Negative regulation of Smad2 by PIASy is required for proper *Xenopus* mesoderm formation

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Summary

Mesoderm induction and patterning are primarily regulated by the concentration of locally expressed morphogens such as members of the TGFβ superfamily. Smad2 functions as a transcription factor to regulate expression of mesodermal genes downstream of such morphogens. We have identified *Xenopus* PIASy (XPIASy), a member of the PIAS family, by yeast two-hybrid screening using *Xenopus* Smad2 (XSmad2) as a bait. During mesoderm induction, *XPIASy* is expressed in the animal half of embryos with a ventral high-dorsal low gradient at the marginal zone. *XPIASy* expression is positively and negatively regulated by activities of the XSmad2 and Wnt pathways, respectively. Interestingly, inhibition of XPIASy by morpholinos induces elongation of animal caps with induction of mesoderm genes even in the absence of their morphogen-mediated activation. In addition, their introduction into the ventral marginal zone results in a secondary axis formation. Gain-of-function analysis revealed that XPIASy inhibits mesoderm induction by specific and direct downregulation of XSmad2 transcriptional activity. These observations indicate that XPIASy functions as an essential negative regulator of the XSmad2 pathway to ensure proper mesoderm induction at the appropriate time and in the appropriate region, and suggest that both the initial step of morphogen-mediated activation of the XSmad2 pathway and regulation of the final downstream transcription step have crucial roles in mesoderm induction and patterning.

Key words: Smad2, Mesoderm induction, PIASy, *Xenopus*

Introduction

Mesoderm is formed at the marginal zone between the ectoderm and endoderm from late blastula to early gastrula stages. Early studies using *Xenopus* embryos showed that mesoderm formation results from inductive signals released mainly from the underlying endoderm (Gimlich and Gerhart, 1984; Harland and Gerhart, 1997; Nieuwkoop, 1973). The released signals activate downstream signalling cascades, including the Smad2, the Smad1 and Wnt pathways, to induce transcription of mesodermal genes.

The importance of Smad2 in mesoderm induction and patterning was demonstrated in *Xenopus* embryos by observations that inhibition of *Xenopus* Smad2 (XSmad2) at the dorsal marginal zone (DMZ) results in loss of expression of mesodermal markers in association with a defect in dorsal structure. Furthermore, activation of XSmad2 induces mesoderm markers in a dose-dependent manner and the formation of a secondary axis (Graff et al., 1996; Hemmati-Brivanlou and Melton, 1992; Hoodless et al., 1999). During mesoderm induction and patterning, the Smad2 mRNA and protein are widely distributed mainly in the animal half of *Xenopus* embryos. However, interestingly, activation of endogenous XSmad2, which is monitored by phosphorylation of XSmad2, occurs at the marginal zone in a dorsal-to-ventral direction (Faure et al., 2000; Lee et al., 2001; Schohl and Fagotto, 2002). To explain this localized activation, a morphogen model of activin-like ligands is widely accepted (Green et al., 1992; Gurdon and Bourillot, 2001; McDowell and Gurdon, 1999). In this model, locally activated secreted factors diffuse in the embryo and induce specific fates and proper patterning of embryos in a concentration-dependent manner. However, the activation of endogenous XSmad2 is excluded from the prospective ectoderm at the animal pole. This spatial exclusion permits formation of ectoderm at the animal pole, by a mechanism that is not well understood. Application of activin-like ligands to animal caps between late blastula and early gastrula stages converts prospective ectoderm into mesodermal fate by activation of Smad2 (Eppert et al., 1996; Graff et al., 1996). However, if activin-like ligands are applied to animal caps before or after these stages, their application does not induce mesoderm, suggesting that competence towards XSmad2 is temporally and spatially regulated (Grimm and Gurdon, 2002; Lee et al., 2001). Despite the well-characterized mechanism of mesoderm induction and patterning, it is not yet clear how this temporally and spatially restricted competence, which is required for the precise patterning of germ layers, is regulated. Nor is it understood

what prevents the vegetally produced activin-like signal from inducing mesoderm all the way up into the animal hemisphere.

The Smad2 pathway is activated by activin-like molecules of the transforming growth factor β (TGFβ) superfamily (Kofron et al., 1999; Osada and Wright, 1999; Thomsen and Melton, 1993) through their receptor-mediated phosphorylation of Smad2. The activated Smad2 makes a complex with Smad4 and then translocates from the cytoplasm to the nucleus. By recruiting other transcription activators such as FAST1, the Smad2-Smad4 complex activates transcription of target genes (Baker and Harland, 1996; Chen et al., 1997; Graff et al., 1996; Green et al., 1992; Harland and Gerhart, 1997; Horb and Thomsen, 1997; LaBonne and Whitman, 1994; Nomura and Li, 1998). Smad2 protein has three major domains: MH1, linker and MH2 domains (reviewed by Fortuno et al., 2001; Lutz and Knaus, 2002). The MH1 and MH2 domains are highly conserved in members of the Smad family. The MH1 region has a role in autoinhibition by physically interacting with the MH2 domain (Kim et al., 1997). The linker regions among Smad proteins have diverse structures but are well conserved through evolution. The linker domain of Smad2 has three serine phosphorylation sites, which regulate its nuclear exclusion and contribute to the competence of precursor cells to activin-mediated mesoderm induction (Grimm and Gurdon, 2002). The MH2 domain is crucial for regulation of its activity and has three phosphorylation sites for ligand-mediated activation (Abdollah et al., 1997). So far, many Smad2-interacting proteins, including receptors (Ro et al., 1995), other Smad proteins (Wu et al., 1997), and many positive and negative transcription factors [such as FAST and Mixer (Germain et al., 2000; Watanabe and Whitman, 1999; Yeo et al., 1999), Swift (Shimizu et al., 2001), p300/CBP (Janknecht et al., 1998; Pouponnot et al., 1998), Ski and SnoN (Macias-Silva et al., 2002; Stroschein et al., 1999)] have been identified mainly from studies using mammalian cell lines. Each protein interacts with a specific domain of Smad2 and functions at a specific position in the Smad2 signalling pathway. However, their regulatory mechanisms during early development still remain to be solved.

In order to understand the regulation of the Smad2 complex and the importance of Smad2 regulation in early embryogenesis, we performed a yeast two-hybrid screen using XSmad2 as a bait and have identified *Xenopus* PIASy (protein inhibitors of activated STAT y), which is a member of the PIAS family. Recently, members of the PIAS family have been shown to interact with several transcription factors, including Smad1, Smad2, Smad4, Lef1 and androgen receptors, and to be involved in their modification with SUMO (small ubiquitin-like modifier) and transcriptional regulation of the interacting proteins in mammalian cell lines (Jimenez-Lara et al., 2002; Kahyo et al., 2001; Lin et al., 2003a; Long et al., 2003; Sachdev et al., 2001). However, its role and physiological targets in development remain to be elucidated. Therefore, in this paper, we have analyzed the role of XPIASy in mesoderm induction and patterning using *Xenopus* embryos by gain- and loss-of-function approaches. Our analysis has revealed that XPIASy negatively regulates transcription activity of XSmad2 as a main physiological target during mesoderm induction by their direct interaction, but not by its SUMOylation activity. Moreover, we have found that transcription of XPIASy is positively and negatively regulated by XSmad2 and β-catenin, respectively, and, consistent with this regulation, endogenous XPIASy expression is largely overlapping with that of Smad2 in the animal half of embryos, but its expression in the DMZ is significantly reduced at the stage of dorsal mesoderm induction. These observations provide a possible mechanism by which XPIASy ensures the zone of Smad2 activation required for mesoderm induction and patterning: by its developmentally regulated expression and by the inhibition of Smad2 activity in appropriate regions and with appropriate timing.

Materials and methods

Xenopus laevis embryos

Xenopus laevis embryos obtained by in vitro fertilization were dejellied in 2% cysteine and allowed to develop in 0.1×MBS. Embryos were staged according to Nieuwkoop and Faber (Nieuwkoop and Faber, 1994).

The yeast two-hybrid screen

The C-terminal region of XSmad2 (amino acids 180 to 432) was subcloned into a *pBTM116* bait vector (Vojtek et al., 1993). The yeast two-hybrid screening was performed as described (Shimizu et al., 2001) using a *Xenopus* egg cDNA library as many components of the TGFβ signalling pathway are maternally expressed (Horb and Thomsen, 1997; Koyano et al., 1997; Shimizu et al., 2001).

Constructs

The XSmad2-myc construct and the XSmad1 cDNA are from Dr J. Graff. The dominant-negative XSmad2 (P445H) was constructed by PCR mediated mutagenesis. XSmad1-pCS2 construct was made by PCR. The XSmad4-myc constructs are from Prof. E. Nishida, and T7-mPIASy is from Dr Grosschedl. The wild-type and deletion constructs of XPIASy were made using pCS2+ derivatives or the pACTII vector. For in situ hybridization, the cDNAs were subcloned into the pBSSK+ vector (Stratagene). The *Xenopus* β-catenin and Tcf3 constructs were gifts from Dr Van de Wetering. pLexA-Smad2 (amino acids 180 to 432), pLexA-Ras (G12V), pACTII-HK-Swift and pVP-Raf were prepared as described (Shimizu et al., 2001). Flag-tagged SUMO-1 construct was made using *Xenopus* SUMO-1 cDNA (AW767329) and pCS2+.

mRNA injection and animal cap assay

Capped mRNAs were produced from linearized constructs using the relevant promoter according to the manufacturer's instructions (mMessage mMachine, Ambion). The mRNAs were injected at the given concentrations into the indicated regions. For the animal cap assay, the embryos were grown until stage 8 in 0.1×MBS and the animal caps were dissected in 1×MBS. The caps were then cultured in 0.5×MBS either up to stage 10.5 and used for RT-PCR analysis or western blotting, or up to stage 27 to analyse animal cap morphology.

In situ hybridization

Digoxigenin-labelled antisense RNA probes were produced from the corresponding constructs. Hybridizations were performed on whole embryos according to standard protocols (Harland, 1991).

Immunoprecipitation and western blotting

Lysate of injected embryos or animal caps in a modified RIPA buffer was pre-cleared with Protein G fast flow agarose (Sigma) for 1 h at 4°C. The supernatant was then subjected to incubation with anti-Flag (M2, Sigma), anti-Myc (9E10) or T7 antibody (Novagen) for 2 hours at 4°C followed by a 1 hour incubation with protein G agarose. Immunoprecipitated samples were separated by SDS-PAGE gel. The blotted membrane was probed with primary antibodies [anti-Smad2/3

(BD transduction lab), anti-Myc, anti-T7 or anti-Flag antibody] and then with secondary antibody (HRP-conjugated anti-mouse IgG).

Semi-quantitative RT-PCR analysis

cDNAs were made from the extracted mRNA as described (Daniels and Brown, 2001). All primers, except for *XSmad2*, *XPIASy* and *Xvent1*, were used according to previous publications (Gawantka et al., 1998; Xanthos et al., 2002). The primer sequences for *XSmad2* are: forward, 5′-agtcatcatgaactgaaagc-3′; reverse, 5′-ggttccgaataggtgacagg-3′. For *XPIASy*: forward, 5′-agcctatcacatcatgcacc-3′; reverse, 5-’caatctctgtaatagctcgg-3′. The primers for *Xmsx1* are based from http://www.hhmi.ucla.edu/derobertis/index.html. Quantitative ranges were determined before the final analysis. All reactions were normalized against *ODC* product.

Luciferase assay

The reporter construct (50 pg) of pARE-luc or p3TP-lux and mRNAs indicated were injected into both blastomeres at the two-cell stage. For analysis using animal caps, the animal caps were dissected at stage 8. The luciferase activity was measured using caps at stage 10.5 according to the manufacturer’s protocol (Dual-Luciferase, Promega).

Morpholino

The sequences of XPIASy related morpholino are indicated in Fig. 5A. The control morpholino is 5′-cctcttacctcagttacaatttata-3′.

Results

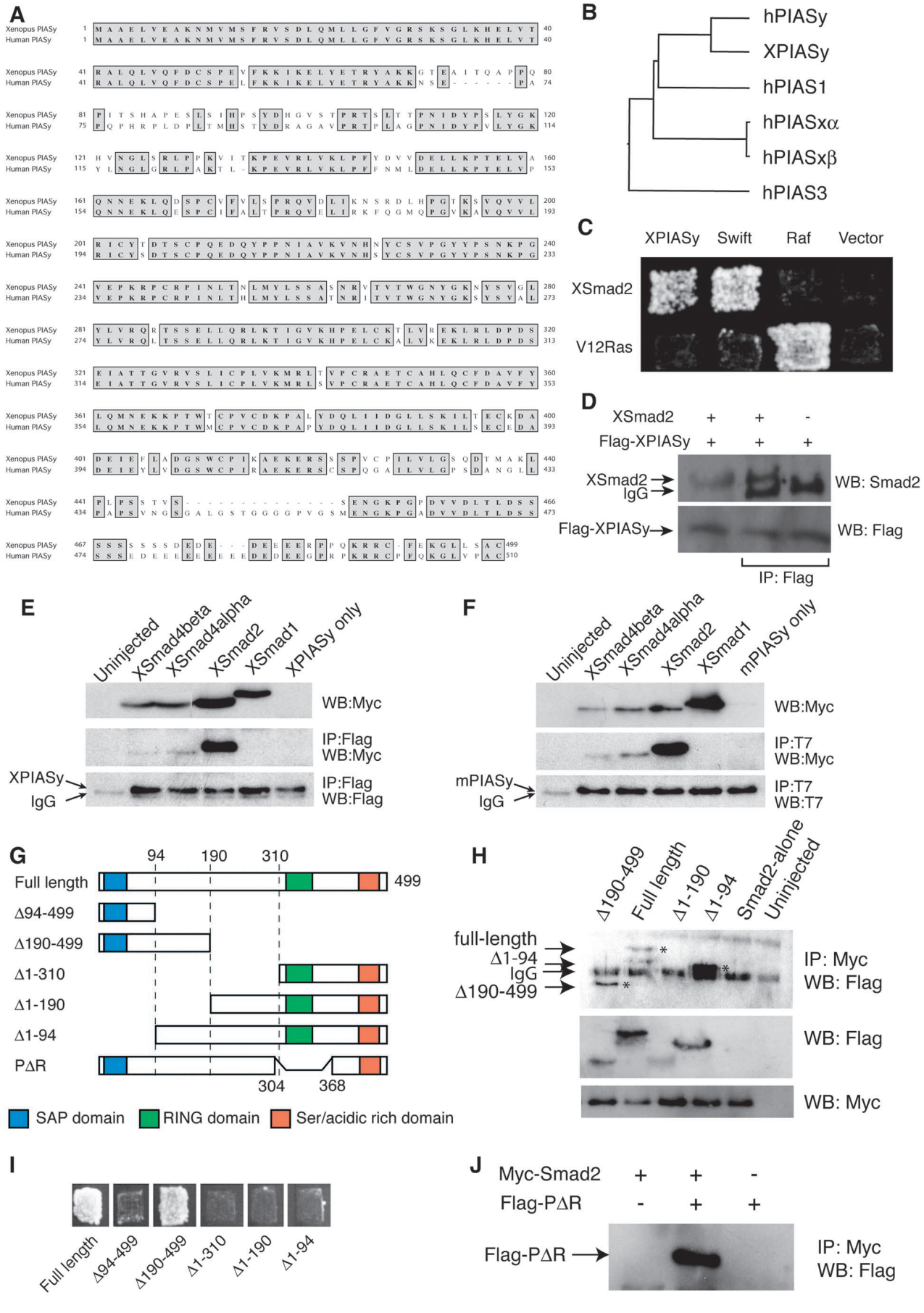
Xenopus Smad2 binds XPIASy via its C-terminal domain

In order to isolate potential regulatory components of the Smad2 mediated pathway, yeast two-hybrid screening was performed using the C-terminal region of XSmad2 as a bait and a *Xenopus* egg cDNA library. Two identical full-length cDNAs were identified that encode a protein that has a high homology with members of the PIAS family. The PIAS family consists of five members in mammals (PIAS1, PIAS3, PIASxα, PIASxβ and PIASy) (Greenhalgh and Hilton, 2001; Starr and Hilton, 1999). As shown in Fig. 1A,B, the identified protein has the highest homology (75% identical) with human PIASy among human PIAS family members. Therefore, we named it XPIASy (*Xenopus* PIASy). Fig. 1C shows that the interaction between the full-length XPIASy and the C-terminal region of XSmad2 in the yeast two-hybrid system. Next, to confirm this interaction in *Xenopus* embryos, we performed immunoprecipitation assays after overexpression of these proteins by injecting their mRNAs (Flag-tagged *XPIASy* and *XSmad2* or Myc-tagged *XSmad2*) in *Xenopus* embryos. As shown in Fig. 1D,E,H, full-length XPIASy interacts with the XSmad2 protein. In parallel with our work, mammalian PIASy has been reported to interact with all members of the Smad family (Imoto et al., 2003; Long et al., 2003). Therefore, interaction of XPIASy with other *Xenopus* Smad proteins was analyzed. Interestingly, XPIASy interacts more strongly with XSmad2 than XSmad4α and XSmad4β, but has no interaction with XSmad1 in *Xenopus* embryos (Fig. 1E), which is different from interaction between mouse PIASy and mouse Smad proteins in mammalian culture cells (Imoto et al., 2003; Long et al., 2003). A further analysis using mouse PIASy in *Xenopus* embryos revealed that the mouse PIASy still hardly interacts with Smad1 (Fig. 1F), although XPIASy overexpression with human Smad1 in COS7 cells shows their weak interaction (data not shown). As sequences of Smad1 are highly conserved in organisms, these observations indicate that the interaction is highly context dependent and suggest that *Xenopus* embryos may have an inhibitor of the interaction.

As mentioned, members of the PIAS family have been recently found to be SUMO E3 ligases (Jackson, 2001; Kahyo et al., 2001; Kotaja et al., 2002; Sachdev et al., 2001; Schmidt and Muller, 2002). These enzymes catalyze the addition of SUMO to lysine residues of target proteins in a similar mechanism as observed in ubiquitin modification. Recently, it has been shown that some members of the PIAS family, such as PIAS3 and PIASy, and Ubc9 (E2 SUMO conjugation enzyme) are binding partners of Smad proteins in mammalian cell cultures (Imoto et al., 2003; Long et al., 2003; Long et al., 2004b) and that Smad proteins are SUMOylated by their activities (Imoto et al., 2003; Lee et al., 2003; Lin et al., 2003a; Lin et al., 2003b; Long et al., 2003; Long et al., 2004a; Long et al., 2004b), although their roles in development are unknown. Members of the PIAS family have three characteristic domains: the SAP domain, the RING domain and a serine/acidic-rich domain (located in the N-terminal, the central and the C-terminal regions, respectively). The SAP domain and the RING domain have roles in DNA binding and SUMO ligase activity, respectively (Aravind and Koonin, 2001; Jackson, 2001). The serine/acidic-rich domain is essential for interaction with certain proteins such as the INF regulatory factor 1 (Nakagawa and Yokosawa, 2002) and TIF-2 (Jimenez-Lara et al., 2002). To elucidate the region responsible for the interaction with XSmad2, several deletion constructs were made (Fig. 1G) and analyzed for their binding abilities to XSmad2, using immunoprecipitation and the yeast two-hybrid system. In immunoprecipitation assays, Δ190-499 and Δ1-94 showed interaction with XSmad2, while Δ1-190 did not (Fig. 1H), suggesting importance of the region between 94 and 190. In yeast two-hybrid assay, Δ190-499, but not Δ90-499 or Δ1-190, formed colonies in co-transformation with the C-terminal region of XSmad2 on SD-Trp-Leu-His plates supplemented with 5 mM 3-AT (Fig. 1I), supporting the results of immunoprecipitation. Interestingly, Δ1-94 did not show interaction in the yeast two-hybrid system. This might result from the structural change associated with fusion to the activator domain. Additionally, we analyzed the importance of the RING domain for the interaction. A mutant with a deletion of the RING domain, PΔR, showed the same degree of interaction as full-length XPIASy in immunoprecipitation assays (Fig. 1J). These observations suggest that the N-terminal region (94-190 amino acids) primarily interacts with XSmad2.

Expression of *XPIASy* overlaps with that of *XSmad2*

The evidence that XPIASy binds with XSmad2 suggests that XPIASy may be involved in XSmad2-mediated developmental events such as mesoderm induction. However, the developmental roles of PIASy have not been studied well except for that of *Drosophila* PIASy in eye development (Betz et al., 2001; Hari et al., 2001). To obtain an insight into the developmental role of XPIASy, its expression pattern was analyzed by RT-PCR and in situ hybridization, and compared with that of *XSmad2*. First, semi-quantitative RT-PCR of whole embryos at different stages was performed (Fig. 2A). Before mid-blastula translation (MBT), maternal mRNA of *XPIASy* is strongly observed. After MBT, the level of *XPIASy*


A
Xenopus PIASy
Human PIASy
B
hPIASy
XPIASy
hPIAS1
hPIASxα
hPIASxβ
hPIAS3
C
XPIASy
Swift
Raf
Vector
XSmad2
V12Ras
D
XSmad2 + + -
Flag-XPIASy + + +
XSmad2
IgG
WB: Smad2
Flag-XPIASy
WB: Flag
IP: Flag
E
Uninjected
XSmad4beta
XSmad4alpha
XSmad2
XSmad1
XPIASy only
WB:Myc
IP:Flag
WB:Myc
XPIASy
IgG
IP:Flag
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XSmad4beta
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XSmad2
XSmad1
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WB:Myc
IP:T7
WB:Myc
mPIASy
IgG
IP:T7
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Δ190-499
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Δ1-94
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368
SAP domain
RING domain
Ser/acidic rich domain
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Δ190-499
Full length
Δ1-190
Δ1-94
Smad2-alone
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Δ1-94
IgG
Δ190-499
IP: Myc
WB: Flag
WB: Flag
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Full length
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Δ1-310
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Myc-Smad2 + + -
Flag-PΔR - + +
Flag-PΔR
IP: Myc
WB: Flag

Fig. 1. Identification of XPIASy as a Smad2-interacting protein. (A) An alignment of human and *Xenopus* PIASy (GenBank AF077952 and AF397163). Shaded amino acids are conserved residues (75% identical). (B) Phylogenetic tree of human PIAS family members and XPIASy. (C) The C-terminal region of XSmad2 binds to XPIASy in a yeast two-hybrid assay. L40 cells were transformed with pLexA-Smad2 (amino acids 180 to 432) or pLexA-Ras(G12V) and with pACTII-XPIASy, pACTII-HK-Swift (Shimizu et al., 2001), pVP-Raf or a vector, pACTII-HK. The interaction was tested by growth on SD-Trp-Leu-His plates supplemented with 10 mM 3-AT for 3 days. (D) Full-length of XSmad2 interacts with full-length XPIASy in immunoprecipitation assay. The mRNA of Flag-tagged *XPIASy* was injected alone (lane 3) or with *XSmad2* (lanes 1,2). (E) XPIASy interacts weakly with XSmad4α or XSmad4β but not with XSmad1. The mRNA of Flag-tagged *XPIASy* was injected with the indicated Myc-tagged *Xenopus* Smad members, and interaction was analyzed by immunoprecipitation using anti-Flag antibody. (F) Mouse PIASy interacts strongly with XSmad2 but weakly with XSmad4α or XSmad4β. The mRNA of T7-tagged mouse PIASy was injected with the indicated Myc-tagged *Xenopus* Smad members, and interaction was analyzed by immunoprecipitation using the anti-T7 antibody. (G) Structures of XPIASy deletion constructs. (H) Immunoprecipitation of XPIASy deletion constructs with full-length of XSmad2. (Upper panel) A western blot of immunoprecipitated samples. The mRNA of Flag-tagged *XPIASy* construct was injected with Myc-tagged *XSmad2* in *Xenopus* embryos. After immunoprecipitation against Myc, precipitated proteins were analyzed by anti-Flag staining. (Lower two panels) Before immunoprecipitation, expressed proteins were confirmed by Flag and Myc staining. (I) The N-terminal region of XPIASy interacts with the C-terminal region of XSmad2. A yeast two-hybrid assay was performed supplemented with 5 mM 3-AT, using pLexA-Smad2 (amino acids 180 to 432) and XPIASy deletion constructs subcloned into pACTII. (J) Immunoprecipitation of RING domain deleted construct, PΔR. The indicated constructs were injected into *Xenopus* embryos. After immunoprecipitation against Myc, western blotting was performed with anti-Flag antibody.

mRNA is quickly downregulated, while the level of XSmad2 is more gradually downregulated (Howell et al., 1999; Howell et al., 2001; Schohl and Fagotto, 2002). The expression of *XPIASy* remains constant throughout late development until stage 34 and then is activated again after stage 38. Next, to analyze the spatial distribution of *XPIASy* at early gastrulation, we dissected out the animal pole, vegetal pole, DMZ and ventral marginal zone (VMZ) from stage 10 embryos, and *XPIASy* expression was analyzed by semi-quantitative RT-PCR (Fig. 2B). The analysis revealed that *XPIASy* is largely distributed in the animal side and the VMZ with less expression in the vegetal side and the DMZ (Fig. 2B). *XSmad2* is also highly expressed in the animal side but its expression at the VMZ is slightly less than the DMZ (Fig. 2B). In situ hybridization of *Xenopus* embryos was carried out to elucidate in more detail the temporal expression patterns of *XSmad2* and *XPIASy* (Fig. 2C). At early stages, the maternal mRNA of *XPIASy* is detected to the animal side (Fig. 2C, part a). At the neurula stage, *XPIASy* exclusively distributes within the neural ectoderm, with strong expression in the anterior region including the eye primordium (Fig. 2C, parts b,c). Later in the development, its expression in neural tissues continues and the expression in the eye continues to be strong (Fig. 2C, parts d-g). This restricted expression pattern is very similar to that of *XSmad2* (Fig. 2C, parts h-n). These synchronized expression patterns of *XPIASy* and *XSmad2* support the idea that they functionally interact with each other during embryogenesis.

Overexpression of XPIASy inhibits XSmad2 activities

Our observations suggest that XPIASy may regulate XSmad2 activity by a direct interaction. In early *Xenopus* gastrulation, XSmad2 is activated during mesoderm formation, mainly in the dorsal mesoderm, which is required for patterning of mesoderm and organizer induction (Faure et al., 2000; Lee et al., 2001; Schohl and Fagotto, 2002). Therefore, to explore the function of XPIASy, *XPIASy* mRNA was injected into the DMZ of four-cell stage embryos and analyzed for its effect on *Xenopus* embryogenesis. The overexpression of *XPIASy* results in a ventralized structure with a slightly reduced anterior region in a dose-dependent manner (Fig. 3A, parts a-e; Table 1). At 1 ng, the majority of embryos resemble a dorsoanterior index

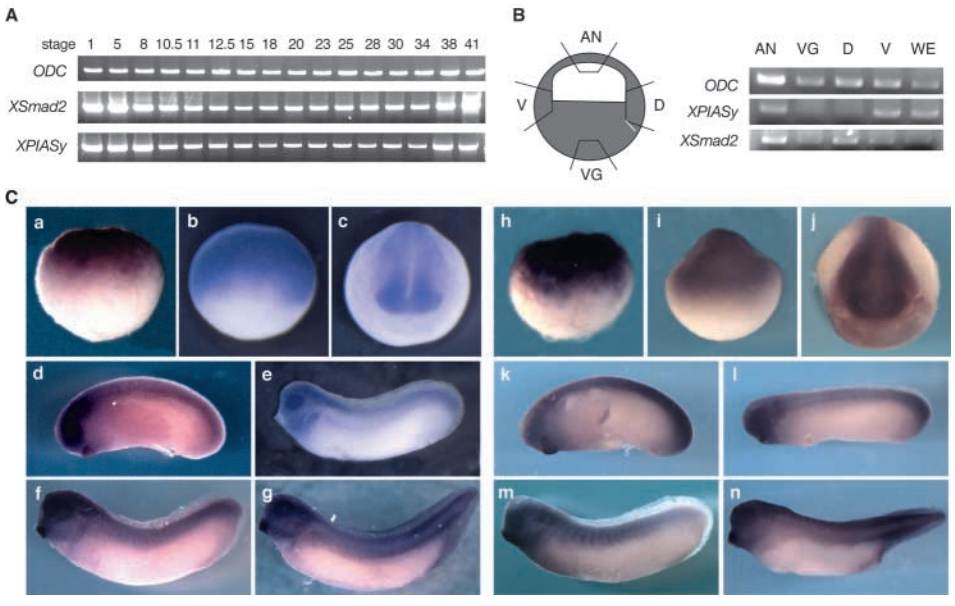

Fig. 2. Expression patterns of *XPIASy* and *XSmad2* during *Xenopus* development. (A) Semi-quantitative RT-PCR analysis of *XPIASy* and *XSmad2* expression was performed using whole embryos at different stages. *ODC* (*Ornithine decarboxylase*) was used as a control. (B) *XPIASy* and *XSmad2* show different temporal expression in the marginal zone at the early gastrula stage. Stage 10 embryos were dissected as shown into animal pole (AN), vegetal pole (VG), DMZ (D) or VMZ (V) explants, or whole embryos (WE), and subjected to RT-PCR analysis of *XPIASy* and *XSmad2*. *XPIASy* is expressed strongly in the VMZ compared with the DMZ. (C) In situ hybridization of *XPIASy* (a-g) and *XSmad2* (h-n) during development. (a,h) Stage 6-1/2, lateral view; (b,i) stage 10; (c,j) stage 18, anterior view; (d,k) stage 25; (e,l) stage 27; (f,m) stage 29/30; and (g,n) stage 37/38.

(DAI) 2 type, with no visible cement gland (Sive et al., 1989). This phenotype is similar to that obtained by overexpression of a dominant negative form of XSmad2 (Fig. 3A, parts f,g) (Hoodless et al., 1999). This suggests that XPIASy might inhibit the endogenous XSmad2 pathway. To determine whether or not this phenotype is produced by XSmad2 inhibition, a rescue experiment was performed by co-overexpression of *XPIASy* with wild-type *XSmad2*. As shown in Fig. 3B, part b and Table 2, the ventralized phenotype induced by XPIASy was largely rescued by XSmad2.

Next, we analyzed the effect of XPIASy on XSmad2-mediated morphogenetic movements using the animal cap assay. Overexpression of XSmad2 in early *Xenopus* embryos exhibits convergent extension movement of animal caps by inducing dorsal mesodermal tissues from ectodermal tissues with epidermal fate (Fig. 3C, part a) (Eppert et al., 1996). A

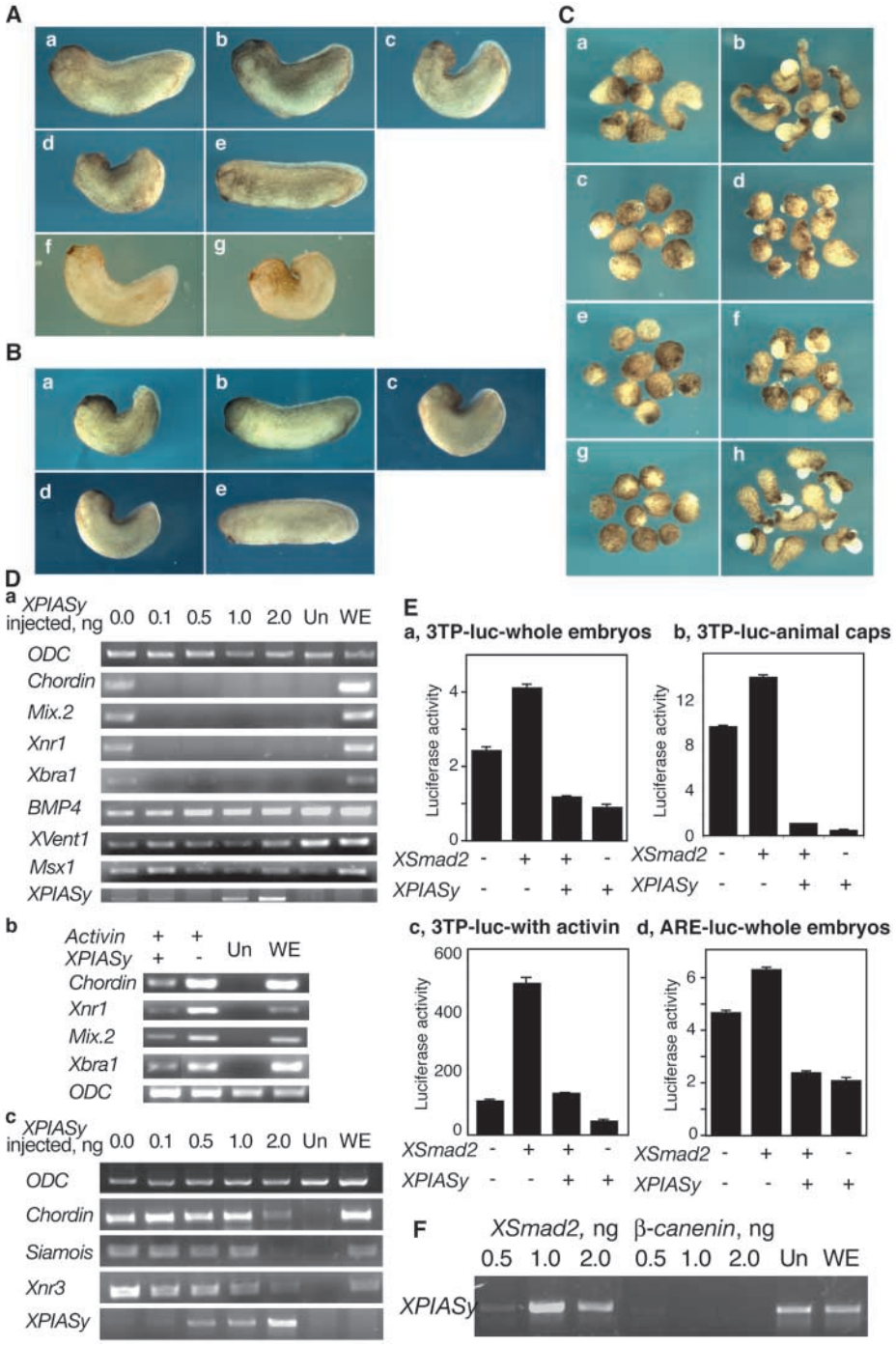

Fig. 3. XPIASy functions as an inhibitor of XSmad2. (A) The effect of XPIASy on embryogenesis. Embryos were injected into the dorsal midline at the four-cell stage with *XPIASy* mRNA at a concentration of 0.5 ng (a), 1 ng (b), 5 ng (c) or 10 ng (d), or with dominant-negative *XSmad2* mRNA at 5 ng (f) or 8 ng (g), and developed until stage 27. (e) Uninjected control. (B) The ventralized phenotype induced by XPIASy is largely rescued by co-injecting with XSmad2, but not with β-catenin. Embryos were injected into the DMZ at the four-cell stage with 1 ng *XPIASy* (a), 1 ng *XPIASy* and 0.1 ng *XSmad2* (b) or β-catenin at 0.1 ng (c) or 0.5 ng (d). (e) Uninjected embryo. (C) XPIASy inhibits animal cap elongation, mediated by the Smad2/activin pathway. Both blastomeres of two-cell stage embryos were injected with 0.5 ng of *XSmad2* (a), *XSmad2* and 0.2 pg of *activin* (b), 0.5 ng of *XPIASy* (c), *XPIASy* and *activin* (d), *XSmad2* and *XPIASy* (e), *XSmad2*, *XPIASy* and *activin* (f), nothing (g), or *activin* (h). At stage 8, animal caps were dissected and cultured until stage 23. (D) XPIASy inhibits mesoderm marker transcription activated by the Smad2 pathway but only slightly inhibits transcription induced by the Wnt pathway. Semi-quantitative RT-PCR was performed to analyze the effect of XPIASy on transcription of the targets of activin/XSmad2 and Wnt pathways. The mRNAs (0.5 ng) of *XSmad2* (a), *activin* (b) or *β-catenin* (c) and/or *XPIASy* were injected into both blastomeres at the two-cell stage. RT-PCR analysis was performed using animal caps as described in the Materials and methods. XPIASy largely reduces the transcription of XSmad2 or activin target genes (*Chordin*, *Mix.2*, *Xbra1* and *Xnr1*) but not Smad1/5 target genes (*BMP4*, *Xvent1* and *Msx1*), (a) while it only slightly reduces the level of β-catenin target genes (*Siamois*, *Xnr3* and *Chordin* (c). Un, uninjected caps; WE, whole embryos. (E) XPIASy inhibits the transcriptional activity of XSmad2 in luciferase assays using 3TP-luc (a-c) and ARE-luc (d). Reporter plasmid (50 pg) and 0.5 ng of mRNAs of *XSmad2* or *XPIASy*, or both, were injected into both blastomeres of two-cell stage embryos. Luciferase assay was performed using whole embryos (a,c,d) or animal caps (b) in the presence (c) or absence (a,b,d) of activin (0.2 pg) as described in the Materials and methods. (F) *XSmad2* and *β-catenin* regulate expression of *XPIASy* in positive and negative ways, respectively. The indicated amount of *XSmad2* or *β-catenin* mRNA was injected in the animal side of two-cell stage *Xenopus* embryos. *XPIASy* level in animal caps was analyzed as described in the Materials and methods. This PCR-cycle number for XPIASy is higher than that in D.

Table 1. Overexpression of XPIASy in dorsal marginal zone ventralizes embryos in a dose-dependent manner

Dose of XPIASy mRNA (ng)	Phenotype (%)			
	Normal	Mild (DAI4 or DAI3)	Strong (DAI2)	Death
0.0	100	0	0	0
0.01	96	4	0	0
0.1	72	28	0	0
0.5	0	96	4	0
1.0	0	15	85	0
5.0	0	0	97	3
10.0	0	0	97	3

The ratio of different phenotypes (see Fig. 3A) is listed according to normal, mild (DAI4 or DAI3, Fig. 3A, parts a,b), strong (DAI 2, Fig. 3A, parts c,d) or death. In all cases, *n*=36 and experiments are repeated three times.

similar phenotype is observed in animal caps treated with activin, the downstream effect of which is to activate XSmad2 activity (Fig. 3C, part h) (Baker and Harland, 1996). First, we overexpressed XPIASy alone in animal caps and checked the effect on convergent extension movement. As shown in Fig. 3C, part c, overexpression of wild type XPIASy did not show any elongation of animal caps, suggesting that XPIASy is not an activator of the XSmad2 pathway. This is consistent with the observation that XPIASy and Smad2 are expressed together in the animal half of embryos, where Smad2 is not activated. However, interestingly, when co-overexpressed with XPIASy and XSmad2, XPIASy completely inhibited the XSmad2-mediated elongation (Fig. 3C, part e). In a similar way, activin mediated elongation was inhibited by XPIASy (Fig. 3C, part d). These observations support the possibility that XPIASy inhibits activin-mediated mesoderm formation by downregulating XSmad2 activity.

Smad2 activates many downstream targets, most of which are important for dorsal mesoderm induction. Thus, to confirm the activity of XPIASy as a negative regulator of XSmad2, we analyzed its effect on expression of downstream targets induced by 0.5 ng of *XSmad2* mRNA co-injection in animal caps using semi-quantitative RT-PCR analysis and the luciferase assay. The downstream targets include *Chordin* (dorsal mesoderm marker) (Sasai et al., 1995), *Xenopus nodal related 1* (*Xnr1*) (Lowe et al., 1996), *Xbrachyury 1* (*Xbra1*, pan-mesoderm marker) (Cunliffe and Smith, 1992) and *Mix.2* (early mesoderm and endoderm marker) (Vize, 1996). XSmad2 induces expression of these mesoderm markers (Fig. 3D-a). As expected, XPIASy co-expression strongly inhibits the induction of their transcriptional levels even at a relatively low concentration of *XPIASy*, whereas target genes in the Smad1/5 pathway (*BMP4*, *Xvent1*, and *msx1*) are not affected by XPIASy overexpression (Fig. 3D, part a) (Kim et al., 1998; Xanthos et al., 2002). In addition, activin-mediated activation of mesoderm markers is inhibited by XPIASy (Fig. 3D, part b). Next, the effect of XPIASy on transcriptional activity of XSmad2 was analyzed using reporter constructs of p3TP-luciferase and pARE-luciferase (*Mix.2* promoter region), which respond specifically to Smad2, Smad3 and Smad4 activities, but not to Smad1 or β-catenin (Carcamo et al., 1995; Yeo et al., 1999). As shown in Fig. 3E, XSmad2-mediated activation of luciferase activity in whole embryos or animal caps was strongly inhibited by XPIASy in both reporter constructs.

Overexpression of XPIASy in the DMZ suppresses dorsalization of embryos, which is similar to the inhibition of XSmad2 (Fig. 3A, parts f,g) and can be largely rescued by co-overexpression with XSmad2 (Fig. 3B, part b). However, there is a small difference between embryos injected with XPIASy (Fig. 3A, parts a-d) and dominant-negative Smad2 (Fig. 3A, parts f,g). In XPIASy-injected embryos, head formation is slightly inhibited, while Smad2 inhibition does not affect head formation. Mouse PIASy was reported in cell culture experiments to inhibit activity of LEF1, a downstream component of the canonical Wnt pathway (Sachdev et al., 2001). It is well known that the activation of the maternal Wnt pathway is required for dorsal mesoderm induction, in particular for head induction (Sokol et al., 1991). Therefore, in order to determine whether XPIASy also regulates the transcriptional activity of the Wnt pathway, the expression of Wnt targets, *Siamois* and *Xnr3* was analyzed (Xanthos et al., 2002). Injection of *XPIASy* itself cannot induce expression of these markers in animal caps (data not shown). However, XPIASy weakly inhibits β-catenin induced expression of the Wnt targets *Siamois*, *Xnr3* and *Chordin* (Fig. 3D, part c), although much higher concentrations of XPIASy are required than that for downregulation of XSmad2 target genes, suggesting that XSmad2 is the primary target of XPIASy in *Xenopus* embryogenesis.

Even though a large amount of XPIASy was injected, the effect on the head formation was much weaker than the phenotype induced by inhibition of Wnt activity, which frequently shows a headless phenotype (Brannon et al., 1999;

Table 2. The ventralized phenotype caused by XPIASy is rescued by XSmad2, but not by Wnt pathway molecules

mRNA treatment	Sample number (*n*)	Phenotype (%)		
		Normal	Mild (DAI4 or DAI3)	Strong (DAI2)
XPIASy (1 ng)	36	0	15	85
XPIASy (1 ng)+*XSmad2* (0.1 ng)	30	66	34	0
XPIASy (1 ng)+*Activin* (0.1 pg)	20	0	20	80
XPIASy (1 ng)+*Activin* (0.5 pg)	20	0	75	25
XPIASy (1 ng)+*β-catenin* (0.1 ng)	20	0	10	90
XPIASy (1 ng)+*β-catenin* (0.5 ng)	20	0	5	95
XPIASy (1 ng)+*Tcf3* (0.1 ng)	20	0	30	70
XPIASy (1 ng)+*Tcf3* (0.5 ng)	20	0	35	65
XPIASy (1 ng)+its morpholino (10 ng)	30	100	0	0

The ratio of different phenotypes (see also Fig. 3B, Fig. 5B) is listed above. Smad2 and morpholino can effectively rescue the phenotype caused by overexpression of XPIASy in dorsal marginal zone.

Heasman et al., 2000). To further confirm that the phenotype induced by *XPIASy* is mainly caused by inhibition of XSmad2 and not by effect on Wnt signalling, we compared the morphology of XPIASy injected embryos with those injected with a β-catenin morpholino (Heasman et al., 2000; Hino et al., 2003). The inhibition of β-catenin at the DMZ results in headless embryos with normal axes, which are different from the phenotype induced by XPIASy (Fig. 3A, parts a-d). Next, to determine the degree of involvement of the Wnt pathway, a rescue experiment was performed using wild-type *Xenopus* β-catenin. As shown in Fig. 3B, parts c,d, and Table 2, these constructs could hardly rescue the XPIASy phenotype. Moreover, in activin-treated animal caps, inhibition of the canonical Wnt pathway, which includes TCF/LEF1 transcription factors, hardly inhibits the induced elongation (Tada and Smith, 2000). However, as discussed above, XPIASy does effectively inhibit the elongation (Fig. 3C). These observations clearly indicate that XPIASy primarily functions as an inhibitor of XSmad2 in association with a weak inhibitory activity of the canonical Wnt pathway.

Zygotic expression of *XPIASy* is activated by XSmad2

The importance of positive and negative feedback has been reported in many developmental systems to ensure the precise timing of the degree of activation. For example, activation of activin-like signals induces expression of negative regulators of the pathway such as antivin and cerberus (Agius et al., 2000; Cheng et al., 2000; Lee et al., 2001; Piccolo et al., 1999). Conversely, expression of *Chordin*, a BMP inhibitor, at the chick organizer is inhibited by the activity of BMP4 (Streit et al., 1998). Therefore, the effects of XSmad2 and β-catenin on the expression of *XPIASy* were examined. As shown in Fig. 3F, overexpression of XSmad2 increased the expression level of *XPIASy* in a dose-dependent manner, whereas overexpression of β-catenin inhibits transcription of *XPIASy*. The feedback induction of *XPIASy* by *XSmad2* is consistent with the synchronized expression of *XPIASy* and *XSmad2* during development (Fig. 2C). However, the inhibition by *β-catenin* may explain the reason why *XPIASy* expression is downregulated at the dorsal mesoderm compared to ventral side because β-catenin is activated at the dorsal side (Larabell et al., 1997).

How does XPIASy inhibit XSmad2 activity?

As mentioned above, PIASy has been identified as a SUMO E3 ligase for LEF1 and Smads (Imoto et al., 2003; Sachdev et al., 2001). SUMO has been implicated in several mechanisms such as determining the localization, stability and transcriptional activity of target proteins (reviewed by Melchior, 2000; Seeler and Dejean, 2001; Seeler and Dejean, 2003). In cell culture experiments, mouse Smad2 has been shown to be modified by SUMO (Lee et al., 2003). This suggests the possibility that SUMOylation of XSmad2 by XPIASy downregulates its activity. Thus, we analyzed the degree of SUMOylation of XSmad2. The mRNAs of XSmad2 and XPIASy were injected in the animal side of two-cell stage embryos and SUMOylation of XSmad2 was analyzed by western blotting after development until stage 10.5 (Fig. 4A). The molecular mass of XSmad2 is 58 kDa. It is known that SUMOylation generally alters the size of the target protein by about 18 kDa on SDS-PAGE gels (Melchior, 2000). As shown in lane 2 and 3 of the top panel, XPIASy co-overexpression with Smad2 and SUMO-1 showed a weak band around 73 kDa, which was confirmed as SUMOylated XSmad2 by analysis of the immunoprecipitated samples (lower panel). However, although under these conditions, XSmad2-mediated mesoderm induction was strongly inhibited by XPIASy (Fig. 3C,D), the SUMOylated band is too weak to explain the downregulation of XSmad2 activity by its SUMOylation. Analysis by densitometry showed that 98% of XSmad2 is the non-modified form. Similar results were achieved using non-tagged XSmad2 (data not shown). These indicate that if the XSmad2 activity is linearly correlated with the amount of non-modified XSmad2 protein, the SUMOylation of XSmad2 cannot account for the downregulation of XSmad2 activity.

The RING domain of members of the PIAS family is responsible for the enzymatic activity of SUMOylation. Thus, to elucidate the importance of the SUMOylation on regulation of mesoderm formation, we analyzed the effect of PΔR (Fig. 1E), on XSmad2-mediated activities. PΔR cannot SUMOylate XSmad2 (Fig. 4A, lane 1). As mentioned, PΔR can still bind to XSmad2 by immunoprecipitation (Fig. 1H). If the SUMOylation mediated by the RING domain is important, PΔR may not be able to inhibit XSmad2 activity or may function as a dominant-negative form as observed in a RING mutant of PIASxβ on Smad4 in mammalian cell culture (Ohshima and Shimotohno, 2003). Interestingly, this mutant can neither activate XSmad2-mediated induction of mesoderm markers in animal caps (Fig. 4B) nor induce the elongation of animal caps (data not shown). However, PΔR still inhibits the XSmad2-mediated induction of mesoderm markers such as *Mix.2* and *Chordin* without affecting expression of XSmad1 targets. In a whole embryo, overexpression of PΔR in the DMZ will still cause ventralized structures with a slightly reduced anterior region in a dose-dependent manner (Fig. 4C; Table 3), but more weakly than the phenotype caused by wild-type XPIASy (Fig. 3A, parts a-f). These observations suggest that the SUMOylation activity of XPIASy is not absolutely required for the inhibition of XSmad2-mediated mesoderm formation, although we cannot exclude the possibility that the SUMOylation activity of XPIASy may attenuate the XSmad2 activity.

XPIASy prevents mesoderm formation by inhibiting XSmad2 activation in the animal pole to ensure organizer induction at the DMZ

To investigate the endogenous role of XPIASy on mesoderm induction and patterning, we performed loss-of-function experiments using an XPIASy morpholino (Mo-1), which specifically targets the translation initiation site of *XPIASy* (Fig. 5A). First, different amounts of Mo-1 were co-injected with Flag-tagged *XPIASy* into embryos at the two-cell stage and the expression level of XPIASy protein was monitored by western blotting after MBT because an XPIAS antibody for measuring endogenous XPIASy levels was not available. As Fig. 5B shows, the expression level of exogenous XPIASy was significantly reduced by Mo-1 in a concentration-dependent manner but not by the control morpholino. Second, Mo-1 was injected together with the mRNA of wild-type *XPIASy* in the DMZ of four-cell embryos. As Fig. 5C and Table 2 show, Mo-1 inhibits the phenotype caused by overexpression of XPIASy

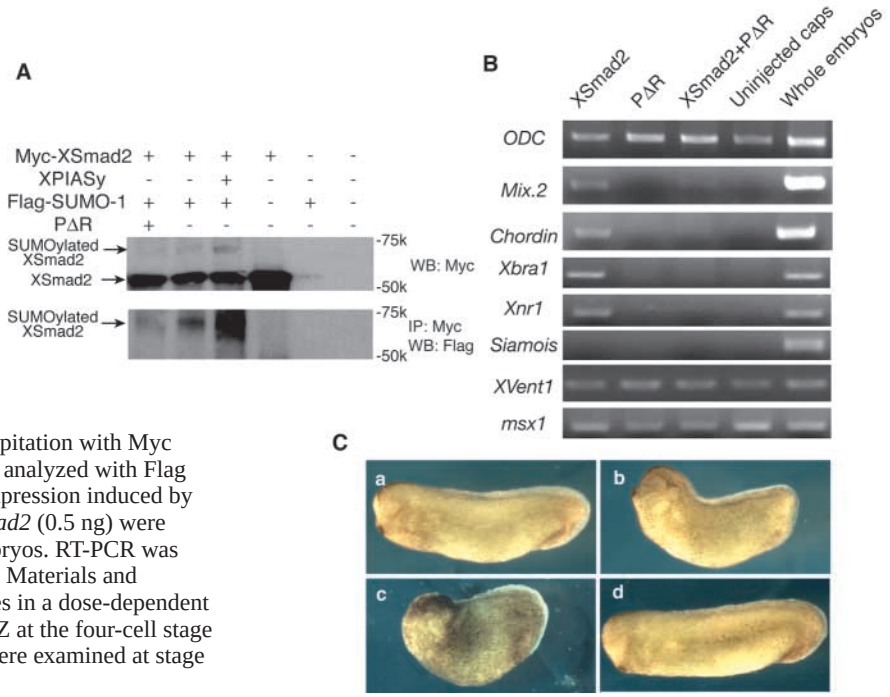

Fig. 4. The RING domain is not absolutely required for the XPIASy activity. (A) XPIASy modifies a small ratio of XSmad2 with SUMO-1. The indicated mRNAs of *Myc-tagged XSmad2* (0.5 ng), *XPIASy* (0.5 ng), *flag-tagged SUMO-1* (0.5 ng) and/or *PΔR* (0.5 ng) were injected into blastomeres at the two-cell stage. Status of XSmad2 protein was analyzed at stage 10.5 by western blotting using Myc antibody. In addition, after immunoprecipitation with Myc antibody, the amount of SUMOylated XSmad2 was analyzed with Flag staining. (B) PΔR still inhibits mesoderm marker expression induced by XSmad2. The mRNAs of *PΔR* (0.5 ng) or/and *XSmad2* (0.5 ng) were injected into both blastomeres at two-cell stage embryos. RT-PCR was performed using the animal caps as described in the Materials and methods. (C) PΔR still induces ventralized structures in a dose-dependent manner. The *PΔR* mRNA was injected into the DMZ at the four-cell stage at 1 ng (a), 3 ng (b) and 6 ng (c). The phenotypes were examined at stage 27. (d) Uninjected embryo.

and does not show any unexpected abnormality. These indicate that Mo-1 can specifically inhibit the expression and the function of XPIASy.

Next, Mo-1 by itself was injected into *Xenopus* embryos, and development was examined. The embryos injected into the DMZ at the four-cell stage exhibited no obvious phenotype until the neurula stage. Interestingly, the embryos injected into the VMZ formed a low frequency of secondary axes (8.2%, *n*=98, Fig. 5D). This effect is similar to that observed when injected with wild-type XSmad2 (100%, *n*=36, data not shown). Moreover, animal cap assays revealed that Mo-1 at 40 ng slightly induces the elongation of animal caps (Fig. 5E). This phenotype, again, is similar to that injected with XSmad2. To determine if Mo-1 activates XSmad2 activity, in situ hybridization of *Chordin* was performed after injecting mRNA of *XSmad2*, *XPIASy* or *Mo-1* into one side of the embryos. As shown in Fig. 5F, the expression of *Chordin* is largely enhanced by overexpressing XSmad2, while slightly reduced by XPIASy. Mo-1, as expected, induced expansion of *Chordin* expression. Next, after injection of Mo-1 into both blastomeres at the two-cell stage, the effect on mesoderm markers in animal caps was analyzed by semi-quantitative RT-PCR. As shown in Fig. 5G, Mo-1 clearly induces the expression of mesodermal markers in a dose-dependent manner although the induction level was weaker than that induced by XSmad2 overexpression, while expression of XSmad1 and β-catenin targets were not affected. Finally, to confirm whether the effects of Mo-1 are specific, we designed a second morpholino (Mo-2) and a mutated Mo-1 (Mo-mut) that has five point mutations (Fig. 5A) and analyzed their function. Effects of Mo-2 on induction of mesoderm markers (Fig. 5F, part d; 5H) and animal cap elongation (data not shown) were almost identical to those by Mo-1, while Mo-mut did not show any effect on our analysis, including mesoderm marker induction (Fig. 5H, data not shown). Moreover, the induction of mesoderm markers by Mo-2 was completely suppressed by co-introduction of *XPIASy* mRNA, which does not have 5′-noncoding region (Fig. 5I). These observations demonstrate that endogenous XPIASy functions as a negative regulator of XSmad2 and that the XSmad1 and Wnt pathways are not main physiological targets of XPIASy. Furthermore, taken together with the *XPIASy* expression pattern and gain-of-function analysis, all observations clearly indicate that XPIASy functions as a gatekeeper in early embryonic patterning to avoid unscheduled activation of XSmad2 in inappropriate places.

Table 3. The RING domain is not necessary for the ventralizing effect

Dose of *PΔR* mRNA (ng)	Sample number (*n*)	Phenotype (%)		
		Normal	Mild (DAI4 or DAI3)	Strong (DAI2)
1.0	22	54.5	45.5	0.0
2.0	24	25.0	70.8	4.2
6.0	30	0.0	13.8	86.2

The ratio of different phenotypes (see Fig. 4C) is listed. The XPIASy mutant without the RING domain can still induce the same phenotype as does by full-length XPIASy injected in dorsal marginal zone in a dose-dependent manner.

Discussion

XPIASy functions as a negative regulator of XSmad2

We have identified XPIASy as a direct interacting protein of XSmad2. At this point, many Smad2-interacting molecules have been isolated (Shi and Massague, 2003). In addition, members of the PIAS family have been reported to regulate several signalling pathways in addition to the Smad pathways in mammalian cell culture systems (Gross et al., 2001; Liu et al., 1998; Sachdev et al., 2001; Schmidt and Muller, 2002). Therefore, to elucidate developmental roles of XPIASy and

biologically relevant pathways involved, we have analyzed the function in early *Xenopus* embryogenesis and shown that XPIASy is an essential component in mesoderm induction by its selective inhibition of XSmad2 activity. For example, overexpression of XPIASy in the DMZ mimics the phenotype of inhibition of the XSmad2 function (Fig. 3A). This phenotype was largely rescued by co-overexpressing XSmad2 (Fig. 3B, part h). XPIASy inhibits the mesoderm induction and convergent extension movement of animal caps mediated by XSmad2 or activin (Fig. 3C). Conversely, downregulation of XPIASy by morpholinos activates the Smad2 pathway (Fig. 5), by inducing the elongation of animal caps, secondary axis formation and the expression of mesoderm markers such as *Chordin*.

How does XPIASy inhibit XSmad2 activity?

All examined members of the PIAS family show SUMOylation activity for various types of proteins, including several transcription factors (Schmidt and Muller, 2003). Many members of the Smad family, such as Smad1, Smad2, Smad3 and Smad4, have been reported to be SUMOylated by members of the PIAS family (PIASy, PIAS1, PIASxβ) or Ubc9 (Imoto et al., 2003; Lee et al., 2003; Lin et al., 2003a; Lin et al., 2003b; Long et al., 2003; Long et al., 2004a; Long et al., 2004b) and PIAS family members are localized in nucleus. These observations suggested that XPIAS might regulate transcriptional activity of XSmad2 through their SUMOylation activities. However, this possibility is disputed by our observations: (1) the majority of XSmad2 is not SUMOylated under conditions where XPIASy shows a developmental phenotype (Fig. 4A); (2) in addition, XPIASy still inhibits the activity of a constitutively active XSmad2 mutant, which lacks the MH1 domain (Baker and Harland, 1996) and the putative consensus SUMOylation site (Lysine-156) (data not shown);

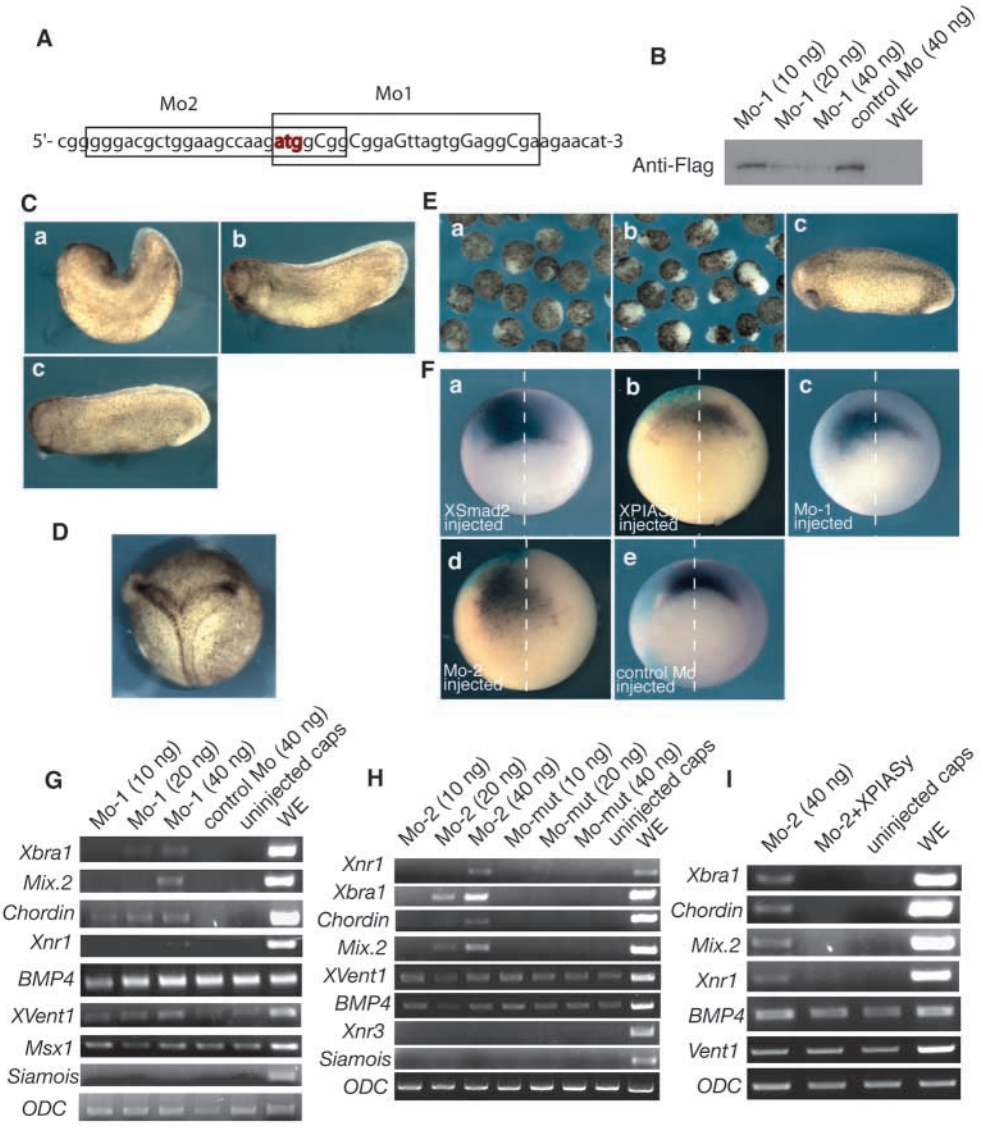

Fig. 5. XPIASy inhibits abnormal mesoderm induction at the animal side. (A) Design of two XPIASy morpholino (Mo-1 and Mo-2) and a mutated Mo-1 (Mo-mut). Mutated positions of Mo-mut are indicated as capital letters. (B) The XPIASy morpholino-1 (Mo-1) specifically decreases the XPIASy protein level. The indicated concentrations of Mo-1 or control morpholino and Flag-tagged *XPIASy* (1 ng) were injected into both blastomeres of two-cell stage embryos. The protein was analyzed at stage 10.5 by western blotting against Flag. (C) The ventralized phenotype caused by XPIASy is rescued by co-injection with Mo-1. The mRNAs of 1 ng *XPIASy* alone (a) or together with 10 ng of Mo-1 (b) were injected into the DMZ of four-cell stage embryos. The phenotype was monitored at stage 28. (c) Uninjected embryo. (D) Secondary axis was induced by Mo-1 injection into VMZ. (E) Mo-1 slightly induces elongation of animal caps. Mo-1 (40 ng) was injected into both blastomeres of two-cell stage embryos, and its effect on elongation was analyzed. (a) Uninjected caps, (b) morpholino injected caps and (c) the sibling embryo. (F) Both XPIASy morpholinos (Mo-1 and Mo-2) induce the expression of *Chordin* as does Smad2. The mRNAs of *β-gal* (0.5 ng) and 0.25 ng *XSmad2* (a), 0.5 ng *XPIASy* (b), 20 ng Mo-1 (c), 20 ng Mo-2 (d) or 20 ng control morpholino (e) were injected in one side of the two-cell stage embryos. At stage 10, embryos were subjected to β-gal staining followed by in situ hybridization against *Chordin*. (G) Mo-1 induces transcription of XSmad2 targets but not targets of XSmad1 and β-catenin. Different concentrations of the indicated morpholino were injected into both blastomeres of two-cell stage embryos. The caps were collected as described in the Materials and methods for RT-PCR analysis. (H) Mo-2 also induces transcription of XSamd2 targets but Mo-mut does not affect transcription of XSmad2 targets. (I) XPIASy inhibits transcription of mesoderm genes induced by Mo-2.

and (3) the XPIASy mutant without the RING domain, PΔR, which has lost its SUMOylation activity (Fig. 4A), still binds to XSmad2 (Fig. 1H) and inhibits the activity of XSmad2 (Fig. 4B,C), although its activity was weaker than wild-type XPIASy. These observations indicate that the SUMOylation activity through the RING domain is not essential for the effect of inhibition of XSmad2 activity, although it might attenuate the activity. This conclusion is further supported by the following functional difference between PIAS family members in cell culture experiments: PIAS1, PIAS3 and PIASxβ activate Smad-mediated activities (Long et al., 2004b; Ohshima and Shimotohno, 2003), while PIASy inhibits them (Imoto et al., 2003; Long et al., 2003; Long et al., 2004b); however, all members of the PIAS family show SUMOylation activity of Smad proteins in overexpression experiments.

PIASy was originally reported to function as a transcriptional co-repressor of Stat1 (Liu et al., 2001a). This activity requires the LXXLL motif in the SAP domain. The LXXLL motif is known to interact with histone deacetylases, HDACs, which function as transcriptional repressors (Ahmad et al., 2003). Recently, PIASy has been reported to directly bind to HDAC1, while PIAS3 binds to p300/CBP transcriptional activators (Long et al., 2003; Long et al., 2004b). Interestingly, Δ1-94, a deletion construct of the SAP domain, which has the RING domain and can bind to XSmad2 (Fig. 1F), did not show any functions similar to the full-length XPIASy (data not shown), indicating the importance of the SAP domain. All these observations strongly suggest that XPIASy might inhibit XSmad2 activity by recruiting HDACs via the SAP domain and not by modulating SUMOylation activity via the RING domain.

Function of XPIASy in the canonical Wnt pathway and the BMP pathway

Recent evidence suggests that the Wnt and TGFβ pathways cooperate to regulate embryonic axis formation and the organizer as β-catenin and Smad2 synergize to transcribe *siamois* and *Xnr3* (Crease et al., 1998; Hussein et al., 2003; Labbe et al., 2000; Letamendia et al., 2001; Nishita et al., 2000; Xanthos et al., 2002). As mentioned above, the SUMOylation activity of PIASy was originally identified using LEF1, a downstream target of the canonical Wnt pathway, as a substrate (Sachdev et al., 2001). In addition, the developmental role of SUMOylation has been reported in the context of the Wnt pathway (Kadoya et al., 2000; Kadoya et al., 2002). Indeed, our gain-of-function analysis shows that XPIASy can negatively regulate the canonical Wnt pathway (Fig. 3A,D). However, this activity seems not to be the primary function in *Xenopus* early embryogenesis based on the following observations. (1) A much higher amount of XPIASy is required for downregulation of gene expression induced by the Wnt pathway compared with its effect on XSmad2 targets (Fig. 3D, part b). (2) β-Catenin cannot rescue the defect in dorsal structure induced by XPIASy (Fig. 3B, parts c,d; Table 2), although the high dose of β-catenin (2 ng) can only rescue head formation (data not shown). Moreover, Tcf3, another binding partner of PIASy and negative regulator of the Wnt pathway, cannot activate the ventralization phenotype induced by XPIASy (Table 2). (3) It has been reported that inhibition of the canonical Wnt pathway does not inhibit activin-mediated convergent extension of animal caps (Vonica and Gumbiner, 2002). However, XPIASy clearly inhibits the extension (Fig. 3C). (4) The XPIASy morpholinos do not induce expression of targets of the Wnt pathway (Fig. 5E,F). Thus, XPIASy is likely to primarily regulate the Xsmad2 pathway but probably secondarily regulates the Wnt pathway during mesoderm formation and patterning. However, we showed that the zygotic expression of XPIASy is negatively regulated by β-catenin, while positively regulated by XSmad2 (Fig. 3F). These observations suggest that XPIASy may monitor and coordinate relative activities of the Wnt and Smad2 pathways to ensure their proper activities during developmental events.

In addition to the Smad2 and Wnt pathways, mouse PIASy has been reported to bind to Smad1, Smad4, Smad6 and Smad7, and the overexpression of PIASy influences the activities of Smads other than Smad2. However, our binding assay in the *Xenopus* system showed that XPIASy preferentially binds to XSmad2 and does not affect expression of targets of the XSmad1/XSmad4 complex. In addition, phenotypes produced by XPIASy modulation are largely different from those expected by modulation of XSmad1 activity. These observations suggest that other Smads are not likely to be physiological targets of XPIASy in early embryogenesis.

The role of XPIASy in mesoderm induction and patterning

How is XPIASy involved in the process of mesoderm induction and patterning? Based on our observations, we propose a 'gatekeeper' model (Fig. 6). XPIASy morpholinos can induce expression of mesoderm markers in the animal cap and formation of a secondary axis by their injection into the VMZ, although these inductive activities are not as strong as observed with XSmad2 overexpression (Fig. 5). In addition, XPIASy is expressed in the appropriate region and at the appropriate time

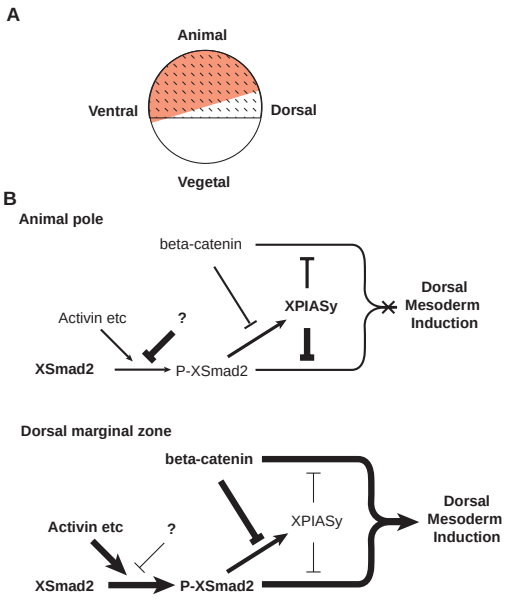

Fig. 6. A possible model of XPIASy activity in mesoderm induction. (A) Expression of *XPIASy* (orange) and *XSmad2* (hatched). (B) A model for the role XPIASy on inhibition of mesoderm induction at the animal pole (top). A model for dorsal mesoderm induction by inhibiting XPIASy expression (bottom).

to play a key role in mesoderm induction (Fig. 2). These observations strongly suggest that XPIASy has an essential role in mesoderm induction and patterning. PIASy largely distributes in the nucleus (Sachdev et al., 2001) and SUMOylation of Smad4 in cell culture was suggested to occur in the nucleus (Lin et al., 2003a). Moreover, as mentioned previously, XPIASy is likely to inhibit XSmad2 activity in the nucleus by recruiting HDACs. These observations suggest that XPIASy regulates mesoderm induction and patterning by acting at the end of the activin-like ligands/Smad2 pathway in nucleus. Therefore, in the gatekeeper model, XPIASy functions as an essential transcriptional regulator (gatekeeper) to ensure the proper initiation (opening) of transcription (gate) of the Smad2 target genes at the end of the signal. This gate is opened by the tightly regulated activity of the gatekeeper (see below).

In regulation of mesoderm formation, the morphogen gradient model has been well accepted in which region specifically expressed secreted ligands such as activin and nodal regulate Smad2 activation through its phosphorylation in the C-terminal region in a concentration-dependent manner (Green et al., 1992; Gurdon et al., 1999; McDowell and Gurdon, 1999; Vincent et al., 2003). Recently, it was reported that the competence of animal caps for activin-mediated conversion of fate from ectoderm to mesoderm is lost after stage 11 in association with acquisition of resistance of XSmad2 translocation from cytosol to nucleus. Further analysis revealed that the resistance of Smad2 translocation is regulated by phosphorylation status of Smad2 linker domain (Grimm and Gurdon, 2002). Our results clearly indicate that, in addition to these mechanisms, the final transcriptional regulation also has a crucial role in mesoderm induction and patterning and suggest that the final monitoring of transcription might be essential to avoid unexpected activation of the targets.

How is the activity of gatekeeper regulated in mesoderm induction and patterning? As semi-quantitative RT-PCR analysis revealed, the ratio of expression level of XPIASy to XSmad2 is well synchronized during embryogenesis (Fig. 2). However, after stage 8, when XSmad2 is activated for mesoderm formation, the total amount of *XPIASy* mRNA is largely reduced compared with that of *XSmad2* (Fig. 2A). The spatial expression pattern in stage 10 embryos shows that the *XPIASy* expression is downregulated in the DMZ but is still expressed in the animal half of embryo (Fig. 2B; Fig. 6A). Our loss-of-function analysis indicates that the animally expressed XPIASy is likely to inhibit ectopic mesoderm induction in ectoderm and that its downregulation in the DMZ promotes organizer formation. Moreover, our analysis of XPIASy expression has revealed that the XPIASy expression is positively and negatively regulated by activities of the Smad2 and Wnt pathways, respectively. These observations suggest that the localized regulation of XPIASy expression is likely to be produced by a combination of local XSmad2 and Wnt activities; the Wnt pathway is activated from the vegetal-dorsal side and XSmad2 is expressed mainly in the animal half including the marginal zone. In addition to XPIASy, other negative regulators of the Smad2 pathway have been identified such as Smad7, Ski and Sno (Liu et al., 2001b; Nakao et al., 1997). Interestingly, these negative regulators as well as XPIASy are induced by activated Smad2 (Nakao et al., 1997; Stroschein et al., 1999). These observations suggest that the precise temporal and spatial regulation of XSmad2 activation seems to be controlled by complex feedback mechanisms including several negative factors such as XPIASy.

Collectively, our data indicate that XPIASy functions as a final ‘gatekeeper’ at the end of the complex XSmad2 pathway in *Xenopus* early embryogenesis and that this gate is opened with appropriate timing and in appropriate regions by the combination of mesoderm induction signals such as the Wnt and Smad2 pathways (Fig. 6).

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