The novel Smad-interacting protein Smicl regulates *Chordin* expression in the *Xenopus* embryo

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Summary

In this paper, we investigate the function of Smicl, a zinc-finger Smad-interacting protein that is expressed maternally in the *Xenopus* embryo. Inhibition of Smicl function by means of antisense morpholino oligonucleotides causes the specific downregulation of *Chordin*, a dorsally expressed gene encoding a secreted BMP inhibitor that is involved in mesodermal patterning and neural induction. *Chordin* is activated by Nodal-related signalling in an indirect manner, and we show here that Smicl is involved in a two-step process that is necessary for this activation. In the first, Smad3 (but not Smad2) activates expression of *Xlim1* in a direct fashion. In the second, a complex containing Smicl and the newly induced Xlim1 induces expression of *Chordin*. As well as revealing the function of Smicl in the early embryo, our work yields important new insight in the regulation of *Chordin* and identifies functional differences between the activities of Smad2 and Smad3 in the *Xenopus* embryo.

Key words: Smicl, *Xenopus*, Chordin, Smad, Nodal, Spemann's organiser

Introduction

Great progress has been made in coming to understand the series of inductive interactions that generates the body plan of the early amphibian embryo (Chan and Etkin, 2001; De Robertis and Kuroda, 2004; Heasman, 1997; Weaver and Kimelman, 2004). Briefly, fertilisation causes rotation of the cortical cytoplasm, which in turn brings about the Wnt11-dependent stabilisation of β-catenin on the dorsal side of the embryo at the blastula stage (Tao et al., 2005). This nuclear β-catenin is involved in the induction of two signalling centres in the blastula. The first, in dorsal ectoderm, is the recently identified BCNE (blastula *Chordin*- and *Noggin*-expressing) centre that is required for proper development of the nervous system. The second is the Nieuwkoop centre, which is formed in dorsal vegetal cells, where the domain of nuclear β-catenin overlaps the vegetally localised maternal mRNAs Vg1 and VegT (De Robertis and Kuroda, 2004).

The Nieuwkoop centre induces the formation of Spemann's organiser in overlying equatorial cells and, through the action of Nodal-related proteins such as Xnr1, Xnr2, Xnr4, Xnr5 and Xnr6, activates the expression of genes such as *Noggin* and *Chordin*, which encode secreted inhibitors of BMP signalling (Agius et al., 2000; De Robertis and Kuroda, 2004). The mechanism by which the Nodal-related proteins induce these genes is poorly understood, although it is known that *Chordin* is an indirect target of the Nodal-related proteins and of Activin, because its activation is inhibited by the protein synthesis inhibitor cycloheximide (Howell and Hill, 1997; Sasai et al., 1994).

In this paper, we provide new insight into the regulation of *Chordin* through our analysis of the novel Smad-interacting protein Smicl (Collart et al., 2005). Receptors of TGFβ family members such as the Nodal-related proteins and Activin signal by phosphorylating, and thereby activating Smad2 and Smad3 (Miyazawa et al., 2002). Once activated, these Smad proteins bind Smad4 and translocate to the nucleus where they regulate gene expression. This is achieved through direct interaction with DNA or by interaction with other transcriptional regulators such as Fast1 (Massague and Wotton, 2000). We show here that *Smicl* is expressed maternally in the *Xenopus* embryo and is required for the expression of *Chordin,* but not of *Goosecoid* or *Xnr3*, in Spemann's organiser. Significantly, the phenotype of embryos lacking Smicl resembles that of embryos in which Chordin is depleted. Smicl interacts specifically with Smad3 and is involved in the second step of an indirect pathway through which the Nodal-related proteins activate *Chordin.* In the first step, Smad3 activates the expression of *Xlim*. In the second a complex containing Smicl, Smad3 and the newly induced Xlim1 activates expression of *Chordin* in a direct manner. Our work defines the role of Smicl in the early *Xenopus* embryo and contributes new findings to the hitherto poorly understood regulation of *Chordin.*

Materials and methods

Xenopus embryos and microinjection

Xenopus embryos were obtained by artificial fertilisation. *X. laevis* embryos were maintained in 10% normal amphibian medium (NAM)

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(Slack, 1984) and *X. tropicalis* embryos in 1% MMR. They were staged according to Nieuwkoop and Faber (Nieuwkoop and Faber, 1975). Embryos at the one-cell stage were injected with RNA or with antisense morpholino oligonucleotides dissolved in water. For animal cap assays, embryos were dissected at stage 8, before the midblastula transition (MBT), and cultured in 75% NAM. Cycloheximide was dissolved in ethanol to a concentration of 10 mM and then diluted to a final concentration of 10 μM in 75% NAM containing 0.1% bovine serum albumin. Animal caps were frozen when sibling embryos reached stage 11.

Western blotting

The efficacy of antisense morpholino oligonucleotide XtMO1, directed against *Xenopus tropicalis* Smicl, was tested by injecting embryos with XtMO1 or the control oligonucleotide coMO followed by RNA encoding an HA-tagged form of XtSmicl. Embryos were allowed to develop to early gastrula stage 10 and were then subjected to SDS polyacrylamide gel electrophoresis and western blotting, using rat monoclonal anti-HA antibody 3F10 (Roche Diagnostics) to detect the HA epitope and a mouse monoclonal anti-GAPDH antibody (HyTest Ltd) as a loading control.

Whole-mount in situ hybridisation

In situ hybridisation was carried out essentially as described previously (Harland, 1991), except that BMP purple was used as a substrate. A *Chordin* probe was as described (Sasai et al., 1994) and expression of *XtSmicl* was detected by transcription of a *Smicl* cDNA in the vector pCS107 derived from the *Xenopus tropicalis* EST database (http://www.sanger.ac.uk/Projects/X_tropicalis/; GenBank Accession Number AL675957). The plasmid was linearised with *Eco*RI and transcribed with T3 RNA polymerase.

Real time RT-PCR

Total RNA was prepared from five pooled *X. laevis* embryos, 30 *X. tropicalis* embryos or 10 *X. laevis* animal caps using the TriPure reagent (Roche), followed by DNAseI digestion, proteinase K treatment, phenol/chloroform extraction and ethanol precipitation. RNA was dissolved in water and used as a template for real-time RT-PCR.

Real-time RT-PCR with the LightCycler (Roche) was carried out using the manufacturer's RNA amplification kit. All determinations included a negative control and a serial dilution of embryo RNA was used to create a standard curve. Primers specific for *Xbra*, *Goosecoid*, *Chordin* and *Ornithine decarboxylase* (*ODC*) were as described previously (Piepenburg et al., 2004), *Sox17* and *Xlim1* were as described previously (Xanthos et al., 2001), *Xnr3* was as described previously (Kofron et al., 1999) and *Siamois* was as described previously (Heasman et al., 2000). *XtSmicl* primers were 5′-AGC-GCAGTCTGGCCATCATC-3′ and 5′-TCGGGAGACATAGACG-TGGC-3′. All values were normalised to the level of ODC in each sample.

Expression constructs and transcription

A mouse Smicl cDNA comprising the entire open reading frame except for the first six amino acids was cloned between the *Eco*RI and *Xba*I sites of pCS3, thereby introducing six N-terminal Myc tags. The construct was linearised with *Asp*718 and sense RNA was transcribed with SP6 RNA polymerase. An *X. tropicalis* cDNA comprising the entire XtSmicl open reading frame and 45 bp of 5′ UTR (GenBank Accession Number AY887083) was provided with a C-terminal HA tag by PCR and cloned between the *Eco*RI and *Xba*I sites of pCS2. Sense RNA was produced using SP6 RNA polymerase after linearisation of the plasmid with *Asp*718. A Myc-tagged Smad2 construct, cloned in pFTX5 (Howell and Hill, 1997), was linearised with *Xba*I and sense RNA was transcribed with T7 RNA polymerase. The open reading frame of *Chordin*, cloned in pSP35T (the gift of E. De Robertis), was linearised with *Xba*I and transcribed with SP6 polymerase. The open reading frame of Xnr1 cloned in pCS2 (Williams et al., 2004) was linearised with *Asp*718, and sense RNA was transcribed with SP6 RNA polymerase. A Smad3 construct in pCS2 was linearised with *Asp*718, and sense RNA was transcribed with SP6 RNA polymerase. Flag-Xlim1/3m, cloned in pCS2 (Yamamoto et al., 2003), was linearised with *Not*I and sense RNA was transcribed with SP6 RNA polymerase. A cDNA encoding Siamois, cloned in pBluescript RN3 (Lemaire et al., 1995), was linearised with *Sfi*I and sense RNA was transcribed with T3 RNA polymerase. A constitutively active β-catenin construct cloned in pSP64T (Domingos et al., 2001), was linearised with *Sfi*I and sense RNA was synthesised using SP6 RNA polymerase. Myc-tagged Smad constructs were a gift from Dr K. Miyazono. Constitutively active forms of ALK6 and ALK4 were as described (Armes and Smith, 1997).

