabelled *in vitro* translations of both the long isoform (LF) of BMPR-II, containing the exon 12 encoded region and the short form (SF) lacking exon 12. In addition, the kinase (KD) and cytoplasmic domains (CD) of BMPR-II expressed as independent domains were similarly analysed to determine the domain responsible for binding Tctex-1. Following incubation with GST-Tctex1, both isoforms of BMPR-II were found to interact with the fusion product but not GST alone. The experiment was repeated five times and, in each case, the binding affinity of the SF for Tctex-1 was markedly lower than the LF. The kinase domain was not capable of binding Tctex-1 whereas the cytoplasmic region demonstrated a high affinity for the light chain (Fig. 2A). The specificity of the interaction between these proteins was supported not only by the absence of binding to the kinase domain of BMPR-II, but also by the observation that the

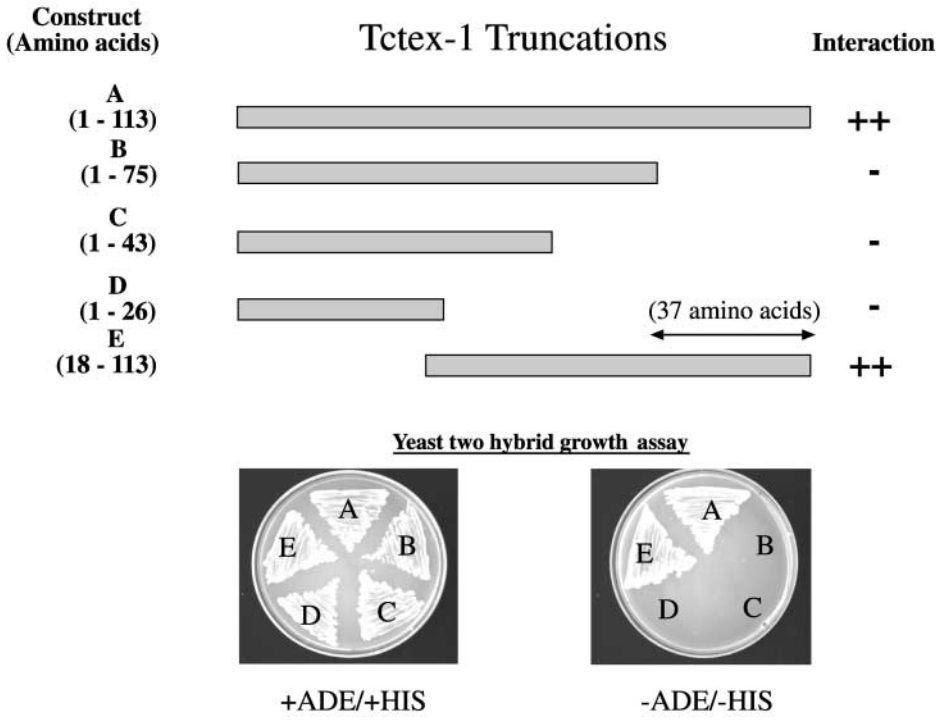

Figure 1. Truncation mapping of the Tctex-1 domain interacting with BMPR-II. Five combinations of Tctex-1 constructs (**A–E**), fused to the Gal4 activation domain in pACT2 were individually transformed into the PJ69-4a yeast strain along with the BMPR-II bait construct. Co-transformants were streaked onto SD media containing (+) or lacking (−) the amino acids adenine and histidine. An interaction between the fusion proteins results in the transcription of the reporter genes, permitting growth. Levels of growth are indicated (++ = strong/− = none). Only constructs A and E interact with BMPR-II, suggesting that the domain of interaction in Tctex-1 is at its C-terminal end.

related TGF-β type I receptor, ALK-1 failed to interact with GST-Tctex1 (data not shown).

We next sought to determine whether the type I receptors, BMPRIA and -IB, which have been shown to complex with BMPR-II *in vitro*, were also capable of interacting with Tctex-1 (Fig. 2B) (1). The ability of both to bind indicates that type I BMP receptors can also associate with this light chain.

Effects of PPH causing *BMPR2* mutation on interaction with Tctex-1

The cytoplasmic domain construct was subjected to site-directed mutagenesis to reproduce three truncating mutations previously identified in subjects with familial and sporadic PPH (14). Two mutations, designated 2292insA and 2386delG, create frameshifts of the open reading frame at the nucleotide positions indicated and result in premature protein truncation after the addition of an aberrant series of amino acids. The third mutation, R899X, causes truncation by the replacement of the native arginine residue with a premature stop codon at amino acid position 899. The ability of these mutant constructs to bind Tctex-1, assessed by the GST pull-down assay, was consistent with the demonstrated binding affinity of the wild-type cytoplasmic domain (Fig. 3A). As before, these experiments were repeated to ensure reproducibility. Varying degrees of truncation of the terminal end of the cytoplasmic tail and the removal of the region encoded by exon 13 of the gene do not cause a reduction in binding affinity. These findings, together with those of the LF and SF, suggest that the region of the receptor capable of maintaining high levels of interaction with Tctex-1 extends from amino acids 509–764 (Fig. 3B).

