Regulation of TGF-\(\beta\) signalling by N-acetylgalactosaminyltransferase-like 1

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The TGF-\(\beta\) superfamily of secreted signalling molecules plays a pivotal role in the regulation of early embryogenesis, organogenesis and adult tissue homeostasis. Here we report the identification of Xenopus N-acetylgalactosaminyltransferase-like 1 (xGalntl-1) as a novel important regulator of TGF-\(\beta\) signalling. N-acetylgalactosaminyltransferases mediate the first step of mucin-type glycosylation, adding N-acetylgalactosamine to serine or threonine side chains. xGalntl-1 is expressed in the anterior mesoderm and neural crest territory at neurula stage, and in the anterior neural crest, notochord and the mediolateral spinal cord at tailbud stage. Inhibition of endogenous xGalntl-1 protein synthesis, using specific morpholino oligomers, interfered with the formation of anterior neural crest, anterior notochord and the spinal cord. Xenopus and mammalian Galntl-1 inhibited Activin as well as BMP signalling in the early Xenopus embryo and in human HEK 293T cells. Gain- and loss-of-function experiments showed that xGalntl-1 interferes with the activity of the common TGF-\(\beta\) type II receptor ActR-IIB in vivo. In addition, our biochemical data demonstrated that xGalntl-1 specifically interferes with the binding of ActR-IIB to Activin- and BMP-specific type I receptors. This inhibitory activity of xGalntl-1 was dependent on mucin-type glycosylation, as it was sensitive to the chemical inhibitor benzyl-GalNAc. These studies reveal an important role of a N-acetylgalactosaminyltransferase in the regulation of TGF-\(\beta\) signalling. This novel regulatory mechanism is evolutionarily conserved and, thus, might provide a new paradigm for the regulation of TGF-\(\beta\) signalling in vertebrates.

KEY WORDS: TGF-\(\beta\), Mucin, O-linked glycosylation, BMP, Nodal

INTRODUCTION
The TGF-\(\beta\) superfamily of secreted growth factors regulates a plethora of biological processes in a highly dose-dependent manner that demands tight regulation of this signalling pathway during embryogenesis and adult tissue homeostasis (Lutz and Knaus, 2002; Shi and Massague, 2003). TGF-\(\beta\) family members bind to heteromeric complexes consisting of type I and type II transmembrane serine/threonine kinase receptors. Upon ligand binding, the type II receptor subunit activates the type I receptor, which subsequently phosphorylates receptor-associated mediators of the Smad family (R-Smads). The phosphorylated R-Smad proteoglycans, including membrane-associated endoglin and betaglycan, act as additional co-receptors (TGF-\(\beta\) type III receptors) that bind TGF-\(\beta\) proteins and facilitate the binding of the ligand to the heteromeric TGF-\(\beta\) receptor complex. In addition, secreted proteoglycans, like Decorin and Biglycan, bind and sequester TGF-\(\beta\)-type cytokines in the extracellular matrix (Gumienny and Padgett, 2002). Modification of epidermal growth factor-like (EGF) repeats by O-fucosyltransferases and \(\beta_{1,3}\)-N-acetylglucosaminyltransferases of the Fringe family regulates Notch as well as Nodal signalling. The O-linked glycosylation of Notch and of Notch ligands modulates the activation of Notch signalling after binding to its ligands Delta and Serrate/Jagged (Haltiwanger and Lowe, 2004). Mutation of an O-fucosylation site in the Nodal co-receptor Cripto interferes with the activation of Nodal signalling (Schiffer et al., 2001; Yan et al., 2002). More recently, a \(\beta_{1,4}\)-galactosyltransferase was shown to modulate doroventral patterning and BMP signalling in the early zebrafish embryo (Machingo et al., 2006).