Cell lines and transfections

HEK293T cells were grown in Dulbecco's modified Eagle's medium containing 10% foetal bovine serum (FBS) supplemented with 4.5 g/l glucose. Cells were grown to 50% confluence in 9 cm dishes and transfected using Fugene (Roche Molecular Biochemicals) according to the manufacturer's protocol.

Co-immunoprecipitation experiments

Transiently transfected HEK293T cells were frozen in liquid nitrogen, thawed on ice and solubilised in lysis buffer containing 1% NP40, 150 mM NaCl, 20 mM Tris pH 7.5, 2 mM EDTA, 50 mM NaF, 1 mM sodium pyrophosphate, supplemented with protease inhibitors (Roche Molecular Biochemicals). Cell lysates were cleared by centrifugation, and precipitations were performed by overnight incubations with beads coupled to mouse monoclonal anti HA (Roche), mouse monoclonal M2Flag or mouse monoclonal 9E10 anti Myc (Santa Cruz). Unbound proteins were removed by washing four times with lysis buffer and once with phosphate-buffered saline at 4°C. Bound proteins were harvested by boiling in sample buffer, and they were resolved by SDS-polyacrylamide gel electrophoresis. Myc-tagged, Flag-tagged, HA-tagged proteins and endogenous Smad3 were visualised after western blotting using mouse monoclonal 9E10 anti-Myc (a gift from Innogenetics), anti-M2Flag (Santa Cruz), anti-HA (Roche) and rabbit polyclonal anti-Smad3 (Abcam) antibodies, in combination with horseradish peroxidase-conjugated anti-mouse and anti-rabbit secondary antibodies (Jackson), and the enhanced chemiluminescence kit (New England Nuclear).

Biotinylated oligonucleotide precipitation assay

DNA precipitations using biotinylated double-stranded oligonucleotides corresponding to base pairs –696 to –621 relative to the translation start site of *X. tropicalis Chordin* (http://genome.jgi-psf.org/Xentr3/Xentr3.home.html) were carried out as described (Hata et al., 2000). The sequence of the wild-type oligonucleotide was 5′ CCATAC*TGATTA*TTCCCCAAATCTTGTCAAATTCTATGTAG-CTTTCCCACATG*CAATTA*TCTGCATGTCCCCCACT 3′. The sequence of a mutated oligonucleotide was 5′ CCATAC*CTTTTA*T-TCCCCAAATCTTGTCAAATTCTATGTAGCTTTCCCACATG*AC-CAAG*TCTGCATGTCCCCCACT 3′. Wild type and mutated Xlim1 binding sites (Mochizuki et al., 2000) are indicated in italics. DNA-bound proteins were collected with streptavidine-agarose beads (Sigma) and analyzed by western blotting.

Results

Cloning and expression analysis of a *Xenopus* homologue of mouse Smicl

Mouse Smicl (Smad-interacting CPSF-like protein) was identified as a candidate Smad-interacting protein in the yeast two-hybrid screen that also identified SIP1 (Collart et al., 2005; Verschueren et al., 1999). A cDNA encoding its

putative *Xenopus tropicalis* homologue (*XtSmicl*) was identified by BLAST searching the *Xenopus tropicalis* EST database (http://www.sanger.ac.uk/Projects/X_tropicalis/), and a full-length version was cloned by reverse transcription PCR using cDNA derived from blastula stage embryos. The deduced amino acid sequence (GenBank Accession Number AY887083), comprising 827 residues, displays 43.1% identity and 44.7% similarity to the mouse sequence (GenBank Accession Number AJ516034), with a domain containing five C3H type zinc fingers being particularly highly conserved.

We first carried out co-immunoprecipitation experiments in HEK293T cells to determine whether XtSmicl, like mouse Smicl, is a Smad-interacting protein. XtSmicl was co-expressed with Smad1 or Smad5 (which act downstream of BMP family members), Smad2 or Smad3 (which act downstream of TGFβ, Activin and Nodal family members), or the common mediator Smad4, in the presence or absence of