Phosphorylation of Tctex-1 by wild-type and mutant BMPR-II

As the BMP receptor complex is known to initiate signalling events by activating intermediaries such as Smad proteins through phosphorylation, we investigated whether BMPR-II was capable of activating Tctex-1 in a similar manner. The two isoforms of wild-type BMPR-II and three LF constructs comprising one kinase domain substitution (D485G), a nonsense (R899X) and a missense mutation (R899P) within the cytoplasmic domain were used in a kinase assay with GST fused Tctex-1. The mutation, R899P, represents the only missense variant observed in exon 12 of *BMPR2* thus far (M. Sankelo *et al.*, submitted for publication). The BMPR-II LF strongly phosphorylated Tctex-1, whereas phosphorylation by the SF was at approximately a third of this level. The kinase mutant abrogated phosphorylation entirely and the distal cytoplasmic domain truncation reduced the efficiency of the process to less than 5% of LF activity (Fig. 4). Interestingly, the amino acid substitution, R899P, also abolished the ability of the receptor to phosphorylate Tctex-1 (data not shown). These data are the first to provide evidence of a differential function between the two isoforms of BMPR-II and suggest that the LF is more efficient at phosphorylating substrate such as Tctex-1.

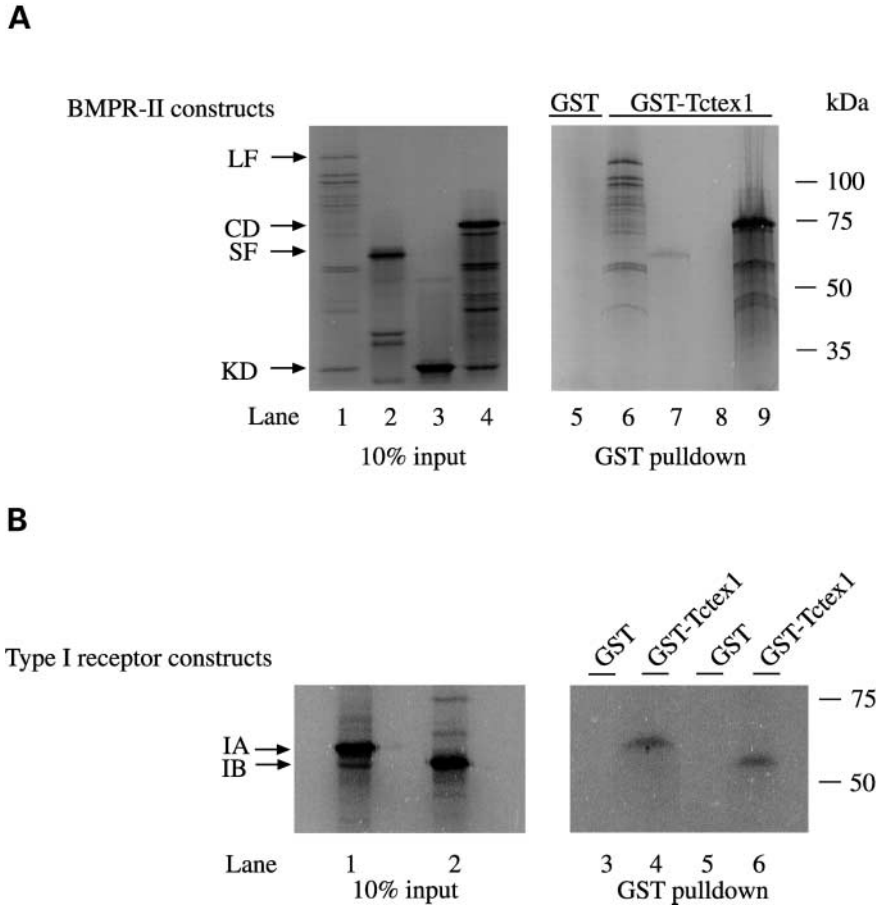

Figure 2. *In vitro* analyses of the interaction between Tctex-1 and the BMP receptors. (**A**) Lanes 1–4 represent ^{35}S-labelled, *in-vitro* translated products of the long isoform (LF), the short isoform (SF), the kinase domain (KD) and the cytoplasmic domain (CD) of BMPR-II. The amount loaded on these lanes was 10% of that incubated with GST alone (lane 5) or GST-Tctex1 (lanes 6–8), immobilized on glutathione–Sepharose beads. Proteins were detected by autoradiography. GST protein alone did not bind the LF (lane 5) whereas GST–Tctex1 interacted with the LF (lane 6), SF (lane 7) and the CD (lane 9) of BMPR-II. (**B**) The type I BMP receptors, BMPRIA (IA) and BMPRIB (IB) were *in vitro* translated (10% of input in lanes 1 and 2) and incubated with purified GST alone (lanes 3 and 5) and GST–Tctex1 (lanes 4 and 6). Both receptors displayed an interaction with Tctex-1. The horizontal arrows to the left of the gels indicate the full-length protein, the lower bands representing degradation products. Size ladder shown in kDa.

Immunolocalization of BMPR-II and Tctex-1 in the lung

To determine the expression patterns of BMPR-II and Tctex-1, immunostaining was performed on normal lung sections. BMPR-II staining was demonstrated within endothelial cells lining pulmonary arteries and arterioles, in vascular smooth muscle cells and within the alveolar wall as reported previously (Fig. 5A and D) (19). Tctex-1 immunolocalized to endothelial and smooth muscle cells with a similar distribution pattern to that seen with the BMPR-II antibody (Fig. 5B and E). Both proteins displayed high levels of expression in cells comprising capillaries in the alveolar walls (Fig. 5G and H). Co-incubation of the Tctex-1 antibody, in both pulmonary arterioles and parenchyma, with a ten-fold excess of blocking peptide produced no staining, confirming its specificity (Fig. 5C, F and I).

DISCUSSION

To identify proteins that interact with the cytoplasmic domain of BMPR-II, we performed a yeast two-hybrid screen of a lung cDNA library. The strongest protein interactor, and the most frequently observed, was Tctex-1, a 14 kDa light chain of dynein.

Cytoplasmic dynein is a retrograde microtubule motor that is required in a variety of cellular functions including mitotic cell division, positioning of cellular organelles and intracellular targeting of proteins (17). This multimeric complex is composed of two heavy chains with force-generating ATP-ase activity and a variety of intermediate and light chains (20). There is likely to be considerable subunit heterogeneity in different complexes as at least three heavy chains, five intermediate chains and three classes of light chains (LC8, LC7/roadblock, Tctex-1/Tctex-2) have been described, many of which have varied cellular expression patterns (20–22). Several of the intermediate and light chains are involved in cargo-binding activities, predominantly in concert with an accessory protein, dynactin, which associates with the IC74 class of intermediate chains (23). However, light chains can act as specific and direct adaptors for cargo, as demonstrated by Tctex-1, which interacts with and translocates the photoreceptor rhodopsin along microtubules by binding to its cytoplasmic tail (24). The TGF-β receptor complex has

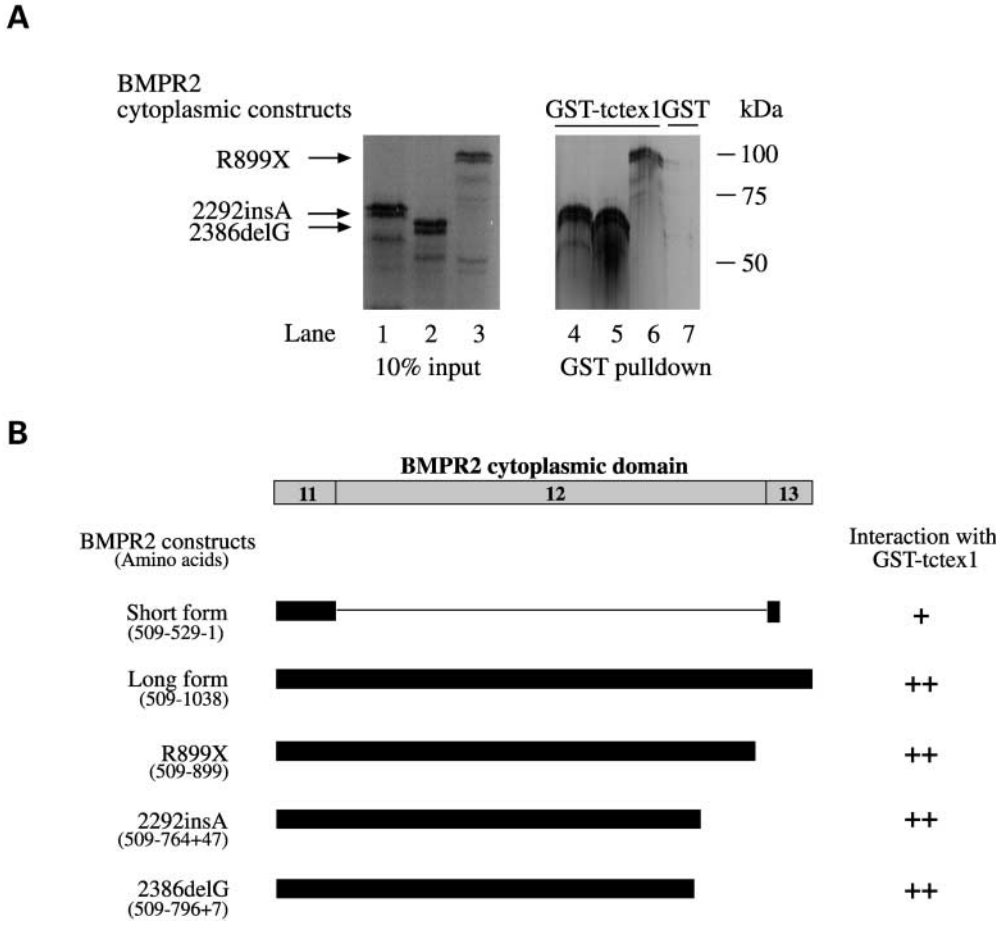

Figure 3. Interaction between BMPR-II mutants and Tctex-1. (**A**) *In vitro* translated, ^{35}S-labelled mutants within the cytoplasmic domain (CD) construct of BMPR-II (lanes 1–3) were incubated with purified GST-Tctex1 (lanes 4–6). A non-mutated version of the BMPR-II CD similarly translated (not shown) was incubated with GST alone (lane 7). The left panel shows 10% of the translated product used in the GST pull-down experiment. The right panel shows that all the mutated forms of the cytoplasmic domain were bound by the GST-Tctex1, whereas the wild-type CD had no affinity for GST alone. The horizontal arrows to the left of the gels indicate the full-length protein, the lower bands representing degradation products. Size ladder shown in kDa. (**B**) The filled grey bar represents the exons encoding the full-length cytoplasmic domain of the receptor. The horizontal black bars depict the two naturally occurring isoforms of BMPR-II as well as the three tested mutants. The predicted length, in amino acids, is presented in parentheses below the name of the construct. In the case of the mutations resulting in frameshifts, the expected number of aberrant amino acids added before the presence of a stop codon is indicated by the + sign. The observed magnitude of binding between Tctex-1 and the BMPR-II constructs is indicated (++ = strong/+ = weak).

also been shown to interact with cytoplasmic dynein by phosphorylating an 11 kDa light chain mLC7-1 on serine residues, to facilitate its regulation of TGF-β signalling by the activation of Jun N-terminal kinase (JNK), phosphorylation of c-Jun, and inhibition of epithelial cell growth (25).

Using a series of truncated constructs we inferred that Tctex-1 bound BMPR-II at its carboxy terminal end (Fig. 1). This region of the receptor contains consensus sites for casein kinase II and protein kinase C. The presence of amino acid sequence resembling the SS×S motif, phosphorylated in Smads by TGF-β receptors, led us to question whether the cargo specificity of this light chain might be a consequence of phosphorylation by BMPR-II and, hence, a mechanism by which this multi-functional pathway might be regulated (18).

The cargo binding and motor activities of dynein are known to be highly specific, both spatially and temporally. The molecular basis of this regulation is not fully understood, yet a number of mechanisms have been postulated (26). Functional specificity of dynein is dependent upon the subunit composition of the complex. For example, Tctex-1, but not its homologue RP3, is capable of binding the cytoplasmic tail of rhodopsin, ectopic expression of RP3 displaces Tctex-1 from the dynein complex, resulting in the disruption of rhodopsin trafficking (27). Regulation is also achieved by the phosphorylation of dynein subunits by proteins with kinase activity. Casein kinase II, a serine/threonine kinase, binds and phosphorylates the intermediate chain, a process believed to disrupt the association of dynactin with the intermediate chain and likely to lead to a loss of specificity for cargo recognized by the dynactin/dynein complex (28).

We next performed *in vitro* GST pull-down assays using a GST–Tctex1 fusion protein and a series of wild-type and mutant BMPR-II constructs. These data demonstrate that motifs of the cytoplasmic tail, encoded by exon 11 (amino acids 509–529) and exon 13 (amino acid 530) and present in the BMPR-II SF, are capable of binding Tctex-1, albeit at a

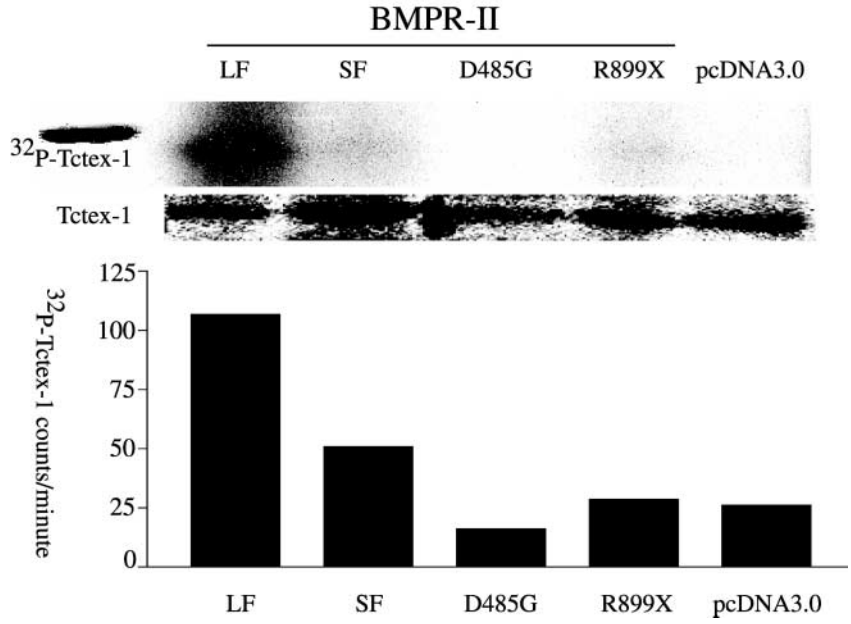

Figure 4. Phosphorylation of Tctex-1 by wild-type and mutant BMPR-II. The two isoforms of BMPR-II, LF and SF, a kinase mutant, D485G, and a truncation mutant of the cytoplasmic domain, R899X, were assayed for the ability to phosphorylate GST–Tctex1. GST–Tctex1 was resolved on a 12% polyacrylamide gel and incorporated ^{32}P detected by autoradiography. As indicated by the lower panel, equal concentrations of GST–Tctex1, stained with Coomassie blue, were used in each reaction. The level of phosphorylation, shown by the bar chart, was quantified by a measurement of the ^{32}P count/min. Relative to the empty vector pcDNA3.0, the long form phosphorylates Tctex-1 strongly, the short form has 30% this level of kinase activity and the mutant receptors are either incapable of (D485G), or highly deficient in phosphorylating Tctex-1.

much lower affinity than the LF (Fig. 2A). These results provide, for the first time, a function for the large cytoplasmic tail domain of BMPR-II, a unique feature of this receptor within the context of the TGF-β superfamily. Importantly, we show that constructs of truncating mutations of the cytoplasmic domain bind Tctex-1, implying that loss of interaction is not required for the pathogenesis of PPH (Fig. 3A).

Using GST binding assays, we have also demonstrated that the type I BMP receptors, BMPRIA and -IB, can bind Tctex-1 (Fig. 2B). These interactions might represent a cooperative intracellular association between independent components of the BMPR heteromeric complex and Tctex-1 that mirrors the mechanism of ligand binding by these receptors (29). However, the structural nature of receptor interaction with Tctex-1 is likely to differ between members of the receptor superfamily as the extraordinarily long cytoplasmic domain appears unique to BMPR-II.

To determine whether Tctex-1 is phosphorylated, and perhaps activated, by the BMP receptors, we performed *in vitro* kinase assays on the two BMPR-II isoforms. The BMPR-II LF phosphorylated Tctex-1 strongly, whereas SF activity was relatively weak. This was consistent with our premise, on the basis of the binding data, that the two isoforms of BMPR-II have distinct cellular profiles and, further, indicated that the entire cytoplasmic domain was required for effective phosphorylation of Tctex-1. To confirm this, we repeated the assay with the mutant receptors, R899X and R899P. The nonsense mutation represents the most distal truncation observed in a PPH patient whilst the amino acid substitution is the sole missense mutation thus far identified within exon 12. These constructs, although capable of binding Tctex-1, demonstrated a near complete loss of kinase activity with regard to the light chain. This observation implies that the intact cytoplasmic domain of the LF generates a three-dimensional conformation conducive to kinase phosphorylation activity. Truncation or the substitution of residues such as proline, an amino acid known to be required for structural integrity, disrupt this process (Fig. 4). As predicted, we found that a receptor with a missense mutation in the kinase domain was incapable of phosphorylating Tctex-1. Based upon these data, we can hypothesise that truncating mutations located proximal to the cytoplasmic tail would not be anticipated to bind dynein through Tctex-1 and we have previously shown that extracellular mutation within the ligand binding domain generates inactive receptor complexes incapable of transducing signal (16). Hence, the majority of pathogenic *BMPR2* mutations are predicted to result in loss of interaction with Tctex-1, suggesting that the functional relationship between these proteins is likely to be critical in the dynamic regulation of BMPR-II mediated signalling and hence of potential significance to the pathogenesis of PPH.

Immunohistochemistry in normal lung sections demonstrated that BMPR-II and Tctex-1 are expressed in the endothelium and smooth muscle of pulmonary arteries and arterioles, both key sites of the pathogenic vascular remodelling observed in PPH. Co-localization of both proteins was also observed in the capillaries of the lung parenchyma. The prominence of Tctex-1 staining, in conjunction with the type II receptor, might reflect the light chain composition of the motor complex in these cell types and facilitate tissue-specific BMPR-II signalling. Additionally, the presence of Tctex-1 within cells pathologically targeted in PPH adds weight to the potential importance of this protein in combination with BMPR-II in maintaining the normal pulmonary vascular state (Fig. 5).

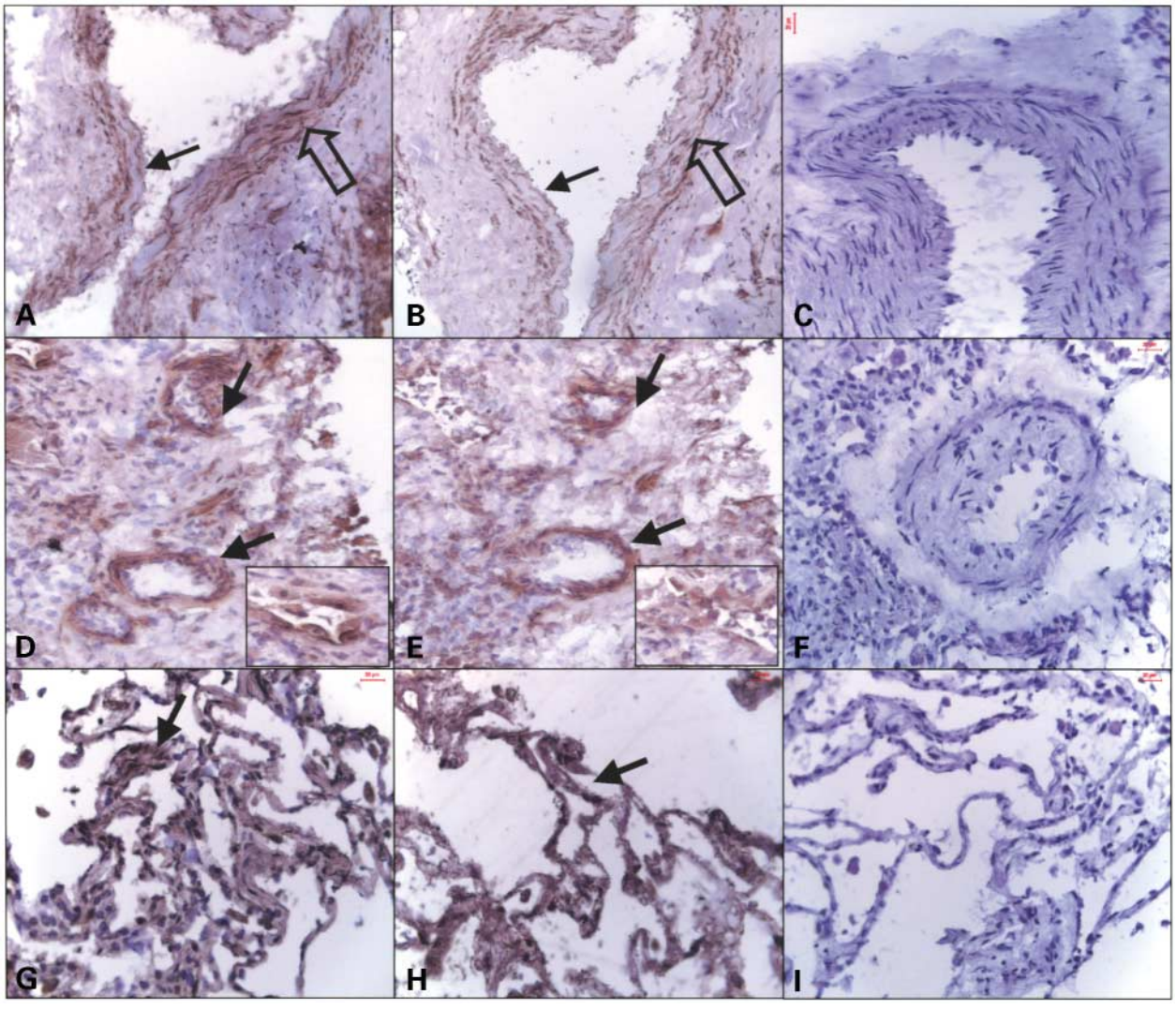

Figure 5. Photomicrographs of frozen sections of human lung immunostained with antibodies to BMPR-II (**A**, **D** and **G**) and Tctex-1 (**B**, **E** and **H**) showing co-localization to smooth muscle (open arrows) and endothelium (arrows) in a muscular pulmonary artery (A and B), in small peripheral arteries (D and E). High power insets (D and E) show endothelial co-localization. Staining is also apparent in the walls of alveoli (arrows) of lung parenchyma (G and H). The specificity of the Tctex-1 antibody is demonstrated by the lack of staining, when incubated with blocking peptide, in pulmonary arteries (**C**) arterioles (**F**) and lung parenchyma (**I**).

In summary, this study describes a novel interaction between BMPR-II and a 14 kDa dynein light chain Tctex-1, identified by means of a yeast two-hybrid screen and confirmed through *in vitro* assays. The region of Tctex-1 required for interaction was refined to the C-terminal 37 amino acids of the protein whilst the cytoplasmic domain of BMPR-II was demonstrated to be necessary for this interaction. Functional differences between the two isoforms of BMPR-II (i.e. SF and LF) were highlighted by the lower efficiency of the SF BMPR-II receptor to both bind and phosphorylate the light chain. We report that cytoplasmic tail mutations in the receptor, known to underlie PPH, do not adversely affect binding, but prevent phosphorylation of Tctex-1 indicating a structural requirement for interaction in this important cellular process. The co-expression of both proteins within endothelial and smooth muscle cells of pulmonary arterioles emphasizes the importance of this interaction in the pulmonary vasculature. As the ability of the dynein motor complex to recognize specific intracellular cargo is dependent on both the composition and phosphorylation of its constituent components, the cellular co-localization and functional interaction between Tctex-1 and BMPR-II supports the hypothesis that this light chain may be an early mediator of BMPR-II signalling (Fig. 6). Evidence exists to suggest that TGF-β signalling can be regulated by the sequestration of Smads 2, 3 and 4 along the microtubules (30). It is therefore conceivable that phosphorylation of the Tctex-1 dynein light chain by the BMPR-II receptor may trigger association with the motor complex, optimize its affinity for other mediators in the TGF-β pathway, notably Smad and $p38^{MAPK}$, facilitating the transport of these proteins along the microtubule network. Furthermore, disruption by mutations of *BMPR2* impede a functional consequence of this interaction, namely light chain

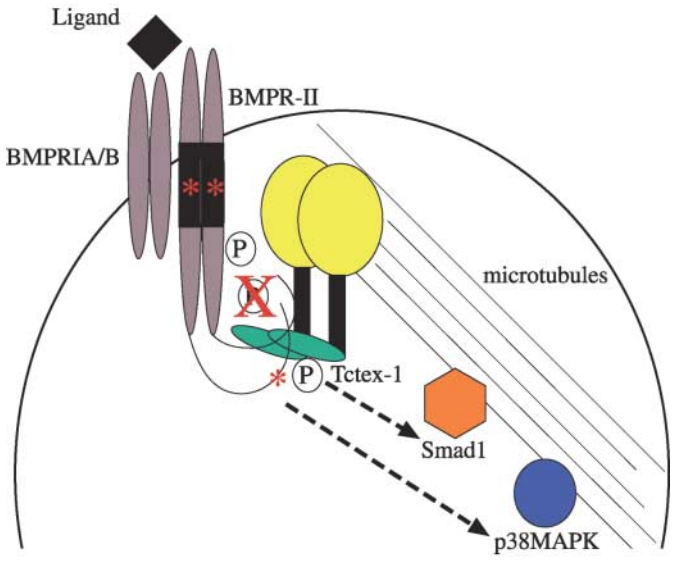

Figure 6. Representation of the interaction between BMPR-II and Tctex-1. The type II receptor, within the BMP receptor complex, depicted by the grey bars on the cell surface, binds Tctex-1 through its cytoplasmic tail. This interaction facilitates the phosphorylation, P, of Tctex-1, in complex with cytoplasmic dynein on the microtubules, through the kinase domain of BMPR-II. Mutations of *BMPR2*, shown by the asterisks, prevent phosphorylation of the light chain. When activated by BMPR-II, Tctex-1 may move along the microtubules and interact with downstream mediators of BMPR-II signalling, for example, Smad1 and $P38^{MAPK}$.

phosphorylation. Taken together these findings imply that the trafficking of intermediaries of BMPR-II signalling to and from the cell surface and the perturbation of these processes warrants further study and specifically in relation to the pathogenesis of the devastating vascular disorder, PPH.

MATERIALS AND METHODS

Plasmid constructs

All primer sequences used for PCR amplification and *in situ* mutagenesis are shown in Table 1. The two isoforms of BMPR-II, LF and SF, in the expression vector pcDNA3.0 were the kind gifts of Drs Kohei Miyazono and Petra Knaus, respectively. The bait construct was generated by PCR amplification through amino acids 209–1038 using the primers Baitf and Baitr with the long isoform of *BMPR2* cDNA as template. This product was digested with *Eco*RI and *Bam*HI and ligated into the same sites in the plasmid pGBDUc1. Full-length Tctex-1, containing the open reading frame alone (amino acids 1–113) was also produced by PCR using the primers Tctex1Af and Tctex1Ar. The template used was the longest Tctex-1 library clone isolated in the yeast two-hybrid screen. The deletion constructs B (amino acids 1–75), C (amino acids 1–43) and D (amino acids 1–26) were amplified using the primer pairs Tctex1Af with -Br, -Cr and -Dr, respectively. Construct E (amino acids 18–113) was produced by use of the primers Tctex1Ef and Tctex1Ar. These products were digested with the restriction enzymes *Eco*RI and *Xho*I and ligated, in the correct frame to the activation domain of pACT2, the lung cDNA library vector. The GST fusion construct of Tctex-1 was produced by generating a PCR product, from the Tctex-1 library clone with the primers GST-tctex1f and GST-tctex1r, which was ligated into the plasmid pGEX-4T1 (Amersham-Pharmacia) using the restriction sites *Eco*RI and *Bam*HI. The BMPR-II kinase domain (KD) was created by PCR, using the primers BMPR2KDf and BMPR2KDr with the LF cDNA as template. This product was ligated into pcDNA3.0 at the restriction sites *Bam*HI and *Xho*I. As the cytoplasmic domain (CD) could not be directly amplified by PCR, it was constructed in two parts by taking advantage of a unique *Hpa*I site 930 nucleotides from the start of the domain. This segment of DNA was amplified

Table 1. Primers used in cloning and mutagenesis

Primer name	Sequence
Baitf	GATCGAATTCATTGGCCGAGGTCGATATGC
Baitr	GATCGGATCCTCACAGACAGTTCATTCCTA
Tctex1Af	GATCGAATTCGCATGGAAGACTACCAGGCTGC GGA
Tctex1Ar	GATCCTCGAGTCAAATAGACAGTCCGAAGGCA
Tctex1Br	GATCCTCGAGTCCAGCTCCATTCTTCTGCAT
Tctex1Cr	GATCCTCGAGGGTCCACTGGTTCACTTTGCT
Tctex1Dr	GATCCTCGAGTTCTATAGCCTCTTTTACAAT
Tctex1Ef	GATCGAATTCGCAACATTGTAAAAGAGGCTATA GAA
GST-tctex1f	GATCGGATCCATGGAAGACTACCAGGCTGC
GST-tctex1r	GATCGAATTCTCAAATAGACAGTCCGAAGG
BMPR2KDf	GATCGGATCCGCCACCATGAATCTGAAACTGT TGGAGCT
BMPR2KDr	GATCCTCGAGTCAAATCATCATAAGTTCAGCCA
BMPR2CDHpaIf	GATCGGATCCGCCACCATGTGGGAAAGAAACA AATCTGT
BMPR2CDHpaIr	GATCGTTAACACTGTGGTTTCTACCTGCCACA
2292insAf	CTTTGAACACCAAAAAATTCAACAAAAGAGCC CCGGC
2292insAr	GCCGGGGCTCTTTTTTTGAATTTTTTGGTGTTC AAAG
2386delGf	GATGAATACAATCAATCAGCAGAACCTCATGTG
2386delGr	CACATGAGGTTCTGCTGATTGATTGTATTCATC

using the primers BMPR2CDHpaIf and BMPR2CDHpaIr. The PCR fragment was digested with *Bam*HI and *Hpa*I and used to replace the preceding 2455 nucleotides from the LF *BMPR2* cDNA insert in pcDNA3.0 to engineer a wild-type cytoplasmic domain construct. The cytoplasmic domain mutant, R899X, was produced by ligating the 930 bp PCR product into a full-length *BMPR2* cDNA containing the mutation. Other CD mutants, 2292insA and 2386delG, were generated by performing site-directed mutagenesis with the QuickChange kit (Stratagene) on the wild-type CD construct using the primer pairs listed in Table 1. All constructs were verified by direct sequencing on an Applied Biosystems 377 sequencer using the Applied Biosystems BigDye Terminator sequencing kit (Perkin-Elmer). The LF mutants, D485G, R899X and R899P were made as previously described (16).

Yeast two-hybrid screen

The yeast strain PJ69-4a (31) was transformed, in a sequential manner, with first the bait plasmid and subsequently lung library cDNA using a protocol devised by Agatep *et al.* (32). The yeast strain, containing the bait plasmid, was maintained by plating onto SD yeast media lacking uracil and transformed with 60 µg of DNA from an adult lung cDNA library (Clontech). Interacting clones were selected for by plating the transformants onto SD/-URA/-LEU/-HIS/-ADE supplemented with 25 mM 3-aminotriazole. To determine the strength of interaction between positive clones, a beta-galactosidase agar overlay assay was performed using the method described by Duttweiler (33). The bait plasmid was removed from the yeast strain by growth on SD/-LEU containing 1 g/l 5-fluoro-orotic acid, which negatively selects against plasmids bearing the uracil encoding gene. The library plasmids were subsequently isolated using a Zymoprep kit (ZymoResearch). All library plasmid inserts were sequenced using a primer to the Gal4 activation domain (5′TACCACTACAATGGATGATGT-3′). The resultant sequences were checked for homology to known transcripts in the GenBank database using the BLAST alogorithm (www.ncbi.nlm.nih.gov/BLAST).

Direct interaction growth assays using this system were performed by transforming Tctex-1 constructs into yeast containing the BMPR-II bait and streaking the transformants onto selective media. Interaction between the clones was inferred by growth on this media.

GST pull-down assays

The GST-Tctex1 construct was transformed into the *E. coli* strain BL21 and expression of the fusion protein induced by the addition of 0.3 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 3 h at 37°C. After lysing the bacterial cells by sonication, the GST fusion protein was purified by incubation with washed glutathione–Sepharose 4B beads (Amersham-Pharmacia) as per the manufacturer's protocol. Using the wheat germ TnT T7-coupled transcription/translation system (Promega), BMPR proteins were labelled with ^{35}S-methionine and *in vitro* translated from the relevant pcDNA3.0 vector following the manufacturer's instructions. Between 10 and 20 µg of the purified GST fusion protein was added to 20 µl of radiolabelled protein, 100 mM PMSF and protease inhibitor cocktail (Roche) at a 7 × concentration. The final volume was adjusted to 750 µl with NETN buffer (0.5% Nonidet P-40, 1 mM EDTA, 20 mM Tris–HCL pH 8, 100 mM NaCl). This mixture was rotated at 4°C for 90 min and subjected to three washes in NETN buffer. The proteins were boiled for 5 min in 2 × Laemmli buffer to disassociate them from the beads and resolved on 10% polyacrylamide gels. Concurrently, a 10% volume of the *in vitro* translated protein used in the GST pull-down was similarly resolved. The gels were stained in Coomassie buffer and exposed to autoradiograph film for up to 5 days.

In vitro kinase assays

HeLa cells were transfected in 10 cm dishes with 20 µg myc-BMPR2-pcDNA3.0 wild type using Lipofectamine 2000 (Invitrogen) as per the manufacturer's protocol. Forty-eight hours post-transfection, cell protein was harvested using kinase lysis buffer (50 mM Hepes, 100 mM NaCl, 5% NP-40, 10 mM EDTA, 1 mM PMSF, 2.5 mM $NaVO_4$, 50 mM NaF, 1mM DTT, 20 mM β-glycerolphosphate, 1 µM pepstatin, and 10 µg/µl each of leupeptin, antipain and aprotinin), incubated on ice for 1 h followed by centrifugation at 13 000 rpm for 20 minutes at 4°C. Samples were then incubated with antibody [mouse anti-myc 9E10 (Santa Cruz), 1:50 dilution; mouse anti-HA clone HA-7 (Sigma), 1:100 dilution] on a rotary mixer for 2 h at 4°C. Pre-washed Protein-G sepharose beads (Amersham) were then added and the mixture incubated on the rotary mixer overnight at 4°C. Protein–bead complexes were washed three times with kinase lysis buffer, excluding detergent at 2500 g for 2 min at 4°C. GST-Tctex1 fusion protein beads were produced as described above.

Kinase reactions were prepared on ice, comprising 10 µl BMP receptor kinase protein, 15 µl Tctex-1 substrate protein, 15 µl kinase assay buffer (20 mM Tris–HCl, pH7.5, 75 mM $NaCl_2$, 100 mM β-glycerolphosphate, 10 mM $MgCl_2$, 10 mM $MnCl_2$, and protease inhibitors as kinase lysis buffer), and 2 µCi of ^{32}P-γATP (Amersham). Reactions were incubated at 30°C for 30 min and subsequently terminated by boiling for 5 min in 5× Laemmli buffer. The protein was resolved on 12% SDS–PAGE gels, fixed and stained in Coomassie buffer, dried and put down to autoradiography for 48 h. Phospho-image analysis was subsequently performed on the specific gel protein substrate bands.

Tissue preparation and immunohistochemistry

Frozen sections of normal lung tissue were cut at 6 µm and fixed in acetone for 6 min at room temperature. Sections were either incubated with the polyclonal anti-Tctex-1 (kindly donated by Dr Stephen M. King) or anti-BMPR-II, which was raised against a peptide corresponding to amino acid residues 185–202 of the 1038-amino acid BMPR-II sequence. Anti-CD31 and anti-smooth muscle actin (SmA) antibodies (DakoCytomation, Ely, UK) were used to localize endothelial and smooth muscle cells, respectively. Primary antibodies were incubated for 1 h at room temperature. Antigens were visualized with a streptavidin biotin peroxidase technique in line with the manufacturer's recommendation (DakoCytomation, Ely, UK). Positive staining was visualized with 3′3 diaminobenzidine, producing a brown reaction product. The specificity of immunostaining was

demonstrated by the absence of signal in sections processed after the omission of primary antibody and by incubation of the Tctex-1 antibody with a 10-fold excess of blocking peptide.

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