The mucin-type of O-linked glycosylation, characterised by \(\alpha\)-N-acetylgalactosamine (GalNAc) attached to the hydroxyl group of serine or threonine side chains, is the most abundant form of O-linked glycosylation in higher eukaryotes (Haltiwanger and Lowe, 2004). Over 150 mucin-type glycoproteins have been annotated in mammals, but a consensus recognition sequence for the O-glycosyltransferases has not been described (Julenius et al., 2005). The initial addition of the GalNAc moiety is catalyzed by members of the UDP-GalNAc:polypeptide N-acetylgalactosaminyltransferase (GalNT) family. Subsequently, downstream glycosyltransferases generate complex O-linked glycans that can modulate a variety of biological processes (Haltiwanger and Lowe, 2004; Hang and Bertozzi, 2005). Interestingly, changes in the expression levels of GalNT family members and in the structures of these O-linked essential co-factors for the Wnt, Hedgehog, Fibroblast growth factor (FGF) and TGF-\(\beta\) signalling pathways in Drosophila as well as mice (Perrimon and Bernfield, 2000). Cell surface proteoglycans, including membrane-associated endoglin and betaglycan, act as additional co-receptors (TGF-\(\beta\) type III receptors) that bind TGF-\(\beta\) proteins and facilitate the binding of the ligand to the heteromeric TGF-\(\beta\) receptor complex.

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glycans have been associated with a number of human diseases, including immunodeficiencies and cancer (Tsuboi and Fukuda, 2001; Hollingsworth and Swanson; 2004; Brockhausen, 2006).

In mammals, 15 members of the GaINt family have been identified that display different substrate specificities and tissue-specific expression patterns (Ten Hagen et al., 2003; Young et al., 2003; Cheng et al., 2004). However, a role for these proteins in embryonic development has not been described. Here, we report the identification of N-acetylgalactosaminyltransferase-like 1 (Galnt1-1) as a novel negative regulator of TGF-β signalling. *Xenopus* Galntl-1 (xGalntl-1) is specifically expressed in neural and dorsal mesodermal tissues and is required for the proper formation of the neural crest, spinal cord and anterior notochord. *Xenopus* and mammalian Galntl-1 can inhibit BMP as well as Nodal signalling in the early *Xenopus* embryo and in human HEK 293T cells. Our biochemical data suggest that Galntl-1 interferes with the formation of heteromeric TGF-β receptor complexes. This study identifies a N-acetylgalactosaminyltransferase as a novel and important regulator of TGF-β signalling in the early *Xenopus* embryo and might provide a new paradigm for the regulation of TGF-β signalling in vertebrates.

**MATERIALS AND METHODS**

**Constructs**

Full-length cDNA encoding *Xenopus*, human and mouse Galntl-1 and *Xenopus* Galntl-6 were obtained from RZPD [IMAGE Consortium (Lennon et al., 1996)]. The open reading frames as well as epitope-tagged versions were cloned by PCR into pCS2+. Human ALK3 (BMPRIA – Human Gene Nomenclature Database), mouse Alk6 (Bmpr1b – Mouse Genome Informatics) and human BMPR-II (BMPR2) expression vectors were kindly provided by Dr L. Attisano (University of Toronto, Canada) and the coding sequences cloned into the EcoRI and Xbal sites of pCS2+. For secreted Galntl-1, the nucleotides encoding amino acids 93-563 of xGalntl-1 were introduced in-frame into a pCS2 expression vector containing the Chordin leader sequence. For recombinant Activin and BMP4 protein (R&D Systems, Minneapolis, MN), transiently transfected cells were incubated overnight in serum-free medium, incubated in serum-free medium containing 2 ng/ml Activin or 40 ng/ml BMP4 protein for 2 hours and lysed in Phosphosafe (Novagen, San Diego, CA) and the transiently expressed FLAG-tagged SMAD proteins immunoprecipitated in lysis buffer. The antibodies specific for phosphorylated SMAD1, SMAD2 and SMAD2 (Cell Signaling Technology, Danvers, MA) were used according to manufacturer’s instructions. For the treatment of HEK 293T with recombinant Activin or BMP4 protein, transfection efficiency was increased by Lipofectamine (Invitrogen). The detection of mucin-type glycosylation in cell membranes and the Golgi compartment using fluorescence-coupled agglutinin from *Helix pomatia* agglutinin (HPA Alexa Fluor 488, Invitrogen) has been described elsewhere (Virtanen, 1990).