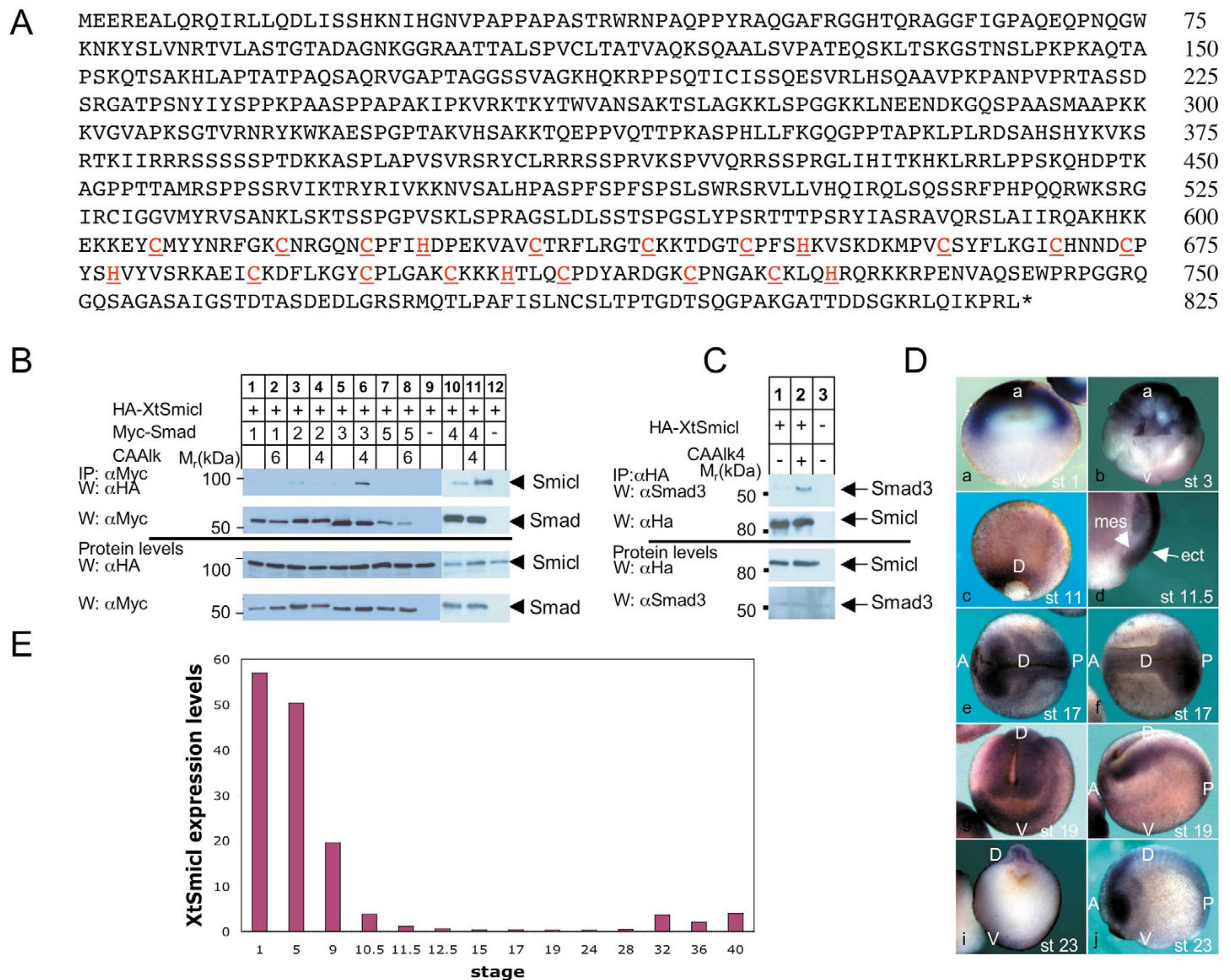

Fig. 1. Smicl is a Smad-interacting protein that is expressed maternally and then zygotically during *Xenopus* development. (A) Amino acid sequence of XtSmicl, a novel Smad interacting protein. Conserved cysteines and histidines in the zinc fingers are underlined. (B) Smicl interacts with Smad3 and Smad4. HA-tagged XtSmicl was expressed in HEK293T cells with plasmids encoding Myc-tagged Smad1, Smad2, Smad3, Smad4 or Smad5 in the presence or absence of the appropriate constitutively active type I receptor. Samples were immunoprecipitated (IP) using an anti-Myc antibody and the presence of HA-XtSmicl was analysed by western blotting (W). (C) Smicl interacts with endogenous Smad3. HA-tagged XtSmicl was expressed in HEK293T cells in the presence or absence of caALK4. Samples were immunoprecipitated with anti-HA coupled beads and the presence of endogenous Smad3 was analysed by western blotting. (D) Expression pattern of *Xenopus tropicalis Smicl* studied by whole-mount in situ hybridisation. Stages (st) are indicated. a, animal; v, vegetal; D, dorsal; V, ventral; A, anterior; P, posterior; mes, mesoderm; ect, ectoderm. *Smicl* is expressed maternally and transcripts are particularly abundant in the animal hemisphere of the fertilised egg. These results were confirmed by in situ hybridisation experiments carried out on bisected embryos to facilitate penetration of the probe in the vegetal hemisphere of the embryo. In situ hybridisation using a sense probe revealed no background staining (data not shown). (E) Quantitative RT-PCR confirms the presence of high levels of *Smicl* RNA in the fertilised egg of *Xenopus tropicalis*. Significant zygotic expression of *Smicl* begins at tadpole stage 32.

their cognate constitutively active (ca) receptors. Smad proteins were immunoprecipitated from cell extracts with anti-Myc antibody and the presence of HA-tagged XtSmicl in the immunoprecipitate was detected by western blotting. XtSmicl proved to interact weakly with Smad2 (Fig. 1B, lanes 3 and 4, either co-transfected with caALK4 or not) and strongly with Smad3 and Smad4 when co-transfected with a constitutively active ALK4 receptor (Fig. 1B, lanes 6 and 11). A strong interaction between overexpressed HA-XtSmicl and endogenous Smad3 in presence of caALK4 could be detected after immunoprecipitation of HA-XtSmicl with anti-HA antibody and analysis of the immunoprecipitate after western blotting with anti-Smad3 antibody (Fig. 1C, lane 2).

The expression pattern of *XtSmicl* during early development was assayed by quantitative RT-PCR and in situ hybridisation. Transcripts are abundant in the animal hemisphere of the fertilised egg (Fig. 1D, part a, Fig. 1E) and decline thereafter, although *XtSmicl* RNA is still detectable at early and mid gastrula stages in involuting dorsal mesoderm (Fig. 1D, parts c,d). By the neurula stage, expression is most abundant in neurectoderm and migrating neural crest cells (Fig. 1D, parts e-h,j), and a section through an embryo at stage 23 shows expression in the notochord, somites and neural tube (Fig. 1D, part i).

Inhibition of Smicl function in *X. laevis* and *X. tropicalis* causes gastrulation defects

To analyse the function of Smicl during early *Xenopus tropicalis* development, we designed two antisense morpholino oligonucleotides: one directed against the start codon of XtSmicl (XtMO1) and the other against a region within the 5′ untranslated region of the mRNA (XtMO2) (Fig. 2A). We also designed a control oligonucleotide (coMO), which differs by eight bases from the sequence of XtMO2 (Fig. 2A). A morpholino oligonucleotide targeted against the start codon of the *X. laevis* homologue of Smicl (XlMO) was based on the

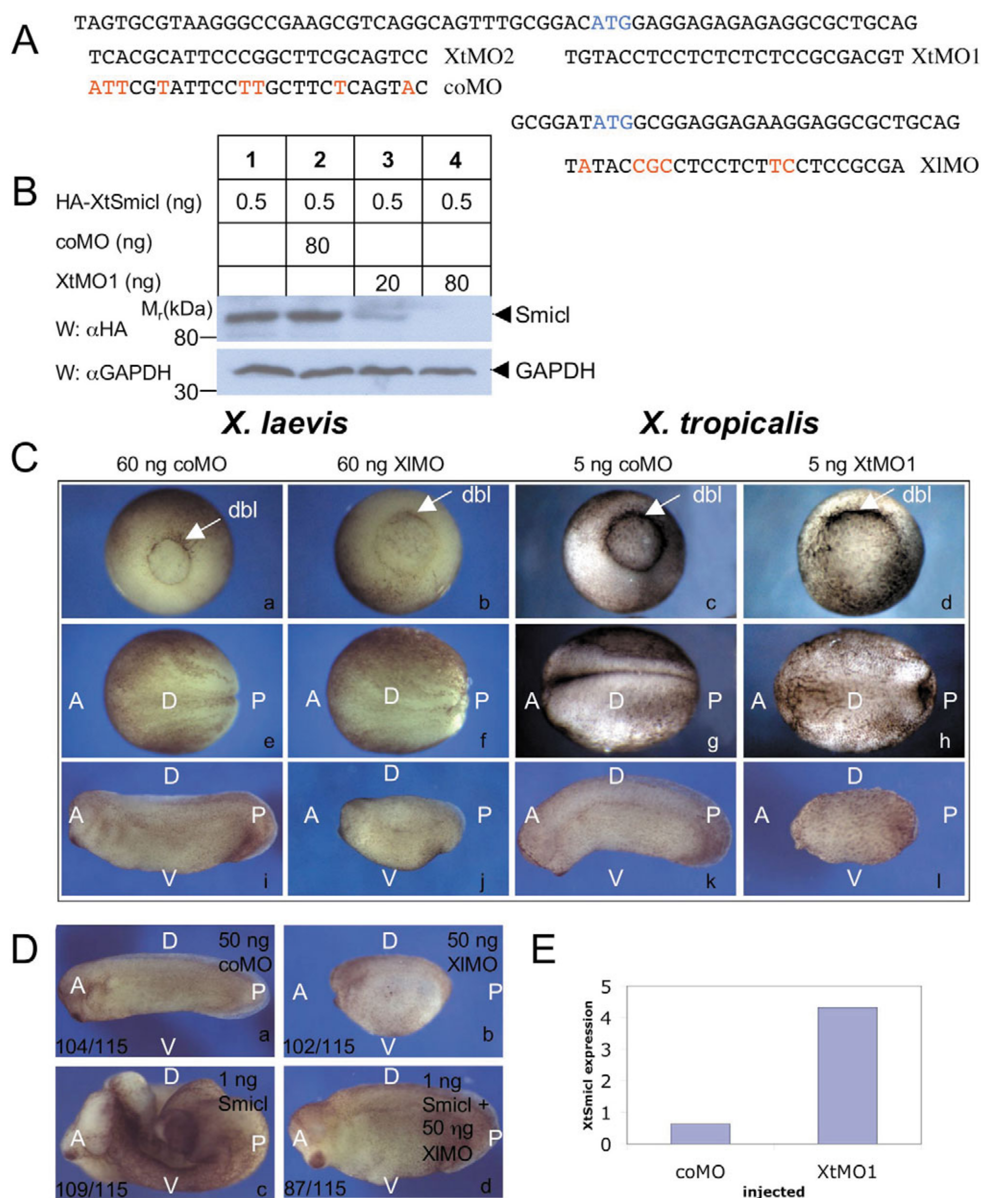

Fig. 2. Analysis of Smicl function in *Xenopus laevis* and *Xenopus tropicalis*. (A) The antisense morpholino oligonucleotides used in this study are aligned with their *Xenopus tropicalis* (Xt) and *Xenopus laevis* (Xl) target sequences. (B) XtMO1 inhibits translation of RNA encoding HA-tagged XtSmicl in a dose-dependent fashion. This is not observed with coMO. XtMO1 and coMO were injected in *Xenopus* embryos at the one-cell stage at the indicated concentrations, followed by RNA encoding HA-tagged XtSmicl. Embryos were cultured to early gastrula stage 10 and subjected to western blotting using an anti-HA antibody and an anti-Gapdh antibody as a loading control. (C) Injection of the morpholino oligonucleotides described in A disrupts gastrulation and axis formation in both *Xenopus laevis* and *Xenopus tropicalis*. Dbl, dorsal blastopore lip; A, anterior; P, posterior; D, dorsal; V, ventral. (D) Injection of mRNA encoding mouse Smicl into embryos of *Xenopus laevis* can ‘rescue’ the phenotype caused by XlMO. Overexpression of mouse *Smicl* alone causes a ‘spina bifida’ phenotype. Quantitation of morpholino defects and rescues are indicated on the figure. (E) Injection of antisense morpholino oligonucleotide XtMO1 causes upregulation of *Smicl* mRNA. RNA was extracted at stage 10.5 from embryos injected with 5 ng XtMO1 or coMO and analysed by quantitative RT-PCR.

