respect the D–V compartment border from either the dorsal (16 out of 16) or the ventral (16 out of 16) side (Fig. 4c), and clones that are produced early enough to be of mixed dorsal and ventral identity can extend across the D–V border without disrupting compartmentalization (14 out of 16) (Fig. 4d).

As the edge of *ap* expression is straight within a broader region of Notch activation (Fig. 4d), there must exist additional mechanisms that contribute to compartmentalization. To examine whether Fng participates in these additional mechanisms, we analysed Flip-out clones co-expressing Fng and activated Notch. If the only role of Fng in D–V compartmentalization were to position Notch activation, then ubiquitous Notch activation should fully suppress the effects of Fng. However, ventral cells co-expressing Fng and activated Notch exhibited a slight but consistent (29 out of 36 clones) extension into the dorsal compartment (Fig. 4e, f). As Wg expression suggests that our activated-Notch clones have levels of Notch signalling similar to that at the normal D–V boundary (Fig. 4c, d), this result suggests that Fng may have an affect on D–V compartmentalization that is separate from its influence on Notch activation.

Studies in the *Drosophila* wing and abdomen have revealed that signalling from posterior to anterior cells has an essential role in effecting anterior–posterior compartmentalization, but that additional factors also contribute to this process[25–27]. Similarly, our results imply that multiple mechanisms contribute to D–V compartmentalization. The primary mechanism is establishment of a stripe of Notch activation along the interface between dorsal and ventral cells. Fng positions this stripe of Notch activation, but it also seems to participate in a secondary mechanism that can influence cell behaviours within a larger region of Notch activation. In the wing, this secondary mechanism may help to ensure the precise register of the compartment border with *ap* expression[28]. Fng-dependent separation of adjacent cell populations will probably also be important for the development of other tissues in which *Fng*-related genes are involved in making developmental boundaries[29]. □

Methods

fng^{13}, Dl^{rev10} Ser^{RX106}, $Notch^{55e11}$ or $Notch^{CO}$ mutant clones were generated 52–72 h after egg laying by Flipase-mediated mitotic recombination[18]. Ectopic expression of Fng, activated Notch or Ser was performed using the UAS-Gal4 and Flip-out systems to make clones of cells expressing Gal4 in animals with *UAS-fng27*, *UAS-Ser* or *UAS-ΔN34a* transgenes[18]. Immunostaining was performed as described previously[10,18], with the addition of *ap* detection using the *ap–lacZ* enhancer trap line *rk568* (ref. 6), and immunostaining of $Notch^{55e11}$ or $Notch^{CO}$ clones with the Notch monoclonal C458.2H.

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Silencing of TGF-β signalling by the pseudoreceptor BAMBI

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Members of the transforming growth factor-β (TGF-β) superfamily, including TGF-β, bone morphogenetic proteins (BMPs), activins and nodals, are vital for regulating growth and differentiation[1]. These growth factors transduce their signals through pairs of transmembrane type I and type II receptor kinases[2–4]. Here, we have cloned a transmembrane protein, BAMBI, which is related to TGF-β-family type I receptors but lacks an intracellular kinase domain. We show that BAMBI is co-

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expressed with the ventralizing morphogen BMP4 (refs 5, 6) during *Xenopus* embryogenesis and that it requires BMP signalling for its expression. The protein stably associates with TGF-β-family receptors and inhibits BMP and activin as well as TGF-β signalling. Finally, we provide evidence that BAMBI's inhibitory effects are mediated by its intracellular domain, which resembles the homodimerization interface of a type I receptor and prevents the formation of receptor complexes. The results indicate that BAMBI negatively regulates TGF-β-family signalling by a regulatory mechanism involving the interaction of signalling receptors with a pseudoreceptor.

In an expression screen for components involved in BMP4 signalling[7], we identified a novel *Xenopus* complementary DNA whose expression pattern is very similar to that of BMP4 (Fig. 1b). The predicted gene product, named BAMBI (BMP and activin membrane-bound inhibitor), shows 89% amino-acid-sequence similarity and 83% identity to *nma*, a human gene without known function which is downregulated in metastatic melanoma cell lines[8]. BAMBI encodes a putative transmembrane protein of 260 amino acids with an extracellular domain that is closely related to those of type I TGF-β receptors, and among these shows most similarity (53%) to *Xenopus* BMP receptor type-I (BMPR-I; Fig. 1a)[9,10]. Unlike all other known TGF-β type I or type II receptors, the intracellular domain of BAMBI is relatively short and does not encode a serine/threonine-kinase domain, although it shares some sequence homology with the E6 and catalytic loops of receptor serine/threonine kinases (see Supplementary Information). The results indicate that BAMBI encodes a novel TGF-β receptor-related protein.