**Embryos and explants**

In vitro fertilisation, embryo and explant culture, microinjection of synthetic mRNA, in situ hybridisation and RT-PCR analysis were performed as described (Yamamoto et al., 2007; Sive et al., 2000). The in situ probes for *msx1*, *slug* and *xbra* have been described elsewhere (Yamamoto et al., 2007; Tribulo et al., 2003). For vibratome sections, embryos were embedded in gelatine-albumin (Gove et al., 1997) and 30 µm sections mounted in Glycergel (DAKO, Denmark). For western blot analysis, proteins were electroblotted onto nitrocellulose membranes (our unpublished results), we isolated a homologue *Xenopus* Galntl-1 of human and mouse N-acetylgalactosaminyltransferase 1 (xGalntl-1) of human and mouse N-acetylgalactosaminyltransferase 1 (xGalntl-1) of human and mouse N-acetylgalactosaminyltransferase 1. The xGalntl-1 protein shares over 72% amino acid identity with mouse and human Galnt1, 56% identity with zebrafish Galnt14 and 47% with *Drosophila* Galnt2 (Fig. 1A and see Fig. S1 in the supplementary material). Members of the GalNAc-transferase family are characterised by an N-terminal transmembrane domain that retains the enzyme in the Golgi compartment, a central catalytic domain and a C-terminal Ricin domain that facilitates binding to partially glycosylated substrates (Fritz et al., 2006; Wandall et al., 2007). The N-terminal transmembrane and the central catalytic domain are highly conserved between *Xenopus* and human Galnt1 homologues (>80%), whereas the C-terminal Ricin domains are more divergent, with only 40% amino acid identity. To test for N-acetylgalactosaminyltransferase activity, we used fluorescence-coupled agglutinin from *Helix pomatia* (HPA) that specifically binds to GalNAc-α-Thr/Ser (Lehtonen et al., 1989; Virtanen, 1990; Hang

**RESULTS**

**Xenopus Galntl-1 is specifically expressed in neural and dorsal mesoderm tissues**

In a screen for genes that are negatively regulated by BMP signals (our unpublished results), we isolated a *Xenopus* homologue (xGalntl-1) of human and mouse N-acetylgalactosaminyltransferase 1. The xGalntl-1 protein shares over 72% amino acid identity with mouse and human Galnt1, 56% identity with zebrafish Galnt14 and 47% with *Drosophila* Galnt2 (Fig. 1A and see Fig. S1 in the supplementary material). Members of the GalNAc-transferase family are characterised by an N-terminal transmembrane domain that retains the enzyme in the Golgi compartment, a central catalytic domain and a C-terminal Ricin domain that facilitates binding to partially glycosylated substrates (Fritz et al., 2006; Wandall et al., 2007). The N-terminal transmembrane and the central catalytic domain are highly conserved between *Xenopus* and human Galnt1 homologues (>80%), whereas the C-terminal Ricin domains are more divergent, with only 40% amino acid identity. To test for N-acetylgalactosaminyltransferase activity, we used fluorescence-coupled agglutinin from *Helix pomatia* (HPA) that specifically binds to GalNAc-α-Thr/Ser (Lehtonen et al., 1989; Virtanen, 1990; Hang
et al., 2003). As expected, we were able to detect enhanced mucin-type O-linked glycosylation in the Golgi compartment and at the cell surface of xGalntl-1-expressing cells (Fig. 1B).