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sequence of an *X. laevis* Smicl EST. Ten bases of XlMO differ from the equivalent *X. tropicalis* sequence (Fig. 2A). The specific morpholino oligonucleotide XtMO1 inhibited translation in a dose-dependent manner of an HA-tagged form of XtSmicl after injection of XtMO1 into *Xenopus* embryos followed by injection of HA-XtSmicl mRNA (Fig. 2B).

Injection of the three Smicl antisense morpholino oligonucleotides causes similar phenotypes in *X. tropicalis* and *X. laevis*. The first observed effect is a delay in the onset of gastrulation (Fig. 2C, parts b,d) and by neurula stages this delay manifests itself as a failure of the blastopore to close (Fig. 2C, parts f,h). At tadpole stages dorsoanterior structures are reduced and ventroposterior structures somewhat expanded; the anteroposterior axis is shortened; and embryos are microcephalic (Fig. 2C, parts j,l). Injection of coMO causes no detectable defects in development.

We note that injection of XtMO1, but not of coMO, causes the upregulation of *Smicl* mRNA at stage 10.5 (Fig. 2E). This elevated transcription may reflect an attempt by the embryo to regulate levels of Smicl protein following inhibition of translation by the antisense morpholino oligonucleotide, or it may be due to stabilisation of the RNA.

The observation that three different morpholino oligonucleotides yield similar phenotypes in two species of *Xenopus* argues that the effects of these reagents are specific. To confirm this impression, we carried out rescue experiments in *Xenopus laevis* using a mouse Smicl construct that lacks the first six amino acids and contains a Myc tag (see Materials and methods), so that its translation is not inhibited by the antisense oligonucleotide. This mRNA caused significant rescue of the phenotype caused by XlMO (Fig. 2D, part d), and indeed XlMO was able to rescue the spina bifida phenotype that is caused by mis-expression of the mouse Smicl construct (Fig. 2D, part c). Together, these experiments indicate that our Smicl antisense morpholino oligonucleotides function in a specific manner and that the mouse and *Xenopus* proteins are functional homologues.

Smicl is required for normal expression of *Chordin* mRNA

One way in which inhibition of Smicl function might disrupt gastrulation is by interfering with gene expression in the organiser, and indeed the Smicl phenotype resembles quite closely that of the organiser-specific gene *Chordin*, obtained by targeting both *Xenopus laevis* Chordin pseudo-alleles with antisense morpholino oligonucleotides (Oelgeschlager et al., 2003). To address this point, we studied expression levels of the pan mesodermal marker *Xbra* (Fig. 3A), the endodermal marker *Sox17* (Fig. 3B) and the organiser-specific genes *Xnr3* (Fig. 3C), *Goosecoid* (Fig. 3D) and *Chordin* (Fig. 3E-G). The only one of these genes to be affected by inhibition of Smicl function, in *X. laevis* and in *X. tropicalis*, was *Chordin*. This was confirmed by in situ hybridisation, which showed that inhibition of Smicl function both reduces the expression level of *Chordin* and decreases the size of its expression domain (Fig. 3H), while the expression pattern of the other organiser markers, also analyzed by in situ hybridisation, is normal (data not shown). As an additional control, we observed that the *X. tropicalis* oligonucleotide XtMO1, which differs by ten bases from XlMO, did not decrease expression of *Chordin* in *X. laevis* (data not shown), and the downregulation of *Chordin* caused by XlMO was rescued by co-injection of mRNA encoding mouse Smicl (Fig. 3H).

To ask whether the Smicl loss-of-function phenotype is caused in part by the downregulation of *Chordin*, we attempted to rescue the effects of the *Xenopus laevis* Smicl antisense oligonucleotide by co-injection of RNA encoding Chordin. This mRNA brought about partial rescue of the anterior structures of the embryos (Fig. 3J, part d), which are significantly reduced in embryos injected with XlMO (Fig. 3J, part c).

Together, these experiments indicate that Smicl is required for expression of *Chordin* in the *Xenopus* organiser, and that the phenotype caused by loss of Smicl function is due in part to the downregulation of *Chordin*. We therefore went on to investigate the role of this Smad-interacting protein in the regulation of *Chordin* in more detail.

Smicl is not involved in β-catenin-mediated induction of *Chordin* via Siamois

Previous work indicates that the expression of *Chordin* in the organiser of *Xenopus* is initiated by β-catenin signalling and that its maintenance depends on high levels of Nodal related proteins such as Xnr1 derived from the Nieuwkoop centre (Wessely et al., 2001). Consistent with this idea, activation of *Chordin* in isolated animal pole regions by members of the TGFβ family is inhibited by cycloheximide (Howell and Hill, 1997; Sasai et al., 1994), suggesting that induction requires the synthesis of intermediate proteins and is therefore indirect.

To investigate the activation of *Chordin* in more detail, we first asked whether its activation by β-catenin is direct or indirect. RNA encoding Xnr1 or β-catenin was injected into *Xenopus* embryos at the one-cell stage, and animal pole regions were dissected before the mid-blastula transition (that is, before the onset of zygotic transcription) and incubated in the presence or absence of cycloheximide until the equivalent of the early gastrula stage. Xnr1 and β-catenin both activate expression of *Chordin* in animal caps, but induction by β-catenin, like induction by Xnr1, is inhibited by cycloheximide and is therefore indirect (Fig. 4A,B).

It is possible that the indirect induction of *Chordin* by β-catenin occurs through Siamois (Lemaire et al., 1995), a transcription factor that is expressed in the organiser in response to β-catenin and that can activate transcription of *Chordin* (Wessely et al., 2004). Further experiments demonstrated that Siamois activates *Chordin* in a direct manner (Fig. 5A). To examine the possibility that Smicl is involved in this process, we asked whether Smicl antisense morpholino oligonucleotides prevent induction of *Chordin* by Siamois in animal caps. This proved not to be the case (Fig. 5C). Moreover, inhibition of Smicl function does not inhibit *Siamois* expression in intact embryos (Fig. 5B). We conclude that Smicl is not involved in the induction of *Chordin* through the β-catenin/Siamois pathway, although we cannot exclude the possibility that β-catenin induces *Chordin* via other genes.

Smicl is involved in the induction of *Chordin* through the Smad pathway

The inductive effects of Nodal-related signalling are mediated by Smad2 and Smad3, which, on receptor activation, associate with a co-Smad and accumulate in the nucleus where they are recruited to particular promoters by specific transcription

factors (Hill, 2001). Preliminary experiments revealed that both Smad2 and Smad3 are able to induce expression of *Chordin* in isolated *Xenopus laevis* animal caps (Fig. 6A). Smad3 proved to be a more powerful inducer of Chordin than did Smad2, and further experiments using Smad3 revealed that induction of *Chordin* by both Xnr1 and by Smad3 requires Smicl (Fig. 6B).

To ask whether a Smicl/Smad3 complex might activate *Chordin* directly, animal pole regions were dissected from embryos expressing exogenous Smad3 and in which endogenous Smicl is also present. The animal caps were incubated in the presence or absence of cycloheximide, and assayed for expression of *Chordin* at the early gastrula stage. Cycloheximide proved to inhibit activation of *Chordin* (Fig. 6A), indicating that Smad3 acts indirectly, presumably through the induction of another gene 'X'. This gene is unlikely to be

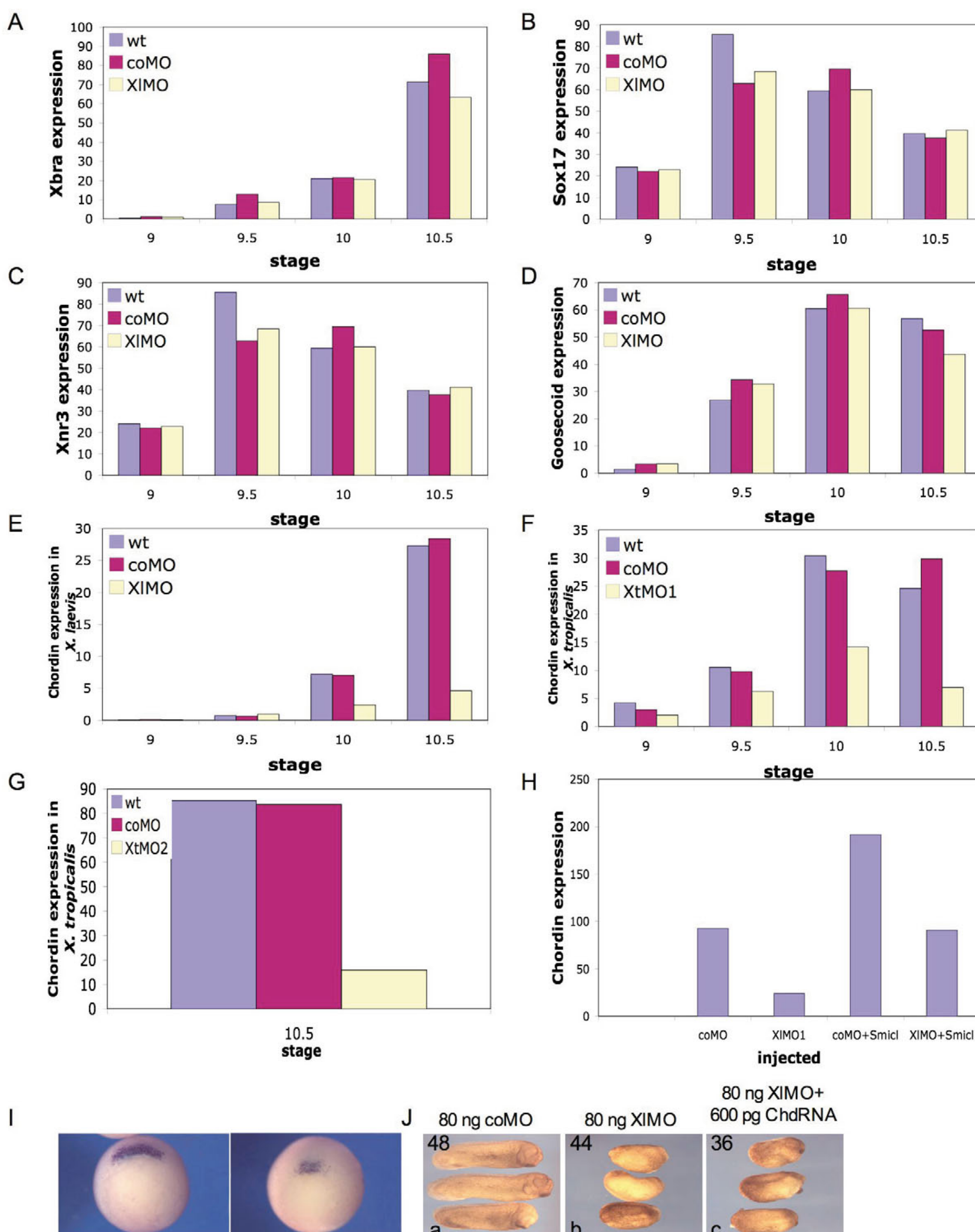

Fig. 3. See next page for legend.

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Smicl, because neither Xnr1 nor Smad3 increases expression of *Smicl* in animal caps or in intact embryos (data not shown).

Xlim1 induces expression of Chordin in concert with Smicl

The experiments described above indicate that Smicl regulates the expression of *Chordin* by functioning in concert with a factor X that is produced in response to signalling by Xnr1 (Fig. 6C). One candidate for X is Xlim1, which is involved in the induction of organiser-specific genes such as *Goosecoid* and *Cerberus* (Mochizuki et al., 2000; Yamamoto et al., 2003), as well as *Chordin* (Taira et al., 1994). Moreover, expression of *Xlim1* is induced in isolated animal pole regions by members of the TGFβ family such as Activin (Taira et al., 1992).

To investigate whether Xlim1 is involved in the Xnr1/Smad3 signalling cascade that leads to induction of *Chordin*, we first tested the abilities of Smad2 and Smad3 to activate *Xlim1* in isolated animal pole regions. Smad3 proved to induce strong expression of *Xlim1* in a direct manner; induction by Smad2 was weaker and indirect (Fig. 7A).

We next asked whether Xlim1, as would be expected of factor X, can induce expression of *Chordin* in isolated animal pole regions. These experiments made use of Xlim1/3m, a constitutively active variant of Xlim1 in which two inhibitory Lim domains are inactivated (Taira et al., 1994). As previously reported (Taira et al., 1994), expression of Xlim1/3m does activate *Chordin* in isolated animal pole regions, and this induction proved to be direct (Fig. 7B). Depletion of *Xlim* in *Xenopus* embryos does not cause downregulation of *Chordin* or other organiser-specific genes at very early gastrula stages, but it remains possible that *Xlim1* plays a role in the maintenance of their expression (Hukriede et al., 2003).

Together, these experiments indicate that *Xlim1* can be induced directly by Smad3 and that Xlim1 in turn can activate *Chordin* in a direct fashion. Smicl is not involved in the first of these steps, because inhibition of Smicl function by injection of XlMO does not affect expression levels of *Xlim1* in *Xenopus laevis* (Fig. 7C). However, use of the same antisense morpholino oligonucleotide shows that Smicl is required for activation of *Chordin* by Xlim1/3m (Fig. 7D). Together, these experiments indicate that the factor X that is required downstream of Xnr1 and Smad3 is Xlim1, and that induction of *Chordin* by Xlim1 requires the Smad-interacting protein Smicl.

Xlim1 is present in a complex with Smad3 and Smicl

The requirement for Smicl in the induction of *Chordin* by Xlim1 suggests that the two proteins might physically interact. This possibility was tested by co-immunoprecipitation experiments showing that XtSmicl associates with Xlim1/3m following expression of the two proteins in HEK293T cells (Fig. 7E, lane 5). Xlim1 does not interact directly with Smad3 (Fig. 7E, lane 4), but the Smad-interacting protein Smicl can recruit Smad3 to create a complex containing these two proteins and Xlim1 (Fig. 7E, lane 1).

Fig. 3. Inhibition of Smicl function causes down regulation of *Chordin* expression, but not of other mesodermal and endodermal markers. (A-E) Expression levels, normalised to that of *ornithine decarboxylase*, of *Xbra* (A), *Sox17* (B), *Xnr3* (C), *Goosecoid* (D) and *Chordin* (E) in uninjected embryos, embryos injected with morpholino oligonucleotide coMO (80 ng), or embryos injected with morpholino oligonucleotide XlMO (80 ng). RNA was extracted at the indicated stages, and RNA levels were analysed by quantitative RT-PCR. (F,G) Inhibition of Smicl function by means of morpholino oligonucleotide XtMO1 (5 ng; F) or XtMO2 (30 ng; G) also causes downregulation of *Chordin* expression in *Xenopus tropicalis*. Embryos were assayed at stages 9, 9.5, 10 and 10.5 (F), or at stage 10.5 (G). (H) Injection of RNA encoding mouse Smicl causes the upregulation of *Chordin* in embryos injected with morpholino oligonucleotide coMO and rescues the downregulation of *Chordin* caused by morpholino oligonucleotide XlMO. Embryos were injected with the indicated RNAs (1 ng) or morpholino oligonucleotides (50 ng). RNA was extracted at stage 10.5 and expression of *Chordin* and *ornithine decarboxylase* was assayed by quantitative RT-PCR. (I) In situ hybridisation of *Xenopus laevis* embryos at the early gastrula stage confirms that inhibition of Smicl function causes the downregulation of *Chordin*. The embryo on the left was injected with coMO (80 ng; *n*=15) and the one on the right with XlMO (80 ng; *n*=15). (J) Injection of *Chordin* mRNA can partially rescue the phenotype caused by XlMO. Numbers indicate how many embryos out of 50 display this phenotype.

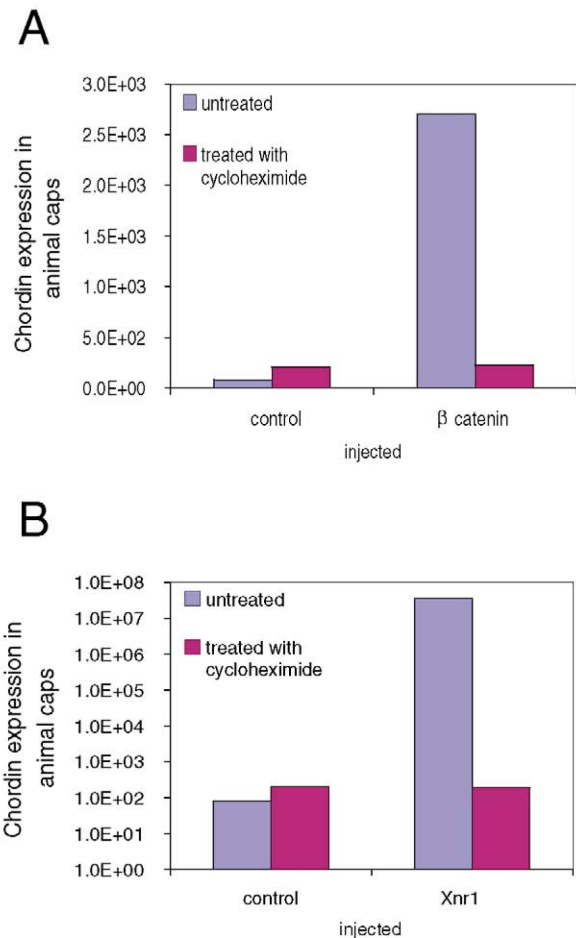

Fig. 4. Indirect induction of *Chordin* by Xnr1 and β-catenin. Induction of *Chordin* by β-catenin (A) and by Xnr1 (B) is inhibited by cycloheximide. RNA (100 pg) encoding Xnr1 or constitutively active β-catenin was injected into *Xenopus laevis* embryos at the one-cell stage and animal caps were dissected at mid blastula stage 8 (before the mid blastula transition). The animal pole regions were allowed to develop for 3 hours in the presence or absence of cycloheximide and RNA was analysed by quantitative RT-PCR for expression of *Chordin*. Induction of *Chordin* by both Xnr1 and β-catenin is inhibited by cycloheximide and is therefore indirect.

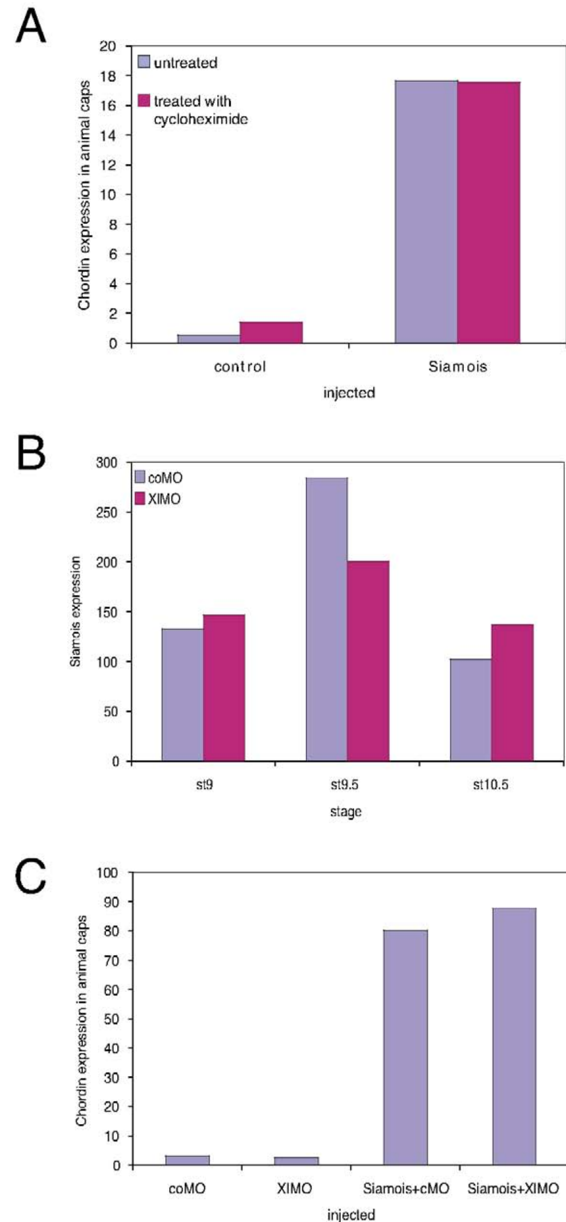

Fig. 5. Siamois induces expression of *Chordin* in a direct manner that does not require Smicl. (A) RNA encoding Siamois (500 pg) was injected into embryos of *Xenopus laevis* at the one-cell stage and animal pole regions were dissected at mid blastula stage 8 (before the mid blastula transition). The animal pole regions were cultured for 3 hours in the presence or absence of cycloheximide, and expression of *Chordin* was assayed by quantitative RT-PCR. Induction of Chordin is not inhibited by cycloheximide. (B) Expression of *Siamois* is not inhibited by antisense morpholino oligonucleotides directed against Smicl. Morpholino oligonucleotide XlMO (80 ng) was injected into *Xenopus laevis* embryos at the one cell stage and expression of *Siamois* was analysed by quantitative RT-PCR at the indicated stages. (C) RNA encoding Siamois (500 pg) was injected into *Xenopus* embryos at the one-cell stage either alone or in the presence of the indicated morpholino oligonucleotides. Animal caps were dissected at mid blastula stage 8 and they were cultured to the equivalent of stage 10.5 before being analysed for *Chordin* expression by quantitative RT-PCR.

Xlim1 binds Chordin promoter sequences comprising two Xlim-binding sites

Our results predict that Xlim1 should bind to the *Chordin* promoter. Previous work has demonstrated that Xlim1 can bind, in either orientation, the sequence TAATXY, where XY is TA, TG, CA or GG (Mochizuki et al., 2000). Inspection of the *Xenopus tropicalis Chordin* promoter region revealed two putative Xlim1-binding sites positioned at nucleotides –638 to –643 and –685 to –690 relative to the translation start site (see Materials and methods). To confirm that these sites can indeed bind Xlim1, we carried out a DNA-mediated pull-down assay (Hata et al., 2000). Flag-tagged Xlim1/3m derived from transfected HEK293T cells was efficiently precipitated by biotinylated *Chordin* promoter oligonucleotides containing the two Xlim1-binding sites (Fig. 7F, lane 3) and further experiments showed that HA-tagged Smicl could be co-immunoprecipitated with Xlim1 (Fig. 7F, lane 5). Mutation of the Xlim1 binding sites abolished binding (Fig. 7F, lane 8).

Discussion

In this paper, we study the function of Smicl, a novel Smad-interacting zinc-finger protein that is expressed maternally in the *Xenopus* embryo. Inhibition of Smicl function by injection of antisense morpholino oligonucleotides causes the loss or reduction of anterior and dorsal structures, and the shortening of the anteroposterior axis. This phenotype is preceded, and is partially caused by, a reduction in *Chordin* expression at the early gastrula stage: we note that the phenotype of embryos in which Smicl function is inhibited resembles that of embryos injected with antisense morpholino oligonucleotides directed against Chordin (Oelgeschlager et al., 2003), and that injection of *Chordin* mRNA rescues, in part, the effects of loss of Smicl function (Fig. 3J).

Our results are of interest for three reasons. First, they define the function of a novel *Xenopus* Smad-interacting protein, and in doing so they reveal a surprising degree of specificity in this protein; although we have not examined a large panel of markers, inhibition of Smicl function does not downregulate all genes expressed in the organiser, just *Chordin*. In this regard, we note that expression of *Chordin* in the embryo is not completely inhibited by antisense morpholino oligonucleotides directed against Smicl. There are four possible explanations for this observation. First, the concentration of the Smicl antisense oligonucleotides used in these experiments may be too low to elicit the most extreme phenotype. Second, maternal protein may persist long enough to provide some 'rescue' of the effects of inhibiting de novo translation. Third, Smicl antisense morpholino oligonucleotides may not inhibit the initial activation of *Chordin* expression, but may prevent its maintenance. And finally, other signalling pathways may be involved in *Chordin* regulation, although it is not clear, at present, whether FGF signalling plays a role (Delaune et al., 2005; Mitchell and Sheets, 2001).

A second point of interest concerns the regulation of *Chordin*, which has long been recognised as an indirect target of TGFβ signalling, to the extent that it is sometimes used as a control for the efficacy of cycloheximide treatment (Howell and Hill, 1997). Our work defines the steps involved in this

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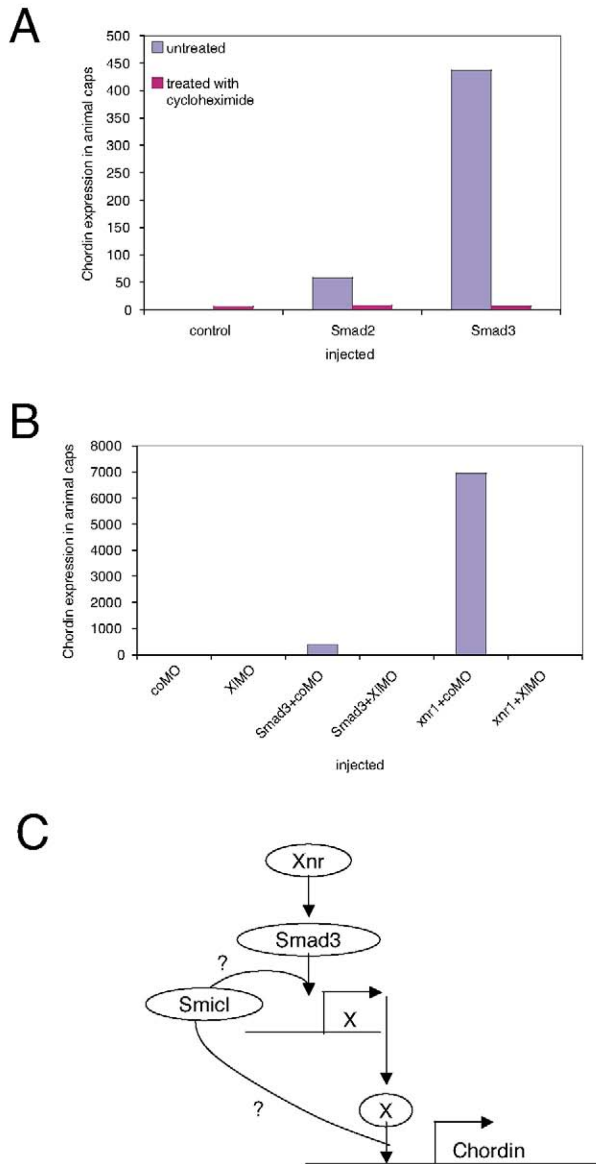

Fig. 6. Induction of *Chordin* by Smad3 is indirect and requires Smicl. (A) RNA (500 pg) encoding Smad2 or Smad3 was injected into embryos of *Xenopus laevis* at the one-cell stage and animal pole regions were dissected at mid blastula stage 8 (before the mid blastula transition). The animal pole regions were cultured for 3 hours in the presence or absence of cycloheximide and expression of *Chordin* was assayed by quantitative RT-PCR. Smad3 is a more efficient inducer of *Chordin* than is Smad2, and the action of both is indirect. In the same experiment, *Xwnt8* and *eFGF* proved to be induced to higher levels by Smad2 than by Smad3, confirming that the two Smad family members have differential effects in the *Xenopus* embryo. Additional experiments showed that *eFGF* and *Goosecoid* are direct targets of Smad2 and Smad3, respectively (data not shown). (B) Inhibition of Smicl function prevents induction of *Chordin* by Smad3 and by Xnr1. Embryos of *Xenopus laevis* were injected with morpholino oligonucleotides coMO or XlMO (80 ng) either alone or in the presence of RNA encoding Smad3 (500 pg) or Xnr1 (100 pg). Animal caps were dissected at mid blastula stage 8 and they were cultured to the equivalent of stage 10.5 before being analysed for *Chordin* expression by quantitative RT-PCR. Both Smad3 and Xnr1 induced expression of *Chordin*, and this was inhibited by a morpholino oligonucleotide directed against Smicl. (C) A model based on the data presented so far: the induction of *Chordin* by Xnr1 and Smad3 is indirect and requires Smicl and the synthesis of another factor X.

indirect activation. And finally, our results are of note because they define differential activities for Smad2 and Smad3 in the early *Xenopus* embryo. This point and the other issues mentioned above are discussed below.

Smicl

Smicl was identified in a yeast two-hybrid screen designed to identify Smad-interacting proteins (Collart et al., 2005; Verschueren et al., 1999). The C terminus of the protein contains five CCCH-type zinc fingers that display homology to a domain in CPSF30, the 30 kDa subunit of cleavage and polyadenylation specificity factor (CPSF). CPSF is required for the cleavage and polyadenylation of pre-mRNA and, like CPSF, the zinc-finger domain of Smicl can bind single-stranded DNA as well as cleave RNA in vitro (Collart et al., 2005).

To investigate the role of Smicl during early *Xenopus* development, we injected specific antisense morpholino oligonucleotides into embryos of *Xenopus laevis* and *Xenopus tropicalis*. Such embryos develop with small heads, reduced dorsal tissues, increased ventral and posterior structures, and shortened trunks. Interestingly, this phenotype resembles that of *Xenopus* and zebrafish embryos in which Chordin function is inhibited or absent (Leung et al., 2005; Oelgeschlager et al., 2003; Schulte-Merker et al., 1997), and indeed of the genes we investigated only *Chordin* proved to be affected by the inhibition of Smicl function (Fig. 3). Consistent with this observation, the phenotype of embryos lacking Smicl can be rescued quite significantly by injection of RNA encoding Chordin (Fig. 3J), although the fact that rescue is not complete suggests that there are other Smicl target genes yet to be identified. Some such genes have been identified in a preliminary microarray analysis, but none of these has yet proved to be organiser-specific (C.C., J. Ramis and J.C.S., unpublished).

The phenotype of embryos lacking Smicl function differs from that of zebrafish embryos lacking no arches, the zebrafish homologue of CPSF30 (Gaiano et al., 1996). Such embryos, as their name implies, lack pharyngeal arches and eyes. Preliminary experiments using an antisense morpholino directed against *Xenopus tropicalis* CPSF30 reveal a more severe phenotype in which epidermal cells no longer adhere to the underlying mesodermal tissue (A.R., C.C. and J.C.S., unpublished). We do not yet know why the two phenotypes should differ; perhaps there is another CPSF30 in the zebrafish genome.

Regulation of Chordin

Chordin is expressed in the organiser of the *Xenopus* embryo. It encodes a secreted factor that binds to, and inhibits the function of, BMP family members such as BMP4, and thereby functions as an important mediator of the inducing and patterning activities of the organiser (Sasai et al., 1995; Sasai et al., 1994). Previous work has demonstrated that *Chordin* is an indirect target of TGFβ signalling and more recent

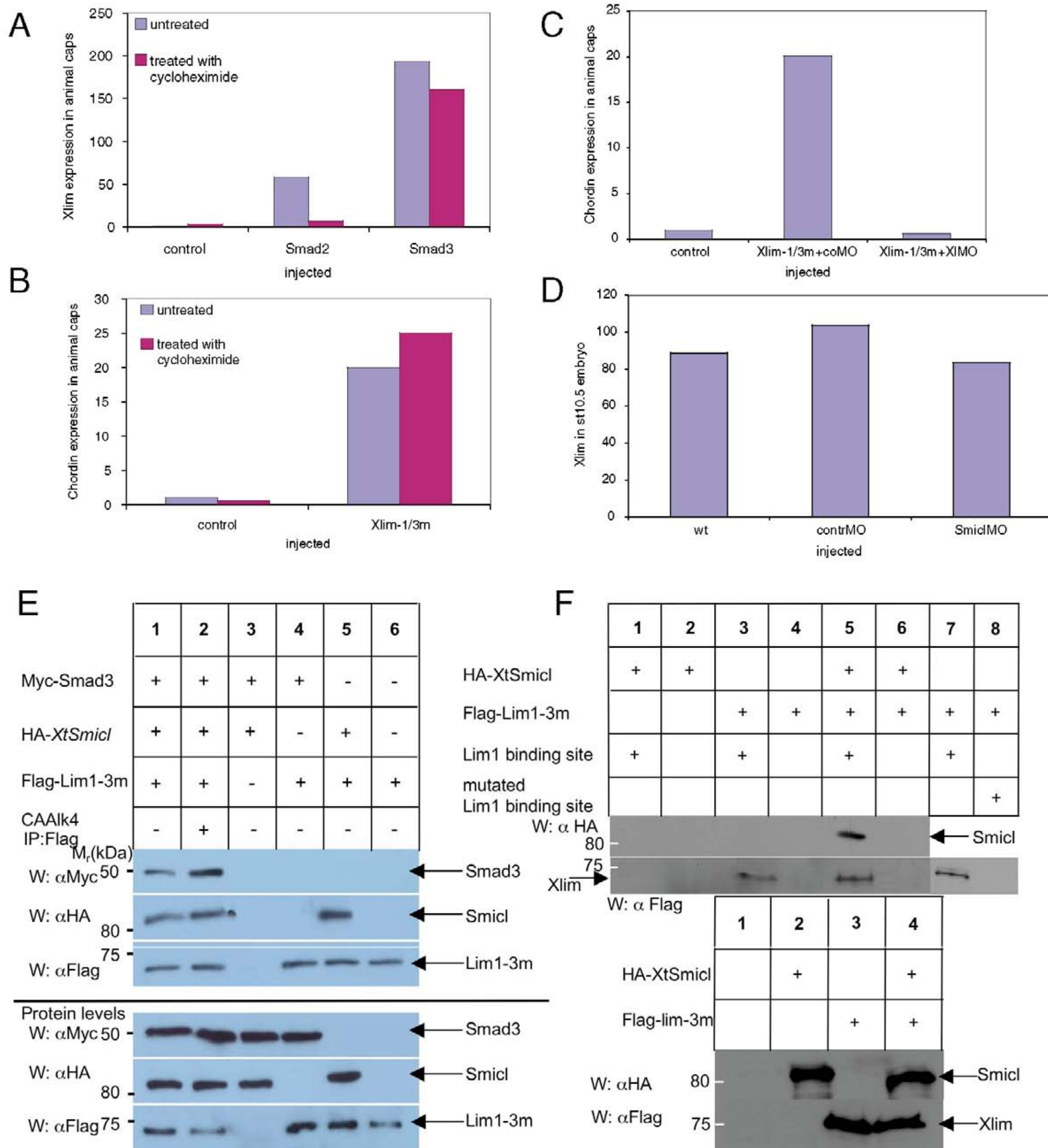

Fig. 7. Smad3 induces expression of *Xlim1* in a direct manner; induction of *Chordin* by Xlim1-3m requires Smicl. (A) Smad3 but not Smad2 is a direct inducer of *Xlim1*. The experimental regime was identical to that described in Fig. 6A, except that expression of *Xlim1* and not *Chordin* was analysed by quantitative RT-PCR. (B) Xlim1/3m is a direct inducer of *Chordin*. RNA encoding Xlim1/3m (400 pg) was injected into embryos of *Xenopus laevis* at the one-cell stage. Animal caps were dissected from such embryos and cultured in control medium or medium containing cycloheximide, as described in Fig. 6A. Induction of *Chordin* is not inhibited by cycloheximide. (C) Smicl is required for Xlim1/3-mediated induction of *Chordin*. RNA encoding Xlim1/3m (400 pg) was injected into embryos at the one-cell stage in the presence of 80 ng morpholino oligonucleotide coMO or XlMO. Animal pole regions were dissected from such embryos at mid blastula stage 8 and cultured until control embryos reached stage 11. (D) Smicl is not required for expression of *Xlim1* in *Xenopus laevis*. Morpholino oligonucleotides XlMO or coMO (80 ng) were injected into *Xenopus laevis* embryos at the one-cell stage and RNA was extracted at early gastrula stage 10.5. Expression of *Xlim1* is not inhibited by XlMO. (E) Smicl and Xlim1/3 can interact. The indicated combinations of expression constructs encoding HA tagged XtSmicl, Flag tagged Xlim1/3m, Myc tagged Smad3 and caALK4 were co-transfected into HEK 293T cells. Extracts were subjected to immunoprecipitation using an anti-Flag antibody and the presence of HA XtSmicl was analysed by western blotting by using an anti-HA antibody. Smad3 can bind Xlim1/3 in a Smic1-dependent fashion as shown by western analysis of Xlim1/3 immunoprecipitates using an anti-Myc antibody. (F) Binding of Flag-tagged Xlim and HA-tagged Smicl to *Chordin* promoter sequences containing Xlim-binding sites. Extracts of HEK293T cells transfected with Xlim and/or Smicl expression constructs were incubated with streptavidin-agarose beads and biotinylated double-stranded oligonucleotides. Upper panel: precipitated complexes were subjected to western blotting using anti-Flag and anti-HA antibodies. Xlim1, but not Smicl, interacts with this region of the *Chordin* promoter, but Smicl can form part of a ternary complex with the *Chordin* promoter region and Xlim1. Xlim does not bind to *Chordin* promoter oligonucleotides with mutations in the Xlim-binding sites (see Materials and methods). Lower panel: western blotting of whole extracts shows similar expression levels of Flag-tagged Xlim and HA-tagged Smicl.

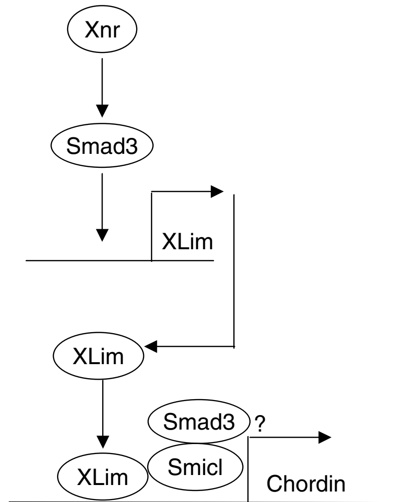

Fig. 8. A model describing the activation of *Chordin*. Briefly, signalling by *Xenopus* Nodal-related proteins such as Xnr1 activates Smad2 and Smad3. Smad3 induces expression of Xlim1 directly and Xlim1 and Smicl, perhaps in a ternary complex with Smad3 (indicated by '?'), then induce expression of *Chordin*.

experiments suggest that its expression is initiated by β-catenin-mediated transcriptional activation via Siamois and maintained by Nodal-related signalling pathways (Wessely et al., 2001).

Our work reveals that Smicl is involved in the Xnr-mediated maintenance of *Chordin* expression, and that it acts in the second component of a two-step pathway. In the first, TGFβ signalling activates the expression of *Xlim1* directly via Smad3. In the second, our results suggest that Xlim1 cooperates with Smicl, perhaps in a ternary complex with Smad3, to activate *Chordin* directly (Figs 7, 8). Consistent with this suggestion, we note that *Chordin* (Sasai et al., 1994), *Smicl* (Fig. 1C), *Smad3* (Howell et al., 2001) and *Xlim1* (Taira et al., 1992) are all expressed in dorsal mesoderm at early gastrulation stages.

The activity of Xlim1 is regulated by Ldb1 (Breen et al., 1998; Jurata et al., 1998), which is believed to counteract the effects of an inhibitory protein and thereby cause Xlim1 to shift to an activated state in which it can bind cell specific transcriptional co-activators (Hiratani et al., 2001). Our data suggest that Smicl is such a co-activator. Indeed, the ability of Xnr1 to induce expression of *Goosecoid* in *Xenopus* animal caps is abolished by inhibition of Smicl function (data not shown), although the fact that loss of Smicl activity does not inhibit expression of *Goosecoid* in the dorsal mesoderm of intact embryos (Fig. 3C) suggests that another factor can substitute for Smicl in this region of the embryo.

Differential activities of Smad2 and Smad3

Our observations suggest that the closely related proteins Smad2 and Smad3 play different roles in the early *Xenopus* embryo and that these roles differ not only because Smad2 is expressed at much higher levels than Smad3 (Howell et al., 2001). In particular, we note that Smad3 activates expression of Xlim1 directly, while Smad2 induces Xlim1 in an indirect fashion, in the sense that activation is inhibited by cycloheximide. Previous work has demonstrated that induction of *Xlim1* by Activin and Nodal-related signalling requires the Smad-interacting protein Fast1. This transcription factor, together with receptor activated Smad proteins, acts as a direct transcriptional inducer of *Xlim1* through a cluster of Fast1/Smad4 sites located in the first intron of the gene (Watanabe et al., 2002). Fast1 contains two Smad-binding domains, the Smad interaction motif (SIM) and the Fast/FoxH1 motif (FM) (Randall et al., 2004). While the SIM can bind both Smad2 and Smad3, the FM binding site is highly specific for Smad2. This observation, together with our own data, suggests that the Smad interaction motif of Fast1 but not the Fast/FoxH1 motif is required for direct transcriptional activation of *Xlim1*. The existence of these distinct Smad-binding motifs in Fast1 might provide the molecular basis for the differential activities of Smad2 and Smad3 in the induction of *Xlim1* transcription.

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