Embryonic expression of BAMBI closely follows that of *Xenopus* BMP4 (Fig. 1b)[11,12]. Using polymerase chain reaction with reverse transcription (RT-PCR) we can detect a maternal BAMBI transcript before the onset of zygotic transcription (stage 9), but the highest expression is observed during gastrulation and neurulation (Fig. 1c). RT-PCR analysis shows that BAMBI is induced in dorsal marginal zone (DMZ) explants from *Xenopus* embryos microinjected with BMP4 messenger RNA (Fig. 1d). BMP signalling is also required for BAMBI, as its expression is downregulated in ventral marginal zone (VMZ) explants from embryos microinjected with a dominant-negative BMP2/4 receptor that prevents BMP signalling[10] (Fig. 1d). We conclude that BAMBI is co-expressed with and regulated by BMP4.

These properties indicated that BAMBI might affect BMP signalling in *Xenopus* embryos. RT-PCR analysis (Fig. 2a) shows that microinjection of BAMBI mRNA interferes in animal cap assays with *Xbra* induction by BMP4 or by activin, but not by FGF. To corroborate these findings we carried out phenotypic rescue assays. Activin can induce mesoderm in animal caps, which leads to a

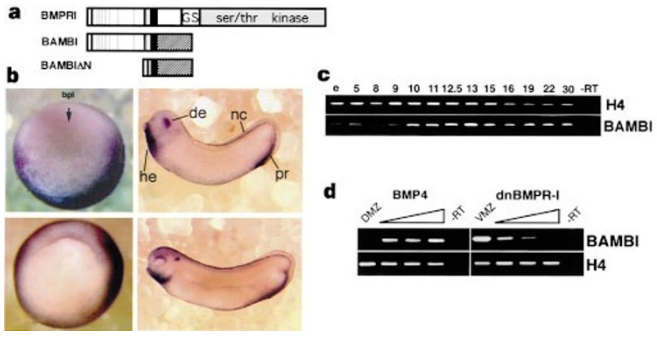

Figure 1 BAMBI is related to BMPR-I and is regulated by BMP4. **a**, Drawing of BMPR-I and BAMBI. Left to right: signal sequence (grey box); putative extracellular domains (white box) containing seven conserved cysteines (vertical lines) and cysteine box (thick vertical line); transmembrane domain (black box); GS and intracellular Ser/Thr-kinase domains of BMPR-I; and intracellular domain of BAMBI (hatched). In BAMBIΔN the extracellular domain is deleted. **b**, Co-expression of *BAMBI* (top) and BMP4 (bottom) in *Xenopus* grastrulae (stage 10; left) and tadpoles (right). Embryos are shown dorsal side up in vegetal view (gastrulae) or lateral view (tadpoles). bpl, dorsal blastopore lip; de, dorsal eye; he, heart, nc, neural crest; pr, proctodeum. **c**, RT-PCR analysis of BAMBI expression in embryos of the indicated stages. –RT, minus reverse transcription control sample; H4, histone H4 for normalization. **d**, DMZ and VMZ assays: 4-cell embryos were uninjected or microinjected into each blastomere with 0.025, 0.1 or 0.3 ng BMP4, or 0.1, 0.25 or 0.5 ng dominant-negative BMP2/4 receptor (dnBMPR-I) mRNA, as indicated. Dorsal (DMZ) or ventral marginal zone (VMZ) fragments were cut from early gastrulae and analysed for induction of BAMBI at stage 10.5 by RT-PCR.

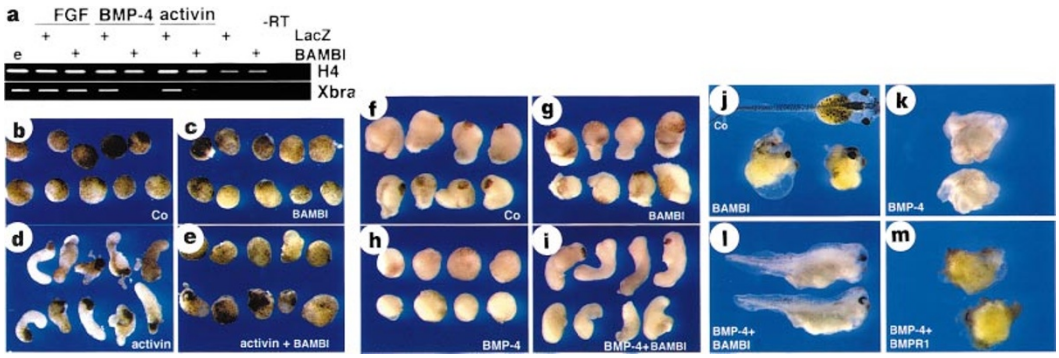

Figure 2 BAMBI inhibits BMP and activin signalling in *Xenopus* embryos. **a**, 4-cell embryos were microinjected into all blastomeres with 0.75 ng per blastomere control (lacZ) or BAMBI mRNA. Animal caps were excised from blastula embryos and cultivated with 1 μg ml^{-1} bFGF, 1 μg ml^{-1} BMP4 or 20 ng ml^{-1} activin as indicated, until stage 10. Expression of histone 4 (H4, for normalization) and the mesodermal marker Xbra were analysed by RT-PCR. **b**–**e**, Animal cap assays. 4-cell embryos were microinjected into all blastomeres with 0.5 ng per blastomere control (lacZ) or BAMBI mRNA with or without 0.1 ng activin mRNA as indicated. Animal caps were excised from blastula embryos and cultivated until stage 20 to score elongation of explants, indicative of mesoderm induction. **f**–**i**, DMZ assays. 4-cell embryos were microinjected into each blastomere with 0.5 ng control (lacZ) or 0.4 ng BAMBI, with or without 0.4 ng BMP4 mRNA per blastomere as indicated. DMZs were excised from gastrula embryos and cultivated until stage 20 to score cement-gland formation (brown tissue) and elongation of explants, indicative of dorsal mesoderm differentiation. **j**, 4-cell embryos were uninjected (top) or microinjected into each blastomere with 0.8 ng BAMBI mRNA, leading to gastrulation defects, spina bifida and enlarged heads (63%; $n = 153$). **k**–**m**, Rescue of BMP4-injected embryos. 4-cell embryos were microinjected into each blastomere with 0.5 ng BMP4, with or without 0.5 ng BAMBI or BMPR-I mRNA. Note loss of head and axial structures following BMP4 injection, which is rescued from a dorsanterior index (DAI) of 1.7 ($n = 68$) to 3.1 ($n = 72$) by co-injection of BAMBI but not BMPR-I (DAI = 1.5; $n = 71$).

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characteristic elongation of the explants (Fig. 2b, d). Co-expression of activin and BAMBI prevents this elongation, indicating inhibition of activin signalling (Fig. 2c, e). In a similar assay, DMZ explants will autonomously elongate and differentiate dark cement-gland tissue, and this can be inhibited by microinjection of BMP4 mRNA (Fig. 2f, h). Co-injection of BMP4 with BAMBI mRNA reverts this phenotype, leading to elongation and cement-gland differentiation (Fig. 2g, i).

As BAMBI can interfere with activin and BMP4 signalling in explant assays, we tested its effects on embryonic development. Embryos microinjected with BAMBI mRNA show a weak dorso-anteriorized phenotype with enlarged heads, and at higher doses they suffer from gastrulation defects resulting in truncated trunk/tail structures (Fig. 2j). The dorso-anteriorized phenotype is consistent with BAMBI's inhibiting BMP signalling, which normally antagonizes dorso-anterior structures[5,6]. Consistent with this, co-injection of BAMBI, but not BMPR-I, mRNA rescues BMP4-ventralized embryos, resulting in fairly normal tadpoles (Fig. 2k–m). In conclusion, the effects observed following overexpression in embryonic explants and whole embryos indicate that BAMBI antagonizes BMP and activin signalling.

To test whether BAMBI has any effect on BMP signalling through endogenous receptors in mammalian cells, the BMP-responsive reporter pVent-Luc[13] was transfected into mouse embryonic carcinoma P19 cells with or without BAMBI. BMP2 induced expression of this reporter in P19 cells, as previously shown[14], and BAMBI significantly inhibited this effect (Fig. 3a). A construct BAMBIΔN with its extracellular domain deleted did not affect endogenous BMP signalling but reversed the inhibitory effects of wild-type BAMBI (Fig. 3a), by acting as a dominant-negative variant (see below). In similar experiments using the activin/TGF-β-responsive Mix.2 ARE reporter pA3-Luc[15], BAMBI blocked the activation of this reporter elicited by activin through endogenous receptors in P19 cells (Fig. 3b).

To extend these observations to other type I receptors, and to distinguish between a ligand-dependent and a ligand-independent mode of action for BAMBI, we tested the effect of BAMBI on signalling by constitutively active forms of the type I receptors. We tested the BMP2/4 type I receptors BMPR-IA and BMPR-IB (also known as ALK3 and ALK6, respectively), the orphan receptor ALK1, the putative BMP7 receptor ALK2, the TGF-β type I receptor TβR-I (also known as ALK5) and the activin type I receptor ActR-IB (also known as ALK4) (Fig. 3c, d). Transfection of constitutively active BMPR-IA, BMPR-IB, ALK1 or ALK2 into P19 cells activated the pVent-Luc reporter[16] (Fig. 3c), whereas transfection of constitutively active TβR-I or ActR-IB activated the pA3-Luc reporter (Fig. 3d). Co-expression of BAMBI decreased the activation of these reporter genes by the constitutively active type I receptors (Fig. 3c, d). The ability of BAMBI to inhibit signalling by constitutively active type I receptors indicates that BAMBI may not need to interfere with ligand binding in order to inhibit receptor signalling.

We used co-immunoprecipitation to investigate whether BAMBI can interact with TGF-β receptors. We found an interaction between BAMBI and all of the type I receptors except ALK2, as

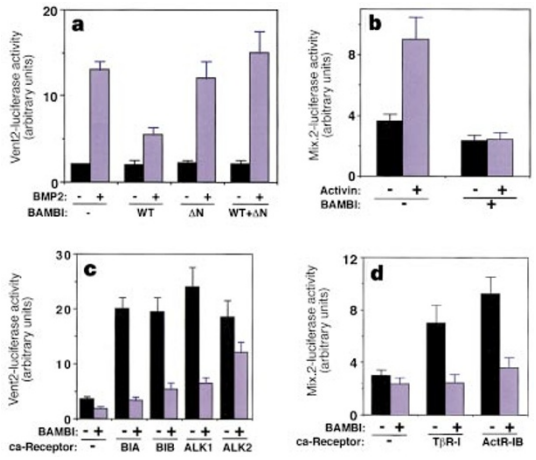

Figure 3 BAMBI inhibits ligand-independent signalling by TGF-β family receptors. The pVent2-Luc reporter (**a**, **c**) or the pA3-Luc reporter (**b**, **d**) were transiently transfected into P19 cells together, with or without BAMBI, BAMBIΔN (ΔN) or constitutively active type I receptor constructs (ca-receptor), as indicated. After 24 h, cells received 0.5 nM BMP2 (**a**), 2 nM activin (**b**) or no additions. Luciferase activity was determined 20 h later. Otherwise, luciferase activity was measured 2 days after transfection (**b**, **d**). Data are the average of three or more assays ± s.d.. BIA, BIB are constitutively active BMPR-IA and BMPR-IB, respectively.

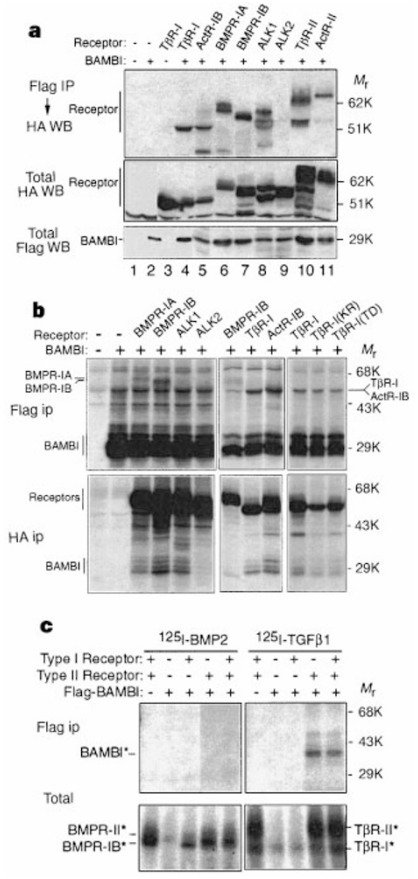

Figure 4 BAMBI interacts with TGF-β receptors. **a**, BAMBI binds to TGF-β receptors. To reveal receptor–BAMBI complexes, the lysates from COS1 cells transfected with the C-terminally HA-tagged type I receptors and C-terminally Flag-tagged BAMBI were immunoprecipitated with anti-Flag M2 antibody and analysed by western blotting with anti-HA polyclonal antibody (top). The expression of receptors and BAMBI was checked by western blotting of total cell lysate (5% aliquots) with anti-HA and anti-Flag antibodies, respectively (middle and bottom). **b**, Cells transfected with various constructs as indicated were labelled with [^{35}S]Met/Cys for 3 h and lysed. Two-thirds of the lysate were subjected to anti-Flag immunoprecipitation and one-third was subjected to anti-HA immunoprecipitation. Proteins were visualized by SDS-PAGE and fluorography for 16 h (middle and right) or 72 h (left). **c**, Transfected cells were incubated with ^{125}I-BMP2 or ^{125}I-TGF-β1 and the crosslinking agent DSS. Affinity-labelled cell lysates were subjected to anti-Flag immunoprecipitation and visualized by SDS-PAGE and autoradiography. The lower panels show receptor labelling by analysing aliquots of total cell lysate. Names with asterisks indicate the positions corresponding to BAMBI–ligand and receptor–ligand crosslinking products (ligand monomer M_r = 13K). BAMBI levels were similar in all conditions, as determined by anti-Flag immunoblotting (not shown).

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well as an interaction with the type II receptors TβR-II and ActR-II (Fig. 4a). Anti-Flag immunoprecipitations from the metabolically labelled transfectants (Fig. 4b) showed that Flag–BAMBI associates with TβR-I, ActR-IB, BMPR-IB and BMPR-IA (in order of affinity). Anti-HA immunoprecipitations revealed that BAMBI associates with TβR-I, ActR-IB, BMPR-IB, ALK1 and BMPR-IA (in order of affinity), and does not interact with ALK2. BAMBI also interacts with the constitutively active TβR-I mutant TβR-I(TD) and with the kinase-inactive mutant TβR-I(KR) (Fig. 4b).

BAMBI alone did not bind [^{125}I]BMP2 or [^{125}I]TGF-β1, as determined using a receptor affinity-labelling protocol[17]. However, BAMBI contacted TGF-β1 when co-expressed with the TGF-β type

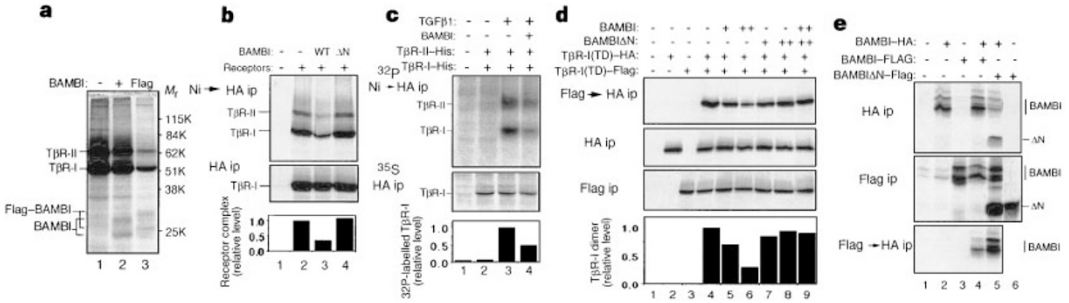
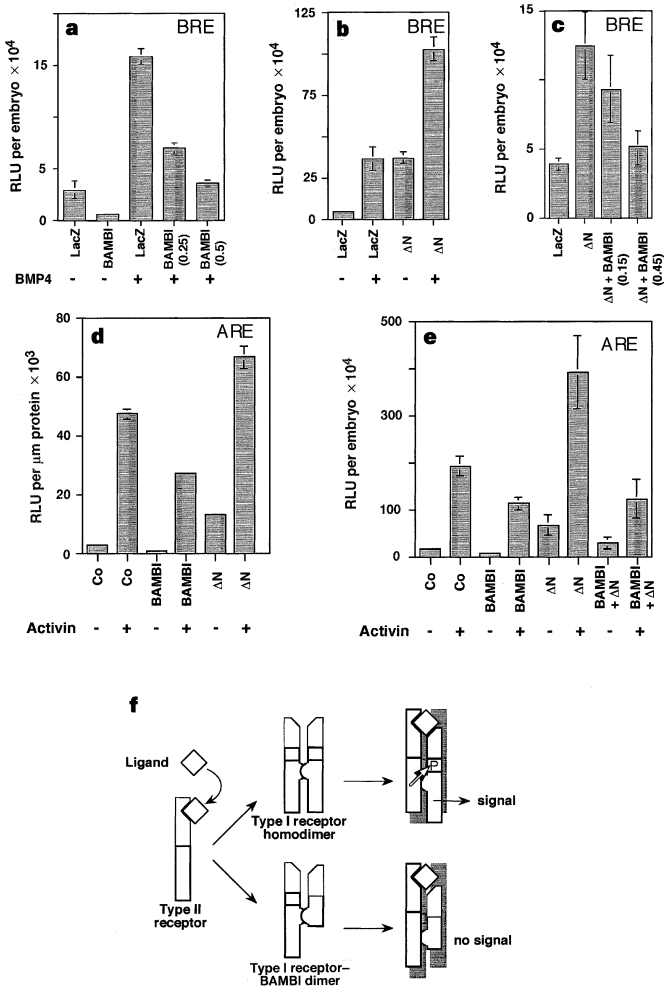


Figure 5 BAMBI inhibits formation of functional receptor complex. **a**, BAMBI is incorporated into a receptor complex. Cells transfected with TβR-II and TβR-I, with or without BAMBI, were treated with TGF-β and labelled with [^{35}S]Met/Cys. Then receptor complexes were isolated by sequential precipitation using a hexahistidine tag in the TβR-II construct (TβR-II–his) and an HA-tag in the TβR-I construct (TβR-I–HA) (lanes 1 and 2), or using a hexahistidine tag in TβR-II and a Flag tag in the BAMBI construct (lane 3). **b**, **c**, BAMBI inhibits receptor-complex formation. Cells transfected with TβR-II–his and TβR-I–HA with various BAMBI, as indicated, were treated with TGFβ and metabolically labelled with [^{35}S]Met/Cys (**b**) or [^{32}P]orthophosphate (**c**). Receptor complexes were purified as in **a**. TβR-I expression was confirmed by anti-HA immunoprecipitation. Histograms show levels of receptor complexes or ^{32}P-labelled TβR-I. **d**, BAMBI inhibits TβR-I(TD) homocomplex formation. Cells transfected with various constructs were labelled with [^{35}S]Met/Cys and TβR-I(TD) homocomplex was purified by sequential immunoprecipitation with anti-Flag and then with anti-HA antibodies. Receptor expression was confirmed by anti-HA or anti-Flag immunoprecipitation. Histograms show quantification of homocomplex level. **e**, BAMBI forms a homocomplex. Cells transfected with various constructs were labelled with [^{35}S]Met/Cys and BAMBI homocomplex was purified by sequential immunoprecipitation with anti-Flag and then with anti-HA antibodies. BAMBI expression was confirmed by anti-HA or anti-Flag immunoprecipitation.



◀ **Figure 6** The intracellular domain of BAMBI mediates inhibition of TGF-β family signalling in *Xenopus*. **a**–**c**, 4-cell embryos were microinjected into each blastomere with 25 pg of the BMP-responsive reporter plasmid pVent2-Luc (BRE) and the following mRNAs (in ng per blastomere) as indicated: 1.0 control (lacZ), 0.5 BMP4, 0.5 BAMBI (unless indicated otherwise in parenthesis), 0.5 BAMBIΔN. At stage 10, luciferase assays were carried out with extracts of whole embryos at least in triplicate. RLU, relative light units. **d**, **e**, 4-cell embryos were microinjected animally (**d**) or equatorially (**e**) into each blastomere with 25 pg of the activin-responsive reporter plasmid pXFD1′Luc-i (ARE) and the following mRNAs in ng per blastomere as indicated: 0.5 control (lacZ or PPL), 0.15 activin, 0.5 BAMBI, 0.25 BAMBIΔN. At gastrula stage, animal caps were excised (**d**) or whole embryos were sampled (**e**) and luciferase and total protein assays carried out. Luciferase activity in RLU is normalized to protein (**d**) or to embryos (**e**). **f**, Model for silencing by BAMBI. Top: following ligand binding, type II receptors associate with preassembled type I receptor homodimers, leading to receptor transphosphorylation and TGF-β signal transduction. Bottom: its extracellular domain allows BAMBI to dock to the receptor complex while the intracellular domain interferes with homodimeric type I receptor interactions[19,20,29,30], by intercalating between and/or outcompeting receptor subunits.

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II receptor (TβR-II) (Fig. 4c, top). As TβR-II is a primary ligand-binding component in the TGF-β receptor complex[18], this result indicates that BAMBI may be incorporated into complexes with TβR-II. We confirmed this by analysing the association of BAMBI with cotransfected TβR-I and TβR-II in the presence of ligand. Using a protocol to isolate the heterotetrameric TβR-I/TβR-II receptor complex[18], we found that BAMBI is coprecipitated with this complex (Fig. 5a, lane 2). Conversely, TβR-I and TβR-II can be co-immunoprecipitated with Flag-tagged BAMBI (Fig. 5a, lane 3).

Expression of BAMBI caused a decrease in TβR-I/TβR-II complex (Fig. 5b) and in the amount of phosphorylated TβR-I in this complex (Fig. 5c). These effects may underlie the ability of BAMBI to inhibit TGF-β signalling, although interference with type II receptors is not excluded. BAMBI pre-assembled with TβR-I may decrease the level of TβR-I/TβR-II complex by taking the place of one TβR-I molecule or decreasing the stability of this complex. Furthermore, the ability of BAMBI to interact with type I TGF-β receptors may also explain its ability to inhibit signalling by constitutively active forms of these receptors: BAMBI inhibits the dimerization of TβR-I(TD) (Fig. 5d). Dimerization is required for signalling by TβR-I(TD)[19,20].

To study the intracellular domain of BAMBI further, we analysed BAMBIΔN (Fig. 1a). BAMBIΔN had no significant effect on the BMP2 response in P19 cells, but neutralized the inhibitory effect of wild-type BAMBI in these cells (Fig. 3a). BAMBI dimerizes with itself and also with BAMBIΔN (Fig. 5e), which indicates that BAMBIΔN may work by sequestering wild-type BAMBI. Indeed, BAMBIΔN does not interact with TβR-I (data not shown), but prevents BAMBI from inhibiting the dimerization of TβR-I(TD) (Fig. 5d, lane 9). Collectively, these results indicate that BAMBIΔN may act as a dominant-negative BAMBI inhibitor.

We used the dominant-negative BAMBIΔN to assess the role of endogenous BAMBI in *Xenopus* embryos. Figure 6a shows that basal luciferase activity due to endogenous BMP signalling and BMP4-stimulated luciferase activity are both inhibited by BAMBI. In contrast to the wild type, BAMBIΔN strongly activates endogenous BMP signalling and BMP4-induced luciferase activity (Fig. 6b). Similarly, wild-type BAMBI inhibits endogenous and activin-induced luciferase activity in animal caps and whole embryos (Fig. 6d, e). In contrast, BAMBIΔN activates endogenous and activin-induced luciferase activity. The activating effect of BAMBIΔN on BMP and activin signalling is specific, as it can be reversed by co-expression with the wild-type mRNA in both cases (Fig. 6c, e). The results indicate that both BMP and activin-like signalling are subject to negative, BAMBI-dependent regulation in *Xenopus* embryos.

Figure 6f shows a model of BAMBI function. Signalling by BAMBI may be relevant for limiting the signalling range of BMPs and activins, which are thought to act as morphogens during early embryogenesis[5]. □

Methods

In vitro fertilization, embryo culture, staging, microinjection, culture of explants and whole-mount *in situ* hybridization were carried out as described[21].

Biochemical assays

Epitope-tag antibodies were from Santa Cruz Biotechnology. Metabolic labelling, receptor-affinity labelling, immunoprecipitation and receptor-complex isolation were performed as described[22]. The cotransfected BAMBI and receptors were expressed at relative levels ranging from 1:1 to 1:3, depending on the receptor. To isolate TβR-I or BAMBI complexes, ^{35}S-labelled cells were lysed with lysis buffer (10 mM Tris, pH 7.8; 150 mM NaCl; 0.5% NP-40) and protease inhibitors. Cell lysates were incubated with anti-Flag M2 antibody (Sigma) and protein A Sepharose for 4 h and the immunoprecipitates were washed with lysis buffer. After boiling in a 1% SDS buffer, eluates were diluted to 0.1% SDS and immunoprecipitated with anti-HA 12CA5 antibody.

Constructs

A full-length BAMBI clone (pBAMBI, in pBSII-KS) was isolated by expression-screening a neurula-stage plasmid library by *in situ* whole-mount hybridization[7] (EMBL accession number AJ243576). BAMBI, BAMBIΔN (lacking amino acids 30–132) and carboxy-terminally FLAG-epitope-tagged BAMBI were subcloned into pCS2+[23] and a C-terminal HA-epitope-tagged form was subcloned into pCMV5. The receptor DNA constructs have been described[16].

Gene expression assays

RT-PCR assays were carried out in the exponential phase of amplification as described[21]. BAMBI primers were (fw)GAGCGACTCG GAGGGATTACAA (r)GTACCCAGCGT CACCATCCAGA (154 base pairs (bp)). Luciferase assays were carried out as described with P19 cells[16] and *Xenopus*[24]. The luciferase reporter constructs employed were the pARE-Luc from the Mix2 promoter[25], pXvent-Luc (BRE)[26] and pXFD1′Luc-i (ARE) encompassing the 1.6-kilobase (kb) intron of the XFD1′ gene, which contains an ARE that is directly activated by Smad2 (ref. 27), fused to the XFD1 minimal promoter (bp −80 to +21 of the transcription start site[28]; a gift from W. Knöchel).

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Tom22 is a multifunctional organizer of the mitochondrial preprotein translocase

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Mitochondrial preproteins are imported by a multisubunit translocase of the outer membrane (TOM), including receptor proteins and a general import pore[1–5]. The central receptor Tom22 binds preproteins through both its cytosolic domain and its intermembrane space domain[6–10] and is stably associated with the channel protein Tom40 (refs 11–13). Here we report the unexpected observation that a yeast strain can survive without Tom22, although it is strongly reduced in growth and the import of mitochondrial proteins. Tom22 is a multifunctional protein that is required for the higher-level organization of the TOM machinery. In the absence of Tom22, the translocase dissociates into core complexes, representing the basic import units, but lacks a tight control of channel gating. The single membrane anchor of Tom22 is required for a stable interaction between the core complexes, whereas its cytosolic domain serves as docking point for the peripheral receptors Tom20 and Tom70. Thus a preprotein translocase can combine receptor functions with distinct organizing roles in a multidomain protein.

Only two Tom proteins, Tom22 and Tom40, have been shown to be essential for cell viability[6,14,15], as determined by a standard assay, namely disruption of one chromosomal copy of a gene in a diploid strain of the yeast *Saccharomyces cerevisiae*, followed by sporulation and analysis to determine whether the resulting haploid cells lacking the gene can grow[16]. Similarly, a sheltered disruption of *TOM22* in *Neurospora crassa* indicated that Tom22 is essential for cell viability[17]. In a second approach using *S. cerevisiae*, we investigated whether *TOM22* could be deleted directly from haploid cells by a one-step disruption, but no viable cells lacking *TOM22* were obtained. In a third approach, a diploid yeast strain (OL551) lacking the *TOM22* coding region in one of its chromosomal copies received a plasmid containing *TOM22* and the *URA3* marker. After sporulation, haploid cells containing the chromosomal disruption of *TOM22* were selected, all of which carried the *TOM22–URA3* plasmid. By adding 5-fluoroorotic acid (5-FOA), we found that cells (OL201) were selected that had lost the *URA3–TOM22* plasmid and seemed to lack *TOM22*.

To exclude the possibility that only the *URA3* marker was inactivated while the *TOM22* gene was still present in OL201, we performed analytical polymerase chain reaction (PCR) with primers flanking the *TOM22* gene. OL201 contained only the disrupted gene (Fig. 1a, lane 3), the control haploid strain OL223 contained the wild-type *TOM22* gene (Fig. 1a, lane 2), and the diploid strain contained both the wild-type gene and the disrupted gene (Fig. 1a, lane 1). The absence of the *TOM22* coding region in OL201 was confirmed by Southern blot analysis, and northern blot analysis revealed the complete lack of *TOM22* messenger RNA (not shown). We conclude that the OL201 strain lacks the *TOM22* gene, and so we now refer to it as *tom22Δ*. The *tom22Δ* cells were devoid of mitochondrial DNA (*rho*^{0}). The control haploid strain (OL223) was converted to the *rho*^{0} state (referred to as wild type with regard to *TOM22*). On fermentable medium, the growth of *tom22Δ* cells was about fourfold slower than that of OL223 cells.

The frequency of spontaneous loss of the *URA3* plasmid from a control strain was about 0.1, whereas the frequency for obtaining OL201 was ~0.02. This indicates that around 20% of the cells could survive the loss of *TOM22*. Moreover, a backcross of OL201 with the wild-type haploid also failed to provide evidence for a rapid generation of extragenic suppressor mutations to explain the viability of *tom22Δ* cells. The analysis of more than 90 tetrads revealed a 2:2 segregation of viability, and all viable spores contained the wild-type *TOM22* gene, indicating that *tom22Δ* spores cannot germinate productively. The strong growth defects of *tom22Δ* cells provide a likely explanation of why the cells did not survive two standard procedures for gene deletion, leading to the previous assumption that Tom22 is essential for viability. Only the mild approach involving plasmid loss allowed viable *tom22Δ* cells to be recovered.

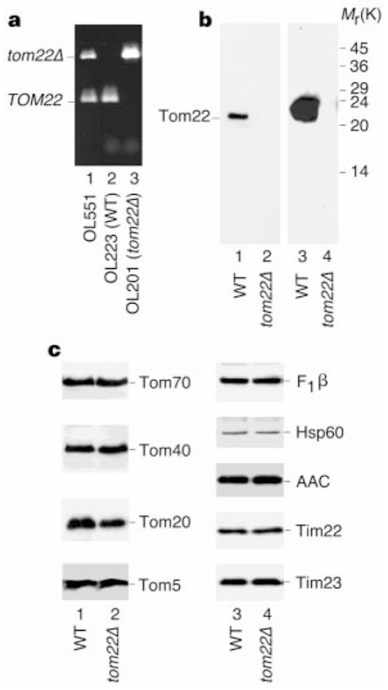

Figure 1 A yeast strain lacking *TOM22*. **a**, Analytical PCR yielding fragments typical for the intact *TOM22* gene (409 base pairs (bp); WT, wild type) and the *HIS3*-disrupted *TOM22* gene (1,1101 bp; *tom22Δ*). **b**, **c**, Analysis of the protein composition of *tom22Δ* mitochondria. Isolated mitochondria (25 μg of protein) were separated by SDS–PAGE and analysed by immunodecoration. In **b**, lanes 3 and 4 received 200 μg of mitochondrial protein.

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