We identified Xenopus homologues of additional N-acetylgalactosaminyltransferases in the EST databases that share significant homology with xGalntl-1. Maternal and zygotic expression of xGalntl-1 and three additional Xenopus N-acetylgalactosaminyltransferases were detectable throughout early development. However, only xGalntl-1 expression was restricted to dorsal marginal zone explants at later developmental stages (Fig. 1C). At neurula stages, the xGalntl-1 transcript levels increased and could be detected in the anterior mesoderm and the neural crest territory (Fig. 1D). At tailbud stages, xGalntl-1 was specifically expressed in the forebrain, mediolateral spinal cord, notochord and in the neural crest cells that are migrating into the head region, branchial arches and the heart anlage (Fig. 1E). The expression of xGalntl-1 in the spinal cord was restricted to the mantle (differentiating) territory of the spinal cord and was excluded from the central area containing proliferating neuronal precursors. In addition, the expression of xGalntl-1 in the spinal cord decreased along the anterior-posterior body axis, whereas the expression in the notochord was highest in posterior regions.

**xGalntl-1 inhibits mesoderm formation and Activin/Nodal signalling**

Marginal microinjection of xGalntl-1 mRNA resulted in severe gastrulation defects (Fig. 2B). Co-injection of β-galactosidase mRNA (lacZ) revealed a cell-autonomous inhibition of mesoderm formation at early gastrula stages, indicated by the reduced expression of the pan-mesodermal marker xbra (Fig. 2C). In marginal zone explants, xGalntl-1 strongly reduced the expression of dorsal (α-actin) and ventral (α-globin, gata1, xvent1) mesodermal marker genes and induced the expression of anterior neural marker genes in ventral marginal zone explants (Fig. 2D). The formation of dorsal and ventral mesoderm in the Xenopus embryo at gastrula stages is mediated by a Nodal activity gradient in the underlying vegetal tissue (Agius et al., 2000; Martello et al., 2007). In addition, members of the FGF family can induce mesodermal cell fate and directly activate transcription of xbra (Latinkic et al., 1997; Wardle and Smith, 2006). The induction of mesodermal (xbra, chordin) and endodermal (sox17β) marker genes in ectodermal explants microinjected with mRNA encoding Activin or Nodal was inhibited by co-injection of xGalntl-1 mRNA. By contrast, the induction of xbra by microinjection of eFGF mRNA was unaffected by xGalntl-1 (Fig. 2E and data not shown). We
conclude from these data that xGalnt-1 interfered specifically with the activity of the Activin/Nodal pathway. The inhibition of Activin signalling by xGalnt-1 appeared to be direct, as the stimulation of Smad2 phosphorylation after treatment of transiently transfected HEK 293T cells with recombinant Activin protein for 2 hours was inhibited in xGalnt-1-expressing cells (Fig. 2F).

To address the in vivo function of xGalnt-1, we designed two specific morpholinos (Summerton, 1999; Heasman, 2002) that inhibited the in vitro translation and in vivo activity of xGalnt-1 from mRNA containing the morpholino target sequences in the 5’-UTR (Fig. 2G and data not shown). To minimise non-specific effects of the morpholinos, we injected a mixture of both morpholinos (half the concentration of each) in all subsequent experiments. To test whether endogenous xGalnt-1 activity modulates Activin/Nodal signalling in vivo, we analysed the effect of the xGalnt-1 morpholinos on mesoderm induction by Activin mRNA in animal cap explants. Co-injection of the xGalnt-1 morpholinos stimulated the induction of mesoderm by Activin. This effect was rescued by co-injection of xGalnt-1 mRNA lacking the morpholino target sequence, at concentrations that did not affect the development of the whole embryo or the differentiation of embryonic explants (Fig. 2H and data not shown). We conclude from these data that xGalnt-1 inhibits the activity of the Activin/Nodal pathway in vivo.

**xGalnt-1 inhibits BMP signalling**

The neuralisation of ventral marginal zone explants by xGalnt-1 (Fig. 2D) implied that xGalnt-1 might also inhibit BMP activity. Microinjection of mRNA encoding xGalnt-1 into animal blastomeres led to a dorsalisised phenotype, with enlarged head and reduced tail and trunk structures (Fig. 3B). In embryonic ectodermal explants, xGalnt-1 induced the expression of otx2 and xag. In contrast to the bona fide BMP antagonist Chordin, xGalnt-1 did not induce the expression of anterior neural marker genes (Fig. 3E), arguing for a mild reduction of BMP activity by xGalnt-1 (Wilson and Hemmati-Brivanlou, 1995). Microinjection of mRNA encoding human or mouse Galnt1 generated similar dorsalisised phenotypes in whole embryos, induced the expression of otx2 and xag in animal cap explants and interfered with mesoderm formation at gastrula stages (Fig. 3B,G and data not shown). Similar to other glycosyltransferases (El-Battari et al., 2003), xGalnt-1 seemed to be cleaved and secreted from transiently transfected HEK 293T cells and microinjected ectodermal explants. We detected a significant amount of xGalnt-1 protein in the supernatant of dissociated animal cap explants that migrated at a slightly lower molecular weight in SDS gels (Fig. 3F). Replacing the N-terminal transmembrane domain of xGalnt-1 with the Chordin leader peptide did not significantly increase the level of secreted xGalnt-1 protein, but instead abolished xGalnt-1 activity in the embryo (Fig. 3D,F). Thus, the N-terminal signal peptide that retains xGalnt-1 in the Golgi compartment is required for Galnt1 activity, whereas the differences in the C-terminal Ricin domain of *Xenopus* and mammalian Galnt-1 proteins had no obvious effect in these assays.

The activation of FGF, Activin/Nodal and Wnt signalling also induces the expression of neural and anterior marker genes such as otx2 and xag. These signalling pathways can interfere with BMP activity, stimulating the expression of BMP antagonists, repressing BMP expression, or by interference with the nuclear translocation of phospho-Smad proteins (Kretzschmar et al., 1997; Baker et al., 1999; Sasai et al., 1994; Oelgeschläger et al., 2003a; Kuroda et al., 2005). Microinjection of BMP7/OP-1 mRNA prevented the induction of otx2 and xag by xGalnt-1 in animal cap assays. By contrast, the inhibition of FGF, Activin/Nodal or Wnt signalling by microinjection of a dominant-negative FGF receptor (dnFGFR-4), dominant-negative Ras (not shown), dominant-negative Activin/Nodal type I receptor (tAlk-4) and dominant-negative TCF-3 had no
Regulation of TGF-β by Galntl-1

**Fig. 3. Inhibition of BMP signalling by Galntl-1.** (A-D) Stage 36 embryos that were injected animaly at the 8-cell stage with a total of 2 ng of mRNA encoding xGalntl-1, human GALNTL1 (hGalntl-1) or secreted Galntl-1 (sGalntl-1), with the N-terminal transmembrane domain replaced by the Chordin leader peptide. (E) RT-PCR analysis of animal cap explants from embryos injected with 2 ng xGalntl-1 or 40 pg chordin mRNA at stage 20 for neural (pax6, ncam, otx2), anterior (xag), muscle (myf5) and epidermal (cytokeratin) marker genes. (F) Western blot of the supernatant (SN) and cell pellet of dissociated ectodermal cells expressing the xGalntl-1 or sGalntl-1 with a C-terminal HA-TAG. (G) RT-PCR analysis of animal cap explants from embryos microinjected with mRNA encoding human, mouse (m) or secreted forms of Galntl-1. (H) RT-PCR analysis of animal cap explants from embryos microinjected with mRNA encoding xGalntl-1 (1 ng) alone or in combination with mRNA encoding BMP7/OP-1 (400 pg), dnFGFR-4 (400 pg), dnAlk-4 (400 pg) or ΔN-TCF-3 (400 pg). (I) Western blot for Smad1 or phospho-Smad1 using animal cap lysates from uninjected embryos or embryos microinjected with 200 pg Smad1-FLAG mRNA alone or together with 200 pg xGalntl-1 mRNA. (J) Western blot analysis for Smad1 and phospho-Smad1 from transiently transfected Hek293T cells treated for 2 hours with 40 ng/ml recombinant BMP4 protein. (K) Western blot for phospho-Smad1 or Smad1 using lysates from HEK 293T cells transiently transfected with the indicated expression plasmids. In the RT-PCR experiments α-actin or myf-5 served as controls for mesoderm contamination; odc as a loading control.

Effect (Fig. 3H see Fig. S2 in the supplementary material). Furthermore, the expression of xGalntl-1 decreased phospho-Smad1 levels in embryonic explants at gastrula stages and in transiently transfected HEK 293T cells treated with recombinant BMP4 protein (Fig. 3I,J). Interestingly, xGalntl-1 did not inhibit phospho-Smad1 induction to the same degree in cells expressing a constitutively active BMP type I receptor (Fig. 3K). We conclude from these data that xGalntl-1 interfered with BMP signalling upstream of, or on the level of, BMP receptor complexes.

**xGalntl-1 is required for the differentiation of neural and mesodermal tissue**

Embryos microinjected with the specific xGalntl-1 morpholinos developed with a smaller head and bent trunk. Paraffin sections revealed that the brain and the spinal cord were reduced in size (Fig. 4A). The patterning of the neural tube is regulated by a dorsoventral BMP activity gradient (Placzek and Briscoe, 2005; Mizutani et al., 2006). However, the overall patterning of the spinal cord was unaffected (data not shown). In more-severely affected embryos, the anterior notochord was hypoblastic (Fig. 4B) and the shape of the anterior somites disturbed, probably reflecting reduced notochord signalling (data not shown). At gastrula stages, we did not observe changes in the expression of the pan-mesoderm marker xbra, indicating that the effect of the xGalntl-1 morpholino on endogenous Activin/Nodal activity was not strong enough to overcome the intrinsic regulatory network, including TGF-β inhibitors such as Ectodermin (Dupont et al., 2005). The earliest phenotype we observed was a thinning of the neural crest territory at neurula stages and reduced expression of the neural crest marker genes msx1 and slug (Fig. 4C). Co-injection of lacZ mRNA revealed a strong reduction of msx1 expression in the injected area and, importantly, this effect was rescued by co-injection of xGalntl-1 mRNA lacking the morpholino target sequence (Fig. 4C). At tailbud stages, the anterior expression of the neural crest marker slug was reduced and the formation of the branchial arches disturbed (Fig. 4D and data not shown). In dorsal marginal zone explants, the xGalntl-1 morpholino generated stronger phenotypes with significant reduction of anterior (six3, otx2) and neural crest (snail) tissue, whereas the expression levels of ventral ectodermal (xvent2) and posterior mesodermal (xbra) marker genes were unchanged (Fig. 4D).

BMP signalling plays an important role in the formation of neural and neural crest tissue. In particular, the expression of msx1 is induced by a sharp threshold concentration of BMP and is expanded in Xenopus and zebrafish embryos with reduced BMP activity (Tribulo et al., 2003). Thus, the morpholino knock-down of xGalntl-1 generated specific phenotypes in the tissues that express endogenous xGalntl-1, which, in support of our gain-of-function data, suggests a regulation of TGF-β signalling by xGalntl-1.
xGalntl-1 inhibits ActR-IIB activity

To test whether the inhibition of BMP activity by xGalntl-1 in ectodermal explants was due to inhibition of type I or type II BMP receptor proteins, we co-expressed xGalntl-1 with Alk-3/BMPR-IA, Alk-6/BMPR-IB, BMPR-IA and ActR-IIB in animal cap explants. As shown in Fig. 5A and Fig. S3 in the supplementary material, only ActR-IIB inhibited the induction of *xag* and *otx2* by xGalntl-1 completely. ActR-IIB overexpression induces the formation of posterior mesoderm in animal cap explants (New et al., 1997). Microinjection of 400 pg *ActR-IIB* mRNA induced the expression of mesodermal marker genes and this activity was strongly inhibited by xGalntl-1 (Fig. 5B). By contrast, similar to the effects observed for Activin, the xGalntl-1 morpholinos stimulated mesoderm induction by low amounts of *ActR-IIB* mRNA that did not induce the expression of mesodermal marker genes alone (Fig. 5C). This effect was apparently due to reduced ActR-IIB activity, as *xbra* induction by xActR-IIB was blocked by xGalntl-1 already at early gastrula stages and, importantly, the induction of Smad2 phosphorylation by ActR-IIB was inhibited by xGalntl-1 in HEK 293T cells (Fig. 5D). By contrast, the stimulation of phospho-Smad1 levels by BMPR-II was hardly affected (Fig. 5D).

A commonly used inhibitor of mucin-type glycosylation is benzyl-N-acetyl-α-galactosaminide (benzyl-GalNAc). This small, chemically synthesized sugar analogue competes for the processing of core GalNAc residues of mucin-type O-linked glycans (Kuan et al., 1989; Prescher and Bertozzi, 2006). We treated transiently transfected HEK 293T cells with 2 mM benzyl-GalNAc to test whether the inhibitory activity of xGalntl-1 was dependent on its glycosyltransferase activity. The benzyl-GalNAc-treated cells had significantly higher phospho-Smad2 levels in the presence of xGalntl-1 (Fig. 5E). Next, we tested whether the reduced expression of *msx1* in cells lacking endogenous xGalntl-1 protein at early neurula stages could be due to enhanced ActR-IIB activity.

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**Fig. 4. Xenopus Galntl-1 loss-of-function phenotype.** (A) Stage 40 uninjected control embryo and embryo injected radially with the xGalntl-1-specific morpholinos at the 2- to 4-cell stage. Numbered lines indicate the positions of the paraffin sections shown on the right that reveal a reduction of neural tissue in the morpholino-injected embryos. (B) Uninjected control embryo and embryos microinjected with xGalntl-1 morpholinos stained with the notochord-specific antibody MZ15 at stage 40 (left) and analysed by in situ hybridisation for *slug* expression at stage 30. The morpholino-injected embryos display defects in the anterior notochord and reduced anterior expression of *slug*. (C) Whole-mount in situ hybridisation for *slug* of stage 15 embryos after radial microinjection of the xGalntl-1 morpholinos at the 2- to 4-cell stage, and stage 13 embryo stained for *msx1* after single injections of the xGalntl-1 morpholinos into a dorsal-animal blastomere at the 8-cell stage together with lacZ mRNA. (D) RT-PCR analysis of stage 20 dorsal marginal zone explants from uninjected embryos or embryos microinjected with xGalntl-1 morpholinos alone or together with 40 pg xGalntl-1 mRNA (20 pg). The expression of *otx2* and *sx3* (forebrain) as well as of *snail* (neural crest) was inhibited by the xGalntl-1 morpholinos, whereas the expression of *dlk5* (epidermis), *xvent2* (ventral mesoderm) and *xbra* (mesoderm) was unaffected.

**Fig. 5. Inhibition of ActR-IIB by Galntl-1.** (A) RT-PCR analysis of animal cap explants analysed at stage 20 from embryos microinjected with 400 pg xGalntl-1 mRNA alone or together with 200 pg mRNA encoding human ALK3, mouse Alk6, human BMPR-II or Xenopus ActR-IIB. (B) RT-PCR analysis of stage 20 animal caps microinