Development 128, 2939-2952 (2001)
Printed in Great Britain © The Company of Biologists Limited 2001
DEV5924

Timing of endogenous activin-like signals and regional specification of the *Xenopus* embryo

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Accepted 16 May 2001

SUMMARY

Signaling by activin-like ligands is important for induction and patterning of mesoderm and endoderm. We have used an antibody that specifically recognizes the phosphorylated and activated form of Smad2, an intracellular transducer of activin-like ligands, to examine how this signaling pathway patterns the early mesendoderm. In contrast to the simple expectation that activin-like signaling should be highest on the dorsal side of the gastrula stage embryo, we have found that while Smad2 phosphorylation is highest dorsally before gastrulation, signaling is attenuated dorsally and is highest on the ventral side by mid-gastrulation. Early dorsal initiation of Smad2 phosphorylation results from cooperation between the vegetally localized maternal transcription factor VegT and dorsally localized β-catenin. The subsequent ventral appearance of Smad2 phosphorylation is dependent on VegT, but not on signaling from the dorsal side. Dorsal attenuation of Smad2 phosphorylation during gastrulation is mediated by early dorsal expression of feedback inhibitors of activin-like signals.

In addition to regulation of Smad2 phosphorylation by the expression of activin-like ligands and their antagonists, the responsiveness of embryonic cells to activin-like ligands is also temporally regulated. Ectopic Vg1, Xnr1 and derrière all fail to activate Smad2 phosphorylation until after the midblastula transition, and the onset of responsiveness to these ligands is independent of transcription. Furthermore, the timing of cellular responsiveness differs for Xnr1 and derrière, and these distinct temporal patterns of responsiveness can be correlated with their distinctive phenotypic effects. These observations suggest that the timing of endogenous activin-like signaling is a determinant of patterning in the early *Xenopus* embryo.

Key words: Smad, Phosphorylation, TGFβ, Nodal, Mesoderm induction, Signal transduction, *Xenopus laevis*

INTRODUCTION

In the developing *Xenopus* embryo, the asymmetric localization of maternal RNAs and proteins in the oocyte leads to the establishment of spatially distinct intercellular signaling centers at late blastula to gastrula stages. With respect to primary germ layer formation, the prospective endoderm is specified maternally by the vegetal localization of RNA determinants, while the mesoderm is specified by intercellular signals in the equatorial region of the embryo that begin after the midblastula transition (MBT) (Kimelman and Griffin, 1998; Zhang et al., 1998). Dorsal or anterior organizing centers have their origin in the vegetal cortex of the oocyte, which is the source of a dorsal determinant. Upon fertilization, the cortex of the egg rotates relative to the inner cytoplasm, distributing the dorsal determinant to the now dorsal side of the egg; cleaving cells that receive this determinant become organizers at late blastula to late gastrula stages (Fujisue et al., 1993; Gerhart et al., 1989; Sakai, 1996). The signaling centers that direct primary germ layer formation and dorsalization act through distinct but overlapping signal transduction pathways (Harland and Gerhart, 1997; Heasman, 1997). Dorsal accumulation of nuclear β-catenin (Larabell et al., 1997; Schneider et al., 1996) is necessary for the induction of dorsal and anterior tissues (Heasman et al., 1994; Wylie et al., 1996; Zorn et al., 1999), and may act in part by positively regulating inhibitors of BMP signaling (Piccolo et al., 1996; Wylie et al., 1996; Zimmerman et al., 1996). BMP signaling is required ventrally in the gastrula to suppress dorsal gene expression and to promote ventral fates (Eimon and Harland, 1999; Graff, 1997; Jones et al., 1996b; Steinbeisser et al., 1995). Activin-like signals are essential for induction of the mesoderm and differentiation of the endoderm (Chang et al., 1997; Hemmati-Brivanlou and Melton, 1992; Henry et al., 1996; Schier and Shen, 1999). Graded doses of activin signaling can, however, also recapitulate dorsal-ventral patterning of mesoderm (Green et al., 1992), raising the possibility that the dorsal-ventral mesodermal axis is specified by a combination of graded activin signaling and overlapping β-catenin and BMP signaling pathways. Thus, overlapping domains of β-catenin, BMPs and the activin-like ligands set up regional gene expression patterns that both reflect and direct early

developmental patterning, but how these signals are integrated is not well understood.

Signal transduction by TGFβ superfamily ligands, including the activin-like ligands and BMPs, is mediated by type I and type II transmembrane receptors and by their intracellular transducers, the Smad proteins (reviewed in Massagué, 1998). In the *Xenopus* embryo, activin-like signals induce phosphorylation and activation of Smad2 and Smad2Δexon3 (here referred to collectively as 'Smad2' for simplicity), while BMP signals phosphorylate and activate Smad1 (Faure et al., 2000; reviewed in Whitman, 1998). Activin-like ligands and activity have been shown to be present maternally (Asashima et al., 1991; Fukui et al., 1994; Oda et al., 1995; Weeks and Melton, 1987), and earlier embryological work has suggested that mesoderm induction is initiated before the MBT (Jones and Woodland, 1987) and hence before the onset of zygotic transcription (Newport and Kirschner, 1982). However, using antibodies specific for the C-terminally phosphorylated, activated forms of the Smads (phosphoSmads), we found that endogenous phosphorylation of Smad2 is not detected before the MBT, and that the onset of Smad2 phosphorylation requires zygotic transcription (Faure et al., 2000). Direct examination of endogenous Smad2 phosphorylation provides a means to assess how regulation of ligand generation and responsiveness to ligands are associated with early steps in mesodermal patterning.

In the embryo, both primary germ layer formation and expression of zygotic activin-like ligands require the activity of the maternal transcription factor VegT. Maternal VegT (originally cloned as VegT, Brat or Xombi) is localized to the presumptive mesendoderm (Horb and Thomsen, 1997; Lustig et al., 1996b; Stennard et al., 1999; Zhang and King, 1996). Depletion of maternal VegT results in greatly decreased expression of dorsal and general mesendodermal genes (Kofron et al., 1999; Xanthos et al., 2001; Zhang et al., 1998), indicating that maternal VegT is required endogenously for formation of both mesoderm and endoderm. Depletion of maternal VegT also significantly reduces zygotic expression of the activin-like ligands Xnrs 1, 2, 4, 5 and 6, and derrière (Kofron et al., 1999; Takahashi et al., 2000). Conversely, overexpression of VegT in animal caps induces expression of each of these ligands, as well as of mesendodermal genes (Chang and Hemmati-Brivanlou, 2000; Clements et al., 1999; Takahashi et al., 2000; Yasuo and Lemaire, 1999). Maternal VegT cell-autonomously activates expression of endodermal genes and of activin-like ligands; activin-like ligands in the endoderm maintain endodermal gene expression and non cell-autonomously induce mesoderm (Chang and Hemmati-Brivanlou, 2000; Clements et al., 1999; Xanthos et al., 2001; Yasuo and Lemaire, 1999). In addition to cell-autonomous activation of activin-like ligands by VegT, expression of several ligands appears also to be regulated non cell-autonomously by positive cross- or autoregulatory loops. For example, transcription of Xnr1 and mouse nodal are stimulated by Smad2 activation via a conserved enhancer regulated by the maternal transcription factor FAST (FoxH1) (Osada et al., 2000; Saijoh et al., 2000), and derrière transcription can also be stimulated by signals that activate Smad2 (Sun et al., 1999). Moreover, a zygotic form of VegT (also known as Antipodean) (Stennard et al., 1996; Stennard et al., 1999) is induced by Smad2 activation through FAST (Lustig et al., 1996b; Stennard et al., 1996; Stennard et al., 1999; Sun et al., 1999; Watanabe and Whitman, 1999), providing an additional potential positive feedback loop for activation of Smad2. While maternal VegT is clearly essential for the zygotic transcription of activin-like ligands, the role of maternally expressed activin-like ligands and of positive feedback loops acting downstream of maternal VegT in the development of localized patterns of Smad2 activation remains to be determined.

Attenuation of activin-like signaling, as well as its activation, is likely to be an important component of mesendodermal patterning. Two endogenous antagonists of activin-like ligands, antivin and cerberus, have been identified in the early embryo and appear to function in anterior patterning (Bouwmeester et al., 1996; Thisse and Thisse, 1999). Antivin opposes the mesoderm-inducing activities of nodal-related ligands in *Xenopus*, zebrafish and mouse (Cheng et al., 2000; Meno et al., 1999; Tanegashima et al., 2000; Thisse and Thisse, 1999); increasing doses of antivin progressively delete posterior fates in zebrafish (Thisse et al., 2000). Cerberus, a multifunctional inhibitor of the nodal-related ligands, BMPs and Wnts, induces ectopic heads, hearts and livers in *Xenopus* (Bouwmeester et al., 1996; Glinka et al., 1997; Hsu et al., 1998; Piccolo et al., 1999). Both antivin and cerberus require activin-like signaling for endogenous expression and can be induced by overexpression of Xnr1/Xnr2 (Agius et al., 2000; Cheng et al., 2000; Piccolo et al., 1999; Tanegashima et al., 2000; Zorn et al., 1999). This suggests that they are regulated as local feedback inhibitors of the Smad2 signaling pathway. The effect of these negative feedback loops on attenuation of the endogenous Smad2 signaling pathway has not been directly addressed.

Activin-like ligands are defined functionally by their common ability to induce both mesodermal and endodermal genes and tissues. While the receptors mediating the action of these different ligands have not been definitively established, several lines of evidence from both *Xenopus* and mouse indicate that ALK4 (ActRIB) is the major type I receptor responsible for Smad2 phosphorylation downstream of the activin-like ligands (Armes and Smith, 1997; Chang et al., 1997; Gu et al., 1998; Yeo and Whitman, 2001). A model in which Smad2 and ALK4 serve as downstream transducers of a range of activin-like ligands is complicated, however, by experiments demonstrating that different activin-like ligands carry out distinct functions in early *Xenopus* development. In embryos depleted of maternal VegT, Xnr1 rescues head formation, while derrière rescues trunks but not heads (Kofron et al., 1999). Consistent with these observations, overexpression of a dominant inhibitory form of Xnr2, cm-Xnr2, results in a preferential loss of anterior tissues (Osada and Wright, 1999), while overexpression of a comparable dominant inhibitor of derrière, cm-derrière, blocks formation of posterior tissues (Sun et al., 1999). While phenotypic assays demonstrate that Xnr1/Xnr2 and derrière carry out distinct functions in anterior-posterior patterning, how these ligands might use the same signaling pathway to achieve different effects on embryonic patterning is not known.

We have used anti-phosphoSmad2 antibodies to investigate mechanisms that regulate endogenous activin-like signaling in the early *Xenopus* embryo. We find that Smad2 activation moves in a wave from the dorsal side to the ventral side of both mesoderm and endoderm. The localized maternal

transcriptional regulators VegT and β-catenin are responsible for the initiation of this wave on the dorsal side of the prospective mesendoderm just after the MBT. Negative feedback mediated through the Smad2 and FAST-dependent activation of the ligand antagonists antivin and cerberus subsequently attenuates Smad2 phosphorylation, also starting from the dorsal side. Initiation and attenuation on the ventral side occur later and require VegT but not dorsal signaling. Finally, different activin-like ligands induce Smad2 phosphorylation at different developmental stages, and this timing difference may explain their differing phenotypic effects. We propose that timing, as well as dose, of activin-like signaling is a crucial determinant of early developmental patterning in the *Xenopus* embryo.

MATERIALS AND METHODS

Embryo manipulation

Embryos were collected, artificially fertilized and dejellied as previously described (Watanabe and Whitman, 1999). Thereafter embryos were cultured in 0.1× MMR. Staging of embryos was according to Nieuwkoop and Faber (Nieuwkoop and Faber, 1967) and is described in Fig. 2A.

For microinjection, embryos were kept in 3% Ficoll/1X MMR and injected at the two cell stage with 5-20 nl RNAs and/or 50 pg α-amanitin (Boehringer Mannheim) (Newport and Kirschner, 1982). α-Amanitin-injected embryos cleaved normally until gastrulation, did not undergo gastrulation movements and died at stage 10.5/11 as previously described (Sible et al., 1997; Stack and Newport, 1997).

Dissections were carried out in 0.7× MMR. Where indicated, animal caps were dissected by stage 9, before significant pregastrulation movements (Bauer et al., 1994). For dorsal-ventral dissections, ventral midlines were marked with Nile Blue (Peng, 1991) at the four cell stage to allow accurate identification before gastrulation. Caps or explants were cultured in 0.7× MMR and were washed in 0.1× MMR before freezing at –80°C for storage.

Ventralization of embryos with short wave ultraviolet (u.v.) light (254 nm) was performed with a hand-held illuminator (Kao and Danilchik, 1991). During the first third of the first cell cycle, vegetal bottoms of healthy, fertilized embryos were exposed through Saran Wrap to light for 1 to 2 minutes based on calibration that gave an average dorso-anterior index (DAI) at late tailbud stage approaching zero (Kao and Elinson, 1988; Scharf and Gerhart, 1983). To prevent nonspecific or toxic effects, only u.v.-treated embryos that showed first and second cleavages within five minutes of those of untreated embryos were analyzed by western blotting (Cooke and Smith, 1987).

Antisense depletion in oocytes

Oocytes were depleted of maternal VegT mRNA by the injection of an antisense oligonucleotide complementary to VegT mRNA, as previously described (Xanthos et al., 2001). Oocytes were cultured for 36 hours and then matured and fertilized by the host transfer technique (Zuck et al., 1998). For rescue of VegT depletion, VegT or activin mRNA was injected into the vegetal poles of VegT-depleted oocytes just prior to maturation.

In vitro transcription

Capped mRNAs were synthesized in vitro using the SP6 mMessage mMACHINE kit (Ambion).

RT-PCR

Total RNA, extracted from whole embryos or dissected explants by the proteinase K/phenol method, was reverse transcribed for PCR reactions that were performed as previously reported (LaBonne and Whitman, 1994; Watanabe and Whitman, 1999). The following primers were used: zygotic VegT/Antipodean (Stennard et al., 1999); Xnr1 (Lustig et al., 1996a); derrière (F, GGTACTGAGGCACTATGAAG; R, TGTGTTTCATCCAGCAGCTC); antivin (F, GCAGGGGCCCATTTCGTAA; R, GGCCCTCCACATCCCTTTGA); cerberus (Bouwmeester et al., 1996); ODC (as described in *Xenopus* Molecular Marker Resource, http://vize222.zo.utexas.edu/); and EF1α (Hemmati-Brivanlou and Melton, 1994).

Antibodies

Affinity-purified anti-phosphoSmad2 and anti-phosphoSmad1 antibodies were prepared as described (Persson et al., 1998; Faure et al., 2000). Anti-Smad2 antibody was obtained from Transduction Labs and anti-actin antibody was obtained from Sigma. Secondary antibodies were goat anti-rabbit IgG-HRP antibodies (Boehringer Mannheim) and donkey anti-mouse IgG-HRP $F(ab')_2$ fragments (Jackson ImmunoResearch Laboratories).

Western blot analysis

Embryos and explants were homogenized in modified RIPA buffer as described (Faure et al., 2000). Preparation of samples and analysis of lysates by western blotting were performed as previously described; Smads run between the 50 kDa and 75 kDa protein markers (Faure et al., 2000). Cytoskeletal actin serves as a loading control for equivalent cellular volume (Tannahill and Melton, 1989). For embryos, 1/4 to 1/8 equivalent was loaded per lane. Two to three animal caps were loaded per lane.

Immunohistochemistry

Embryos were fixed in MEMFA (0.1 M MOPS pH 7.4, 2 mM EGTA pH 8, 1 mM $MgSO_4$, 3.7% formaldehyde) for 3 hours. For cross and longitudinal sections, embryos were rinsed in phosphate-buffered saline (PBS) after fixation and then were soaked in PBS plus 0.3 M sucrose. Next, embryos were embedded in 2% low melt agarose (NuSieve GTG agarose, FMC BioProducts) in PBS plus 0.3 M sucrose and were bisected in a Petri dish under PBS with a disposable micoscalpel (Feather Safety Razor). Bisected embryos were then stored in methanol overnight. Embryos were serially rehydrated into PBS and PBT (PBS, 2 mg/ml BSA, 0.1% Triton X-100) for blocking, incubation with primary and secondary antibodies, and washes as previously reported (Faure et al., 2000). Staining was developed with ImmunoPure Metal-enhanced DAB Substrate Kit (Pierce), as per manufacturer's instructions. The anti-phosphoSmad2 antibody was less sensitive for immunohistochemistry than for western blot analysis, therefore immunohistochemistry is only shown at stages when the phosphoSmad2 signal was detectable by this technique. Images were captured with NIH Image software on a Kodak DCS420 camera attached to a Zeiss Axiophot microscope.

RESULTS

VegT and activin-like ligands induce and maintain Smad2 activation

Maternal VegT regulates zygotic expression of some activin-like ligands (Kofron et al., 1999; Takahashi et al., 2000), while other ligands are present maternally (Fukui et al., 1994; Oda et al., 1995; Weeks and Melton, 1987). To determine whether maternal VegT is required for all endogenous activin-like signaling, we examined Smad2 phosphorylation in its absence by injecting VegT-specific antisense oligonucleotides into the oocyte, causing degradation of maternal VegT in the embryo (Kofron et al., 1999; Xanthos et al., 2001; Zhang et al., 1998). Depletion of VegT eliminates Smad2 phosphorylation in the early gastrula at stage 10+, and phosphorylation of Smad2 is

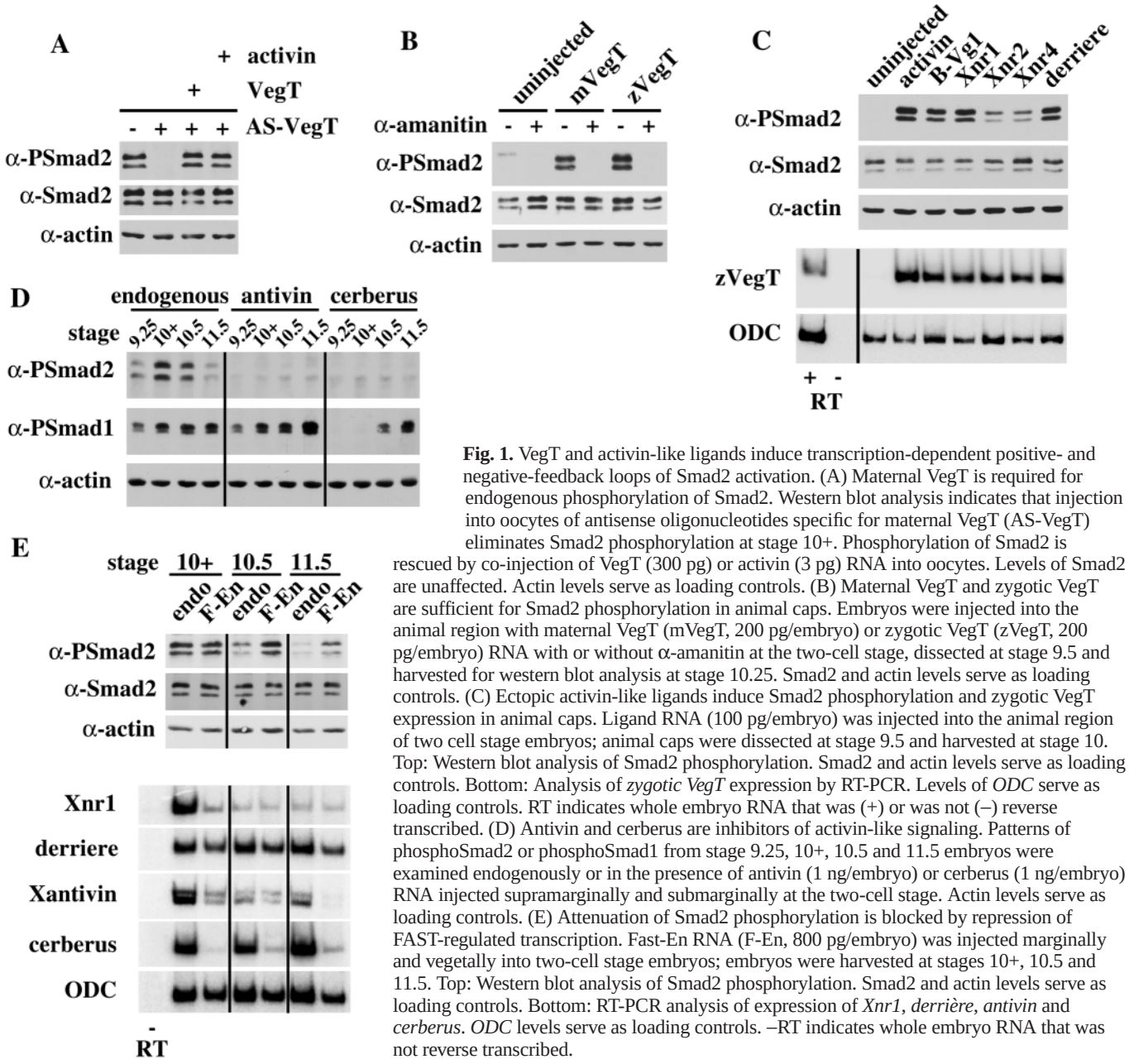

Fig. 1. VegT and activin-like ligands induce transcription-dependent positive- and negative-feedback loops of Smad2 activation. (A) Maternal VegT is required for endogenous phosphorylation of Smad2. Western blot analysis indicates that injection into oocytes of antisense oligonucleotides specific for maternal VegT (AS-VegT) eliminates Smad2 phosphorylation at stage 10+. Phosphorylation of Smad2 is rescued by co-injection of VegT (300 pg) or activin (3 pg) RNA into oocytes. Levels of Smad2 are unaffected. Actin levels serve as loading controls. (B) Maternal VegT and zygotic VegT are sufficient for Smad2 phosphorylation in animal caps. Embryos were injected into the animal region with maternal VegT (mVegT, 200 pg/embryo) or zygotic VegT (zVegT, 200 pg/embryo) RNA with or without α-amanitin at the two-cell stage, dissected at stage 9.5 and harvested for western blot analysis at stage 10.25. Smad2 and actin levels serve as loading controls. (C) Ectopic activin-like ligands induce Smad2 phosphorylation and zygotic VegT expression in animal caps. Ligand RNA (100 pg/embryo) was injected into the animal region of two cell stage embryos; animal caps were dissected at stage 9.5 and harvested at stage 10. Top: Western blot analysis of Smad2 phosphorylation. Smad2 and actin levels serve as loading controls. Bottom: Analysis of *zygotic VegT* expression by RT-PCR. Levels of *ODC* serve as loading controls. RT indicates whole embryo RNA that was (+) or was not (–) reverse transcribed. (D) Antivin and cerberus are inhibitors of activin-like signaling. Patterns of phosphoSmad2 or phosphoSmad1 from stage 9.25, 10+, 10.5 and 11.5 embryos were examined endogenously or in the presence of antivin (1 ng/embryo) or cerberus (1 ng/embryo) RNA injected supramarginally and submarginally at the two-cell stage. Actin levels serve as loading controls. (E) Attenuation of Smad2 phosphorylation is blocked by repression of FAST-regulated transcription. Fast-En RNA (F-En, 800 pg/embryo) was injected marginally and vegetally into two-cell stage embryos; embryos were harvested at stages 10+, 10.5 and 11.5. Top: Western blot analysis of Smad2 phosphorylation. Smad2 and actin levels serve as loading controls. Bottom: RT-PCR analysis of expression of *Xnr1*, *derrière*, *antivin* and *cerberus*. *ODC* levels serve as loading controls. –RT indicates whole embryo RNA that was not reverse transcribed.

rescued by injection of VegT or activin RNA (Fig. 1A). From stage 9.5 to stage 10.5 (data not shown), maternal VegT is required for endogenous Smad2 phosphorylation, but is dispensable for Smad2 phosphorylation in response to ectopic ligands. These results indicate that, despite the presence of maternal activin-like ligands, endogenous phosphorylation of Smad2 is dependent on maternal VegT.

Both maternal and zygotic forms of VegT have been described. Maternal VegT is localized to presumptive endodermal cells, while zygotic VegT is present in the marginal mesoderm after MBT and is transcribed in response to activin-like signals (Horb and Thomsen, 1997; Lustig et al., 1996b; Stennard et al., 1996; Stennard et al., 1999; Sun et al., 1999; Zhang and King, 1996). These two proteins differ at their N termini owing to alternative splicing and possibly also to alternative promoter use (Stennard et al., 1999). In animal caps at stage 10.25, which lack endogenous Smad2 phosphorylation, both maternal and zygotic VegT induce Smad2 phosphorylation that is eliminated by α-amanitin, an inhibitor of transcription (Fig. 1B). Hence, zygotic transcription is required both for endogenous Smad2 phosphorylation (Faure et al., 2000) and for VegT-induced Smad2 phosphorylation. As VegT has been shown to induce expression of zygotic activin-like ligands (Clements et al., 1999; Sun et al., 1999; Takahashi et al., 2000; Yasuo and Lemaire, 1999), we also examined the activities of the activin-like ligands themselves. Activin, B-Vg1, Xnr1, Xnr2, Xnr4 and derrière each induce Smad2 phosphorylation at stage 10+ in animal caps. In addition, each of these ligands also induces expression of *zygotic VegT* (Fig. 1C). Phosphorylation of

Smad2 and induction of *zygotic VegT* by activin-like ligands are thought to be direct, as these activities do not require zygotic transcription or translation, respectively (see below; Watanabe and Whitman, 1999). Endogenous Smad2 phosphorylation is therefore induced and maintained transcriptionally by maternal and zygotic VegT, and is activated post-transcriptionally by the activin-like ligands.

Inhibitors of activin-like ligands attenuate Smad2 activation

The phenotypic effects of ectopic expression of antivin and cerberus strongly indicate that they can antagonize signaling by activin-like ligands. To directly demonstrate the activities of antivin and cerberus with respect to Smad signaling, we expressed these inhibitors in embryos, and then examined endogenous phosphorylation of Smad2 and BMP-regulated Smad1 from late blastula to late gastrula stages (Fig. 1D). Antivin inhibits endogenous phosphorylation of Smad2 but not of Smad1, while cerberus inhibits Smad2 phosphorylation at all stages and Smad1 phosphorylation only at early gastrula stages. As cerberus binds Wnts, nodals and BMPs at distinct sites (reviewed by Piccolo et al., 1999), recovery of phosphorylated Smad1 at late gastrula stages suggests that, as development proceeds, cerberus may be titrated by continuous production of BMPs, different BMP ligands that do not bind cerberus may become active or cerberus itself may become modified in its ability to inhibit Smad1. That both antivin and cerberus entirely inhibit endogenous phosphoSmad2 signaling indicates that, either directly or indirectly, these inhibitors can fully antagonize the function of activin-like ligands in the early embryo.

Both activators and inhibitors of activin-like signaling are induced through the Smad2 signaling pathway by the maternal transcription factor FAST (FoxH1) (Osada et al., 2000; Saijoh et al., 2000; Watanabe and Whitman, 1999). To investigate the importance of FAST in regulating endogenous Smad2 phosphorylation, we examined the effect of expression of a dominant inhibitory FAST, FAST-En, on the expression of activin-like ligands, antagonists of these ligands, and total levels of Smad2 phosphorylation in the early embryo (Fig. 1E). To examine the role of FAST in positive feedback regulation of Smad2 phosphorylation, the effect of FAST-En on the expression of the zygotic ligands *Xnr1* and *derrière* was examined. Endogenous expression of *Xnr1* is highest at stage 10+ and decreases to basal levels thereafter, while endogenous expression of *derrière* is strongly detected from stage 10+ through to stage 11.5. Injection of FAST-En reduces *Xnr1* expression to basal levels, but only partially inhibits *derrière* expression. Although the relative contributions of Xnr1 and derrière to endogenous activin-like signaling are not known, positive regulation through FAST appears to be more important for expression of *Xnr1* than of *derrière*.

A role for FAST in negative feedback regulation of Smad2 signaling was investigated by examining the expression of the ligand inhibitors *antivin* and *cerberus* in the presence of FAST-En. Expression of both *antivin* and *cerberus* is greatly reduced in the presence of FAST-En, indicating that FAST plays a role in mediating negative as well as positive feedback regulation of activin-like signals. Despite its ability to substantially reduce the expression of the ligands *Xnr1* and *derrière*, expression of FAST-En does not significantly affect total Smad2 phosphorylation at stage 10+, indicating that positive feedback through FAST-regulated targets is not required for maintenance of activin-like signaling. At later gastrula stages, when Smad2 phosphorylation has begun to be attenuated in control embryos, FAST-En expression results in an increase in Smad2 phosphorylation, suggesting that expression of FAST-regulated feedback inhibitors such as cerberus and antivin is essential for normal attenuation of Smad2 phosphorylation during gastrulation. That the net effect of the ectopic expression of FAST-En in embryos is an increase in Smad2 phosphorylation by mid-gastrulation suggests that the role of FAST in negative feedback regulation is more important than its role in positive feedback regulation in determining total levels of Smad2 phosphorylation in the gastrula stage embryo.

Endogenous activation of Smad2 is initiated and attenuated from the dorsal side of the *Xenopus* embryo

To visualize activin-like signaling at high spatial resolution, we examined bisected embryos by immunohistochemistry using the anti-phosphoSmad2 antibody (Fig. 2B,C). At stage 9.5, phosphorylated Smad2 is detected as a wedge in the dorsal vegetal region of the embryo but is discernible only faintly in the ventral region (Fig. 2D,E). By stage 9.75, phosphorylated Smad2 is maintained dorsally and is expanding across the embryo to the ventral side (Fig. 2F,G). While, at these late blastula stages, mesodermal cells and endodermal cells are difficult to distinguish, Smad2 phosphorylation appears to be present in both but may be more prominent in the endoderm. In the early gastrula at stage 10+, phosphorylated Smad2 appears equally distributed across the dorsal-ventral axis, with Smad2 phosphorylation present in the ventral mesoderm and dorsal involuting mesoderm but not in dorsal preinvoluting mesoderm (Fig. 2H,I). By stage 10.5, absence of phosphorylated Smad2 is apparent in the prospective deep anterior endoderm and in the dorsal involuting mesoderm (Fig. 2J,K). Faint staining in the stage 11.5 embryo is detected in ventral and posterior endodermal cells but is excluded from cells dorsal to the archenteron and in tissues now specified as anterior (Fig. 2L,M). Taken together, these findings reveal a wave of Smad2 activation in mesendodermal cells passing from the dorsal side of the embryo to the ventral side.

Dorsal-ventral prepattern directs region-autonomous temporal patterns of endogenous activin-like signaling

To examine activin-like signaling intrinsic to dorsal or ventral halves of the embryo, we bisected embryos at stage 8, before Smad2 phosphorylation appears, and harvested cultured halves from late blastula to late gastrula stages for examination of phosphorylation of Smad2 and expression of activin-like ligands and inhibitors. Equivalent material from ventral halves, dorsal halves and whole embryos was compared at each stage. To confirm the accuracy of dissections, ventral halves, dorsal halves and whole embryos were cultured to stage 30. Compared with sibling embryos (Fig. 3C), ventral halves develop no distinctive structures (Fig. 3A), while dorsal halves demonstrate dorsal axes and formation of posterior (tail) and anterior (eyes, cement gland) structures (Fig. 3B) (Kageura and Yamana, 1983).

Analysis of Smad2 phosphorylation in isolated dorsal and

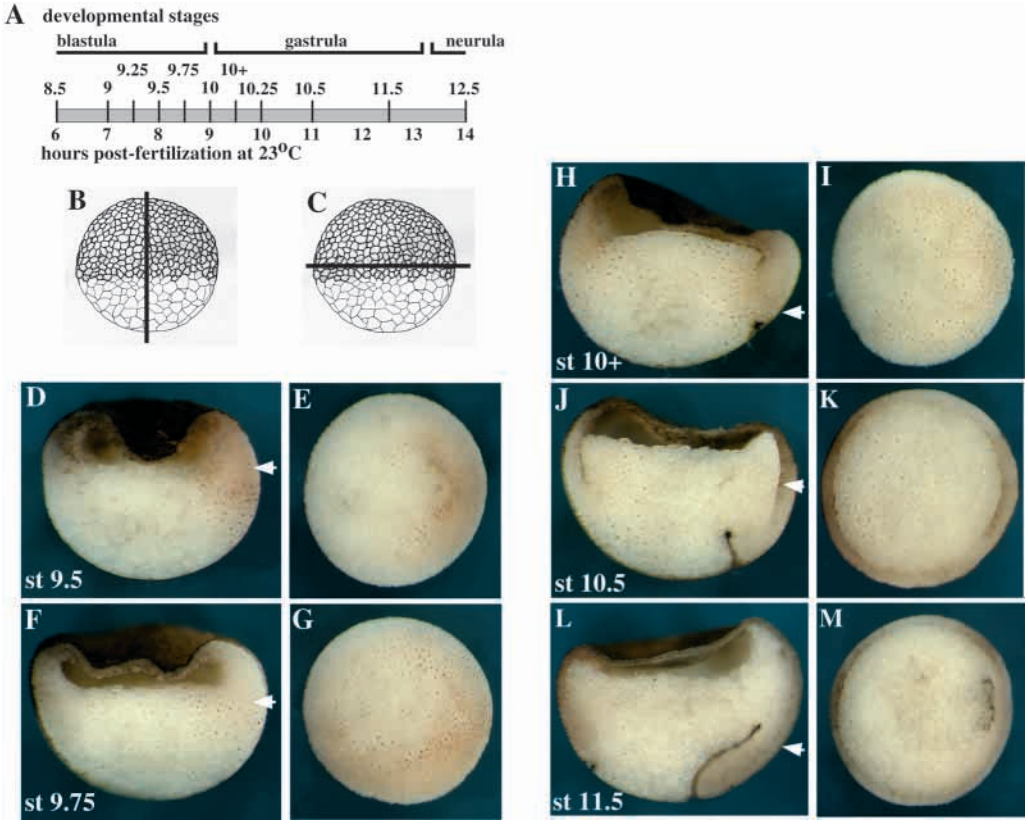

Fig. 2. Endogenous Smad2 activation is initiated and attenuated from the dorsal side of the *Xenopus* embryo. (A) Time course of early development. Developmental stages are correlated with hours post-fertilization at 23°C. Blastula, gastrula and neurula stages are indicated. Most developmental stages are from Nieuwkoop and Faber (Nieuwkoop and Faber, 1967). Intermediate stages are expressed as fractions between established stages (as stage 9.25). (B-M) Immunohistochemical analysis of phosphoSmad2 in the early *Xenopus* embryo. Embryos fixed at the indicated stages were bisected and then processed for immunohistochemistry with the anti-phosphoSmad2 antibody. Orientations of the axes of dissection are indicated (B,C) (Nieuwkoop and Faber, 1967). Representative sections are shown for stages 9.5 (D,E), 9.75 (F,G), 10+ (H,I), 10.5 (J,K) and 11.5 (L,M). Sectioned embryos were photographed en face. Ventral is towards the left; dorsal is towards the right. Animal is towards the top; vegetal is towards the bottom. For cross sections (B,D,F,H,J,L), fixed embryos were bisected through the dorsal-ventral midline. For longitudinal sections (C,E,G,I,K,M), fixed embryos were bisected in a horizontal plane near the equator and below the blastocoel floor; lower sections are shown. Approximate levels and angles of longitudinal sections (estimated from landmarks and the shape of the sections) are indicated with arrows on cross sections.

ventral halves shows that patterns of activin-like signaling are region-autonomous by stage 8 (Fig. 3D). Initiation of activin-like signaling begins dorsally at stage 9, and remains greater in dorsal halves than in ventral halves at stages 9 and 9.5. From stage 10+ to stage 10.5, phosphorylation of Smad2 is nearly equivalent in dorsal and ventral halves. By stage 11.5, Smad2 phosphorylation is greater in ventral halves than in dorsal halves, reflecting attenuation of activin-like signaling dorsally. These results show that the relative levels of Smad2 phosphorylation change over time in isolated dorsal and ventral halves, indicating that Smad2 activation is region-autonomous rather than a result of signals propagated from one side of the embryo to the other.

Activin-like ligands and their inhibitors are also expressed region-autonomously in isolated dorsal and ventral halves (Fig. 3E). Zygotic ligands *Xnr1* and *derrière* are detected strongly from stage 9.5 and stage 9, respectively, and are highest at stage 10+. *Xnr1* expression resembles dynamic phosphorylation of Smad2 in that *Xnr1* is initiated at higher levels on the dorsal side at stage 9.5, is equivalent in dorsal and ventral halves at stage 10+, and is attenuated more on the dorsal side at stage 10.5. *Derrière* expression differs from Smad2 phosphorylation and *Xnr1* expression as *derrière* is expressed at higher levels in ventral halves at stage 9 and stage 9.5, and becomes equally distributed from stage 10+ through stage 11.5. The inhibitors *antivin* and *cerberus* also demonstrate distinct patterns of expression. *Antivin* is detected from stage 9.5, increases through stage 10.5, and then decreases abruptly by stage 11.5; from stage 10+, *antivin* levels are higher dorsally than ventrally. *Cerberus* is detected from stage 10+, increases through stage 11.5 and is expressed at much higher levels dorsally than ventrally at all stages. Antivin activity may contribute to attenuation of Smad2 phosphorylation in both dorsal and ventral tissues of the embryo (Cheng et al., 2000; Tanegashima et al., 2000), while cerberus function is localized to dorsal tissues (Bouwmeester et al., 1996; Schneider and Mercola, 1999; Zorn et al., 1999). In summary, despite equal distribution of maternal VegT across the dorsal-ventral axis of the early embryo (data not shown), region-autonomous expression of the zygotic ligands and the antagonists is integrated at the level of Smad2 phosphorylation and yields distinct patterns of activin-like signaling in dorsal and ventral embryo halves.

β-Catenin alters timing of Smad2 activation

β-Catenin is present as a maternal protein, is enriched in dorsal nuclei after cortical rotation, and is essential for the expression of dorsal-specific genes after MBT (Heasman et al., 1994; Larabell et al., 1997; Schneider et al., 1996; Wylie et al., 1996). To examine whether the dorsal localization of β-catenin after cortical rotation is important in establishing the dorsal initiation of Smad2 phosphorylation, we examined how the inhibition of cortical rotation by u.v. treatment alters the timing of Smad2 phosphorylation, and whether ectopic local

expression of β-catenin can rescue the effects of u.v. treatment. The effectiveness of u.v. treatment and rescue with β-catenin injection was assessed by culturing embryos to stage 40 and examining the resulting phenotypes (Fig. 4A). Embryos treated with u.v. light do not elongate and have no distinctive structures; localized β-catenin injection into u.v.-treated embryos rescues the wild-type body plan (Guger and Gumbiner, 1995).

Smad2 phosphorylation was examined in wild-type, u.v.-treated and u.v.-treated/β-catenin-rescued embryos harvested from stage 8.5 to stage 12.5 (Fig. 4B). In wild-type embryos, Smad2 phosphorylation is initiated before stage 9.5 and is attenuated after stage 10.5. In u.v.-treated embryos, Smad2 phosphorylation is both delayed and prolonged: phosphorylation of Smad2 is initiated later at stage 10+ and is attenuated less, even after stage 11.5. Injection of β-catenin rescues the pattern of early initiation and early attenuation of Smad2 phosphorylation in u.v.-treated embryos. Thus, normal timing of Smad2 phosphorylation requires cortical rotation, and the effects on Smad2 phosphorylation of inhibition of cortical rotation can be rescued by ectopic expression of β-catenin. Maximal levels of Smad2 phosphorylation are not affected by perturbation of the dorsal or vegetal location of endogenous β-catenin, indicating that dorsal β-catenin helps to determine the temporal pattern, but not the maximal level, of activin-like signaling in the developing embryo.

To determine whether u.v. treatment has some more general effect on the timing of signaling rather than a specific effect on Smad2 regulation, we also examined phosphorylation of Smad1 in the same wild-type, u.v.-treated and u.v.-treated/β-catenin-rescued embryos (Fig. 4C). While the levels of phosphorylated Smad1 increase in u.v.-treated embryos in comparison with wild type and β-catenin-rescued embryos, the timing of Smad1 phosphorylation is not altered. A general developmental delay therefore does not explain the differential timing of Smad2 phosphorylation. Rather, timing of Smad2 phosphorylation, but not Smad1 phosphorylation, is developmentally regulated by cortical rotation.

To determine whether β-catenin changes the timing of VegT-induced Smad2 phosphorylation, we examined animal caps dissected before stage 9 and harvested from stage 9 to stage 11.5 (Fig. 4E). Uninjected animal caps do not contain detectable phosphorylation of Smad2 and β-catenin alone is not sufficient to induce Smad2 phosphorylation. VegT induces Smad2 phosphorylation in animal caps by stage 10.5. When injected together, VegT and β-catenin induce by stage 10+ levels of Smad2 phosphorylation seen with VegT alone at stage 10.5. These results demonstrate that β-catenin accelerates the ability of VegT to induce phosphorylation of Smad2 in animal caps, but does not increase the maximal level of Smad2 phosphorylation.

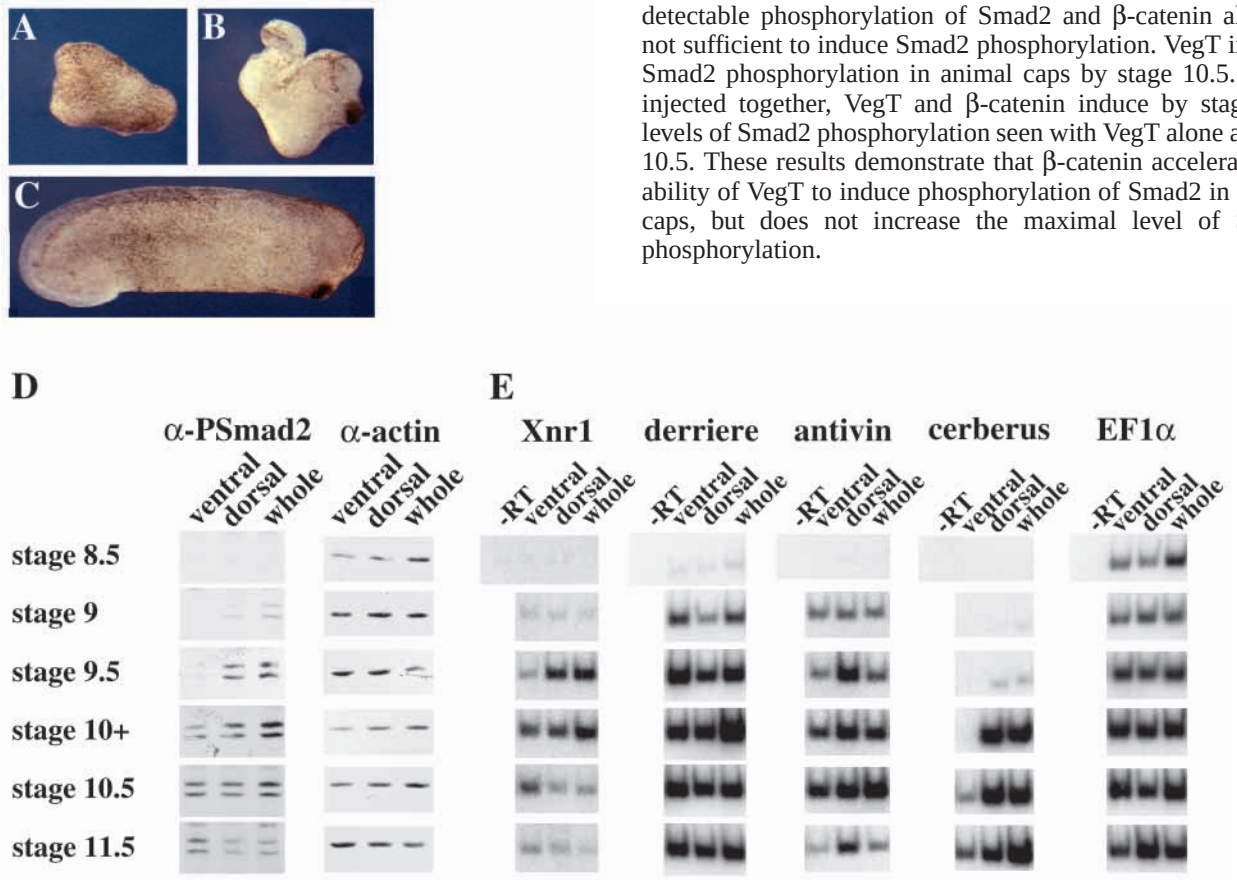

Fig. 3. Dorsal-ventral prepattern directs region-autonomous temporal patterns of endogenous activin-like signaling. (A-C) Phenotype of explants and embryo at stage 30. Embryos were bisected into dorsal and ventral halves at stage 8. Explants were cultured to stage 30. Representative ventral half (A), dorsal half (B) and whole (C) embryos are shown. (D) Endogenous Smad2 phosphorylation is autonomous and distinct in isolated dorsal and ventral halves. Embryos were bisected into dorsal and ventral halves at stage 8. Cultured ventral halves, dorsal halves, and whole embryo controls were harvested at each of the indicated stages for western blot analysis. Each lane contains the same amount of embryonic material; hence, lanes containing whole embryo material should reflect the average amount of signal from ventral and dorsal halves. In general, whole embryos contain more signal, presumably owing to cell loss upon healing after dissection. (E) Autonomous expression of activin-like ligands and inhibitors is distinct in dorsal and ventral halves. Bisected embryos prepared as in D were harvested for RT-PCR analysis of expression of *Xnr1*, *derrière*, *antivin* and *cerberus*. *EF1α* levels serve as loading controls.

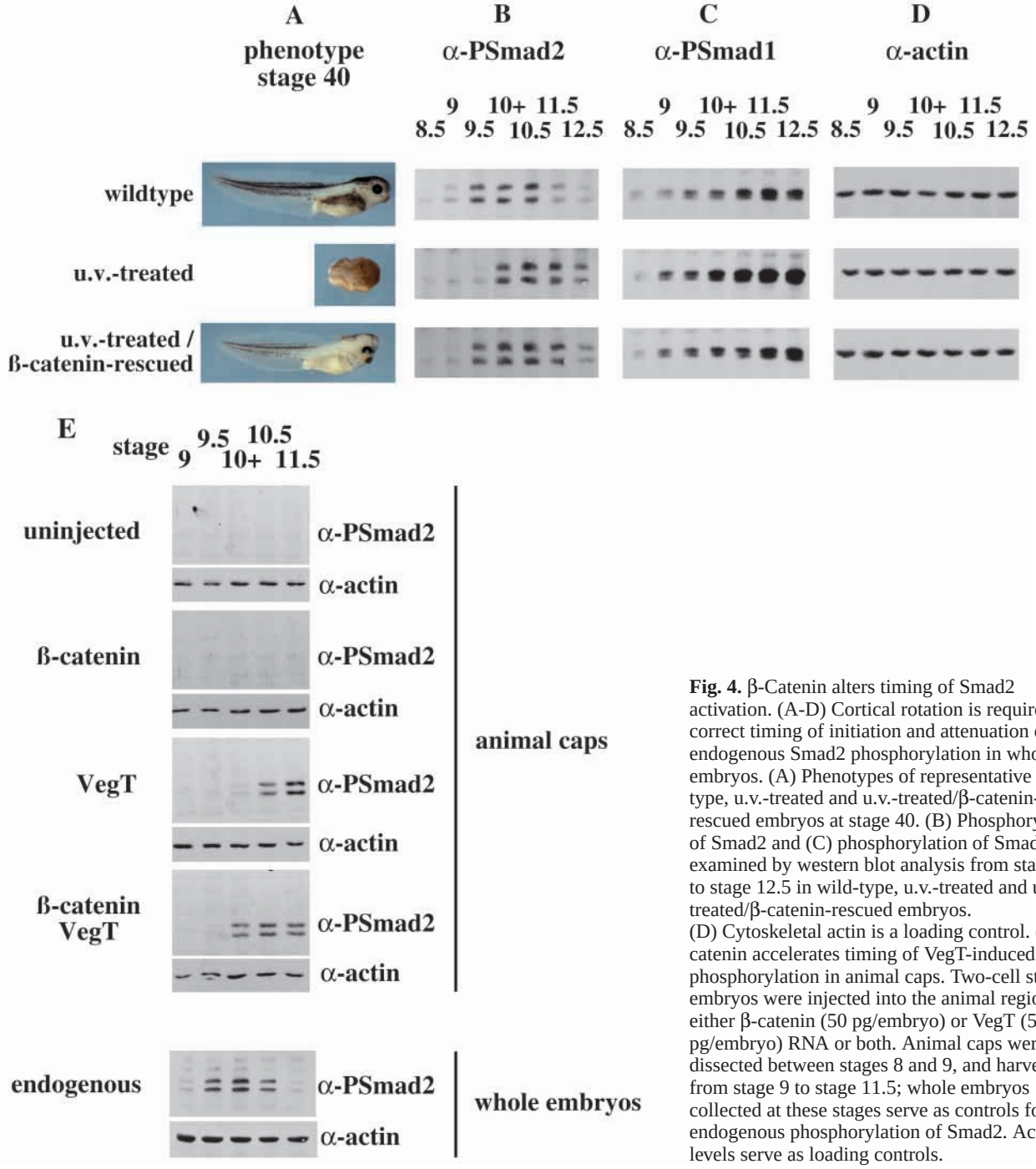

Fig. 4. β-Catenin alters timing of Smad2 activation. (A-D) Cortical rotation is required for correct timing of initiation and attenuation of endogenous Smad2 phosphorylation in whole embryos. (A) Phenotypes of representative wild-type, u.v.-treated and u.v.-treated/β-catenin-rescued embryos at stage 40. (B) Phosphorylation of Smad2 and (C) phosphorylation of Smad1 were examined by western blot analysis from stage 8.5 to stage 12.5 in wild-type, u.v.-treated and u.v.-treated/β-catenin-rescued embryos. (D) Cytoskeletal actin is a loading control. (E) β-catenin accelerates timing of VegT-induced Smad2 phosphorylation in animal caps. Two-cell stage embryos were injected into the animal region with either β-catenin (50 pg/embryo) or VegT (50 pg/embryo) RNA or both. Animal caps were dissected between stages 8 and 9, and harvested from stage 9 to stage 11.5; whole embryos collected at these stages serve as controls for endogenous phosphorylation of Smad2. Actin levels serve as loading controls.

Activin-like ligands induce distinct temporal profiles of Smad2 activation

We have previously shown that, while activin can induce Smad2 phosphorylation before MBT, processed and active maternal Vg1 (Thomsen and Melton, 1993) induces Smad2 phosphorylation only after MBT (Faure et al., 2000). To define responsiveness to zygotic activin-like ligands, we examined the activities of these ligands before or after MBT and in the presence or absence of α-amanitin. While the constitutively active type I receptor ALK4* (Armes and Smith, 1997) can induce Smad2 phosphorylation before MBT, each of the zygotic activin-like ligands Xnrs 1, 2, 4 and derrière (Jones et al., 1995; Joseph and Melton, 1997; Lustig et al., 1996a; Sun et al., 1999) induces Smad2 phosphorylation only after MBT, not before (Fig. 5A). Furthermore, this Smad2 phosphorylation by the zygotic activin-like ligands after MBT is not affected by inhibition of zygotic transcription with α-amanitin (Fig. 5B). Both the zygotic activin-like ligands and the maternal ligand Vg1 therefore appear to be regulated by a post-transcriptional mechanism in their ability to induce Smad2 phosphorylation after MBT.

The activin-like ligands Xnr1 and derrière function

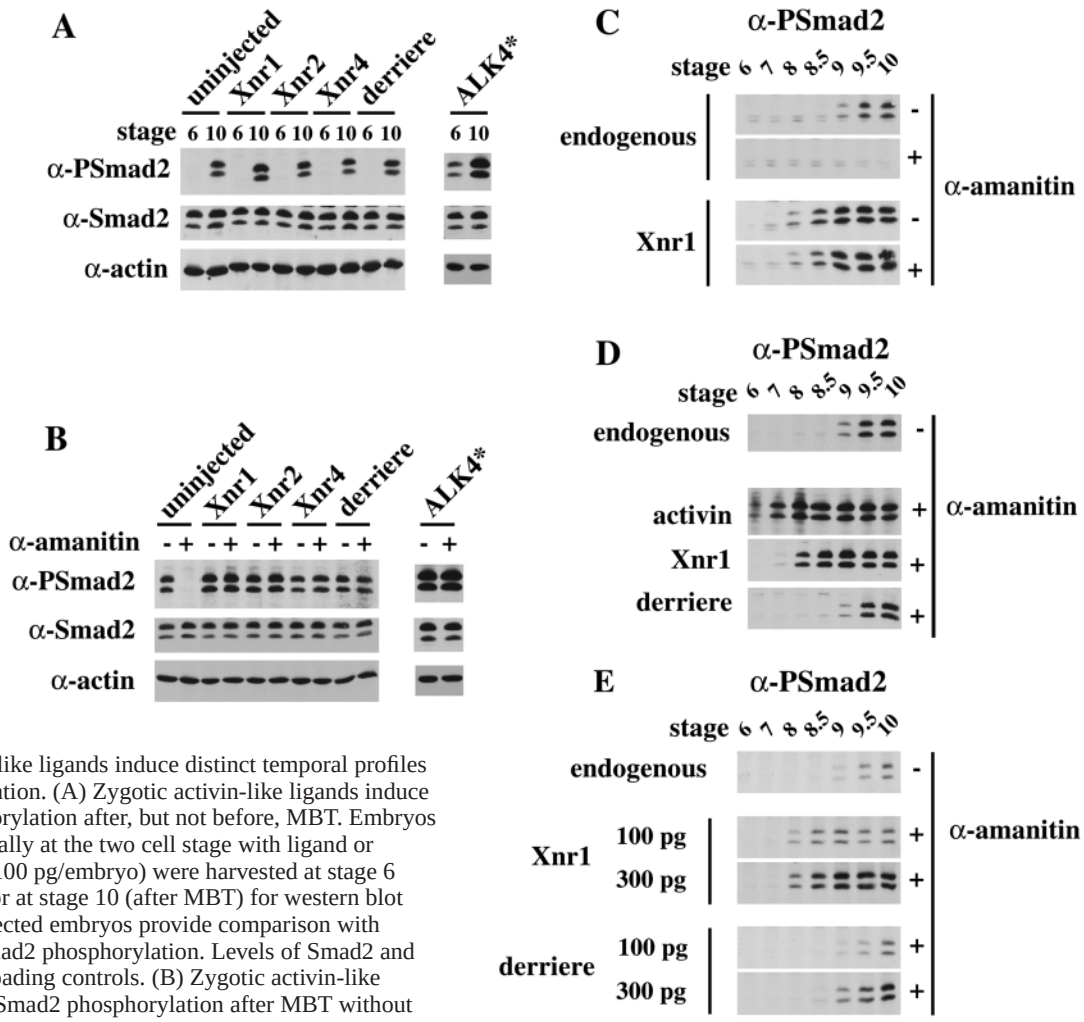

Fig. 5. Activin-like ligands induce distinct temporal profiles of Smad2 activation. (A) Zygotic activin-like ligands induce Smad2 phosphorylation after, but not before, MBT. Embryos injected marginally at the two cell stage with ligand or ALK4* RNA (100 pg/embryo) were harvested at stage 6 (before MBT) or at stage 10 (after MBT) for western blot analysis. Uninjected embryos provide comparison with endogenous Smad2 phosphorylation. Levels of Smad2 and actin serve as loading controls. (B) Zygotic activin-like ligands induce Smad2 phosphorylation after MBT without requirement for zygotic transcription. Embryos injected marginally at the two-cell stage with ligand or ALK4* RNA (100 pg/embryo) alone or with α-amanitin injected vegetally at the four-cell stage were harvested at stage 10+ for western blot analysis. Uninjected embryos provide comparison with endogenous Smad2 phosphorylation. Levels of Smad2 and actin serve as loading controls. (C) Timing of ligand-induced Smad2 phosphorylation is not altered by inhibition of zygotic transcription. Patterns of Xnr1-induced Smad2 phosphorylation were compared with endogenous patterns from stages 6 to 10 in the presence or absence of α-amanitin. Xnr1 RNA (100 pg/embryo) was injected marginally at the two-cell stage; α-amanitin was injected vegetally at the four-cell stage. (D) Responsiveness for Smad2 activation is transcription independent and ligand specific. Patterns of Smad2 phosphorylation induced by activin, Xnr1 or derrière in the presence of α-amanitin were compared with each other and to the endogenous pattern without α-amanitin from stages 6 to 10. Ligand RNA (100 pg/embryo) was injected marginally at the two-cell stage; α-amanitin was injected vegetally at the four-cell stage. (E) Timing of responsiveness to ligands is not altered by increased dose. Patterns of Smad2 phosphorylation induced by Xnr1 or derrière at 100 pg/embryo or 300 pg/embryo in the presence of α-amanitin were compared with each other and with the endogenous pattern without α-amanitin from stages 6 to 10. Ligand RNA was injected marginally at the two-cell stage; α-amanitin was injected vegetally at the four-cell stage.

differently in the early embryo, as induction of anterior and dorsal tissues appears to be mediated by Xnr1/Xnr2 but not by derrière (Kofron et al., 1999; Osada and Wright, 1999; Sun et al., 1999). To attempt to identify a difference in the signaling mechanisms of Xnr1 and derrière, we compared the timing of Smad2 phosphorylation induced by these ligands in embryos harvested from stage 6 to stage 10 (Fig. 5D). To more clearly detect effects of ectopic ligands, endogenous signaling was suppressed by co-injection with α-amanitin (Faure et al., 2000). Activin induces Smad2 phosphorylation before stage 6, the earliest stage examined. Xnr1 induces Smad2 phosphorylation from stage 8, while derrière induces Smad2 phosphorylation strongly from stage 9.5. To confirm that the differences observed between Xnr1 and derrière reflect distinct timing of responsiveness rather than effective dose, we compared timing of Smad2 phosphorylation induced by injected RNA encoding Xnr1 or derrière at two doses: 100 pg/embryo and 300 pg/embryo (Fig. 5E). Increasing the dose of RNA increases the level of Smad2 phosphorylation, but does not alter the time of onset of Smad2 phosphorylation. These results indicate that Xnr1 and derrière are distinguished by the timing of cellular responsiveness to each ligand in the early embryo. The appearance of responsiveness to ligands is unaffected by α-amanatin, indicating the timing of this process is not dependent on zygotic transcription (Fig. 5C).

DISCUSSION

A wave of Smad2 phosphorylation in the prospective mesendoderm: generation of the rising phase

Maternal VegT is a definitive determinant of endogenous activin-like signaling (Fig. 1A). When maternal VegT is depleted, the zygotic activin-like ligands – Xnrs 1, 2, 4, 5 and 6, and derrière (Jones et al., 1995; Joseph and Melton, 1997; Lustig et al., 1996a; Sun et al., 1999; Takahashi et al., 2000) – are not expressed (Kofron et al., 1999). That maternally derived ligands such as Vg1 and activin (Fukui et al., 1994; Oda et al., 1995; Weeks and Melton, 1987) are not sufficient to activate Smad2 to detectable levels raises the possibility that maternal VegT is indirectly required for the post-transcriptional activation of the maternal ligands (e.g. by regulating the expression of processing enzymes). Alternatively, the maternal ligands may act cooperatively with zygotic ligands but are themselves insufficient for Smad2 activation.

Maternal VegT and Smad2 are present at similar levels dorsally and ventrally (data not shown; Faure et al., 2000), but both Smad2 phosphorylation (Fig. 3D) and expression of *Xnr1*, *Xnr5*, *Xnr6*, *derrière* (Fig. 3E; Agius et al., 2000; Sun et al., 1999; Takahashi et al., 2000) and also *Xnr2* (Jones et al., 1995; Chris Wright, personal communication) begin earlier dorsally than ventrally. This suggests that additional maternal factors, differentially distributed or activated along the prospective dorsal-ventral axis, interact with VegT to generate this dorsal-ventral asymmetry in Smad2 regulation. β-Catenin is a likely candidate for such a factor, as it is enriched in nuclei on the dorsal side, and injection of β-catenin can rescue the early initiation of Smad2 phosphorylation in embryos in which cortical rotation has been prevented (Fig. 4B). Analysis of the *Xnr1* promoter has shown that it contains both VegT- and β-catenin-binding sites (Hyde and Old, 2000; Kofron et al., 1999). Antagonism of VegT function blocks activation of this promoter entirely (Hyde and Old, 2000), while antagonism of β-catenin activity blocks *Xnr1* expression at stage 9 but not at stage 9.5 (Agius et al., 2000). These observations are consistent with a model in which VegT and β-catenin cooperate transcriptionally in the dorsal marginal zone to accelerate expression of activin-like ligands, and thus accelerate Smad2 phosphorylation. In addition to Xnr1, four more nodal-related ligands have been identified in the prospective mesendoderm that can act as mesoderm inducers (Xnr2, Xnr4, Xnr5 and Xnr6) (Jones et al., 1995; Joseph and Melton, 1997; Takahashi et al., 2000). Characterization of the regulation and contribution to local Smad2 phosphorylation of each of these ligands will be necessary to develop a complete picture of how this family of ligands participates in mesodermal patterning.

While β-catenin can cooperate with VegT to accelerate the initiation of Smad2 phosphorylation when both are expressed in animal caps, ectopic expression of these two factors is not sufficient to recapitulate endogenous timing of Smad2 phosphorylation in the mesendoderm. Co-injection of β-catenin with VegT induces Smad2 phosphorylation in animal caps at stage 10+ (Fig. 4E), but even high doses of both factors will not induce Smad2 phosphorylation earlier than stage 10+. Endogenous Smad2 phosphorylation begins significantly earlier than this, at stage 9, both in intact embryos or β-catenin-rescued u.v.-treated embryos (Fig. 4B,E). This indicates that either animal caps contain regulators that delay Smad2 activation in the presence of β-catenin and VegT relative to endogenous prospective mesendoderm, or that the prospective mesendoderm contains factors that accelerate Smad2 phosphorylation relative to the effects of β-catenin and VegT alone.

A wave of Smad2 phosphorylation in the prospective mesendoderm: generation of the falling phase

Two inhibitors of activin-like ligands, antivin and cerberus, are rapidly induced by activin-like signaling through Smad2 and FAST. In the early gastrula stage embryo, the same combination of regulators that generates the dorsal 'rising phase' of the wave of Smad2 phosphorylation across the marginal zone also results in the initiation of *antivin* and *cerberus* expression on the dorsal side. Antivin and cerberus proteins therefore accumulate to levels that antagonize activin-like signaling earlier on the dorsal side. Positive regulation by Smad2 activation is probably not the only mechanism that enhances dorsal expression of these inhibitors, as β-catenin may also contribute directly to dorsal *cerberus* expression (Zorn et al., 1999). These observations suggest a relatively simple mechanism by which the activation, and subsequently the attenuation, of activin-like signaling begin from the dorsal side. Within this apparently simple model, however, puzzles regarding specific aspects of dose and timing remain. How are the rates of synthesis of activin-like ligands and inhibitors, and the affinities of each for the other calibrated so that the inhibitors accumulate rapidly enough to efficiently inhibit the ligands? Why does the expression of inhibitors not drop as soon as they begin to inhibit activin-like ligand activity? More quantitative analyses will be necessary to clarify how feedback regulation stably and reliably generates the observed wave of Smad2 signaling.

Is Smad2 signaling propagated across the dorsal-ventral or animal-vegetal axes?

Phosphorylation of Smad2 begins asymmetrically in a region of the late blastula that is both dorsal and vegetal and extends to encompass both ventral and marginal cells by the early gastrula stage. Both diffusion of ligands and local positive autoregulation (relay) of ligand expression have been suggested as mechanisms by which TGFβ superfamily signals might be propagated across the embryo (Jones et al., 1996a; Reilly and Melton, 1996; Osada and Wright, 1999), but it is also possible that changing patterns of signal activation are mediated primarily by differentially timed, cell-autonomous expression of ligands. While tissue recombination experiments have demonstrated that patterning signals can be propagated both between vegetal and animal explants (Nieuwkoop, 1969), and between dorsal and ventral marginal zones (Slack and Forman, 1980; Ding et al., 1998b), it is not clear whether these propagated signals are the predominant ones mediating endogenous patterning. We find that Smad2 phosphorylation can be activated independently, and with distinct timing, in dorsal and ventral embryonic halves isolated at stage 8 (Fig. 3D,E). This indicates that the activation of Smad2 is region-autonomous across the dorsal-ventral axis, but does not rule out the possibility of more locally propagated signaling. The attenuation of Smad2 signaling by feedback inhibitors is likewise region-autonomous (Fig. 3D,E), again suggesting that

these inhibitors are acting at most across relatively short distances rather than globally. The available resolution of dissections do not permit a clear answer to the question of whether Smad2 activating signals are propagated from the vegetal region into the marginal zone. What is clear, however, is that Smad2-activating signals do not propagate towards the animal pole beyond the blastocoel floor. In light of observations that several Smad2-activating ligands are positively autoregulatory, the interesting question is raised of how Smad2 phosphorylation is excluded from the prospective ectoderm. Identification of the mechanisms that inhibit the propagation of a positive feedback loop of Smad2-activating signaling into the animal pole will be an important aspect of understanding the mechanism of germ layer establishment.

Timing of endogenous activin-like signals may direct cell fate specification in the embryo

Can manipulation of the timing of Smad2 phosphorylation direct regionally distinct mesendodermal patterning? The observation that the timing of initiation of Smad2 phosphorylation is regulated across marginal and vegetal regions suggests, but does not in itself demonstrate, a role for this timing in axial patterning. Ectopic Xnr1 induces Smad2 phosphorylation to maximal levels by stage 9, while derrière does not induce maximal levels of phosphorylation until stage 10 (Fig. 5D,E); these differences correspond roughly to the timing of Smad2 phosphorylation in prospective dorso-anterior and ventro-posterior regions, respectively (Figs 2, 3D). Interestingly, Xnr1 is an effective inducer of relatively dorsal and anterior tissues, while derrière induces relatively ventral and posterior tissues and does not induce notochord or heads (Osada and Wright, 1999; Sun et al., 1999). Soluble activin ligand added to progressively older animal caps induces differentiation of progressively less dorsal tissues (Green et al., 1990), further supporting the idea that timing of the initiation of Smad2 phosphorylation is instructive for tissue differentiation. As for early activation of Smad2 signaling, early attenuation of Smad2 phosphorylation is associated with the induction of relatively dorso-anterior structures (Figs 2, 3D, 4B; Gritsman et al., 2000; Thisse et al., 2000), suggesting that signal attenuation, as well as signal onset, may play a role in patterning of the mesendoderm.

Is timing of responsiveness to ligands a point of developmental regulation?

Ectopic expression of activin, Xnr1 or derrière activates Smad2 phosphorylation at distinct developmental stages (Fig. 5D). This regulated timing of responsiveness to ectopic ligand expression may be conferred at the level of production of mature ligands, competence of cells to respond to ligands, or both. TGFβ superfamily ligands must be dimerized and cleaved intracellularly for maturation (Lopez et al., 1992), thereby providing several potential points for regulation (Constam and Robertson, 1999). Recent work demonstrating that the biological activities of nodal orthologs in several species, but not activin, require co-receptors of the epidermal growth factor-cripto, FRL-1, criptic (EGF-CFC) family, raises the possibility that specific EGF-CFC-like molecules are limiting for Xnr1 or derrière signaling in early embryos (Chang and Whitman, 2001; Ding et al., 1998a; Gritsman et al., 1999). The appearance of responsiveness to ectopic nodal-related ligands and derrière is independent of zygotic transcription (Fig. 5B-E), indicating that regulation of EGF-CFC co-receptors, like FRL-1 (Kinoshita et al., 1995), or other limiting components occurs at the level of translation, proteolysis or other post-translational processing. Timing of responsiveness may be a critical mechanism by which different ligands use the same pathway to cause different effects on developmental patterning.

Models of early developmental patterning should consider temporal regulation of activin-like signals

Activin can act as a morphogen on animal cap cells, inducing ventral-to-lateral-to-dorsal mesodermal fates with increasing dose (Green et al., 1992). Activin-like signaling through Smad2 can also act synergistically with β-catenin to induce organizer-specific markers (Crease et al., 1998; Cui et al., 1996; Sokol and Melton, 1992; Watabe et al., 1995). Based on these and related observations, two general models have been proposed for the role of activin-like signals in the dorsal-ventral patterning of the mesoderm. In one model (Agius et al., 2000), activin-like signals are postulated as a static gradient that decreases across the dorsal-ventral axis; the highest level dorsally presumably resulting from overlap with dorsally localized β-catenin. In the alternative model (Clements et al., 1999; Crease et al., 1998), activin-like signaling is represented as evenly distributed across the dorsal-ventral axis of the marginal zone; this signal interacts with dorsal β-catenin to regulate organizer gene expression. In contrast to these models, we find neither an even distribution nor a static gradient of activin-like signaling across the dorsal-ventral axis. Rather, we find that the dorsal-ventral distribution of Smad2 phosphorylation changes dramatically as gastrulation proceeds (Fig. 6). We propose that, while the magnitude of Smad2

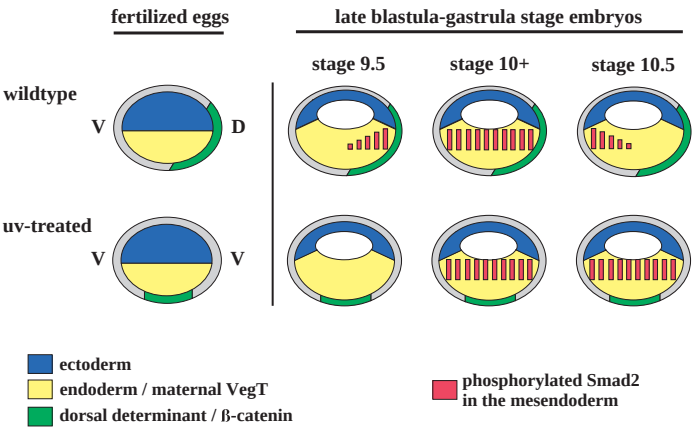

Fig. 6. Dorsal β-catenin modifies timing of Smad2 phosphorylation in early developmental patterning of the *Xenopus* embryo. In the wild-type embryo, early initiation of Smad2 phosphorylation at stage 9.5 in the dorsal vegetal region results from cooperation between dorsal β-catenin and vegetal VegT. At stage 10.5, attenuation of Smad2 phosphorylation on the dorsal side is mediated by expression of negative feedback inhibitors (see text). In u.v.-treated embryos, Smad2 phosphorylation appears later and persists longer than in wild-type embryos. Maximal levels of Smad2 phosphorylation, however, are not affected by perturbation of the location of endogenous β-catenin, indicating that dorsal β-catenin determines the temporal patter, not the maximal level, of endogenous activin-like signaling.

phosphorylation may distinguish the primary germ layers (Faure et al., 2000), the timing of Smad2 phosphorylation determines the role of activin-like signaling in patterning across the dorsal-ventral and anterior-posterior axes.

How might the timing of Smad2 phosphorylation direct cell fate? One possibility is that cells 'take the integral' of dose of signal received over time. In this case it is interesting to note that ventro-posterior regions of the embryo may actually receive more cumulative exposure to phosphorylated Smad2 than dorso-anterior regions, as Smad2 phosphorylation is prolonged on the ventral side. Alternatively, the importance of the timing of activin-like signals may be defined relative to the activities of other signaling pathways. For example, BMP signaling, like activin-like signaling, begins soon after MBT. It is initially ubiquitous, but the expression of dorsal BMP antagonists, combined with positive autoregulation of BMP expression, creates a steep ventral-dorsal gradient in Smad1 phosphorylation by the onset of gastrulation (reviewed in Cho and Blitz, 1998; Faure et al., 2000). In wild-type gastrula stage embryos, the dorsal side is distinguished by inhibition of BMP-regulated Smad1 activation and attenuation of Smad2 activation, while on the ventral side both Smad1 and Smad2 are activated. Smad2 signaling that begins on the ventral side during early gastrulation is acting in cells that have been exposed to BMP signals for several hours, potentially creating a very different cellular context for Smad2 action from that present in dorsal cells several hours before. Cell specification in the early embryo clearly requires the integration of multiple signals, and it may require the integration of a given signal over an extended period of time, but the still more complex possibility is that these two integration functions are tightly linked. In this case, understanding how the timing of Smad2 activation affects patterning will require the molecular definition of how cellular context changes in response to multiple signaling pathways as a function of time.

We thank Peter ten Dijke for crude anti-phosphoSmad antisera. We also thank Doug Melton, Eddy De Robertis, Kristin Kwan, Adrian Salic, Hazel Sive, Fiona Stennard, Jeff Wrana, Minoru Watanabe and Chris Wright for constructs used in this work. We appreciate the helpful comments and review of the manuscript by Charles Farnsworth, Sandrine Faure, Tracy Keller, Kristin Kwan, Aditya Thaduri, Minoru Watanabe, Stephan Wawersik and Chang-Yeol Yeo. We thank Mark Mercola and his laboratory for use of the microscope and digital camera. M. A. L. was supported by a NIGMS grant (GM15389) and a UNCF-Merck Graduate Science Research Dissertation Fellowship. J. H. was supported by NIH RO1 grant HD38272. M. W. was supported by grants from the NICHD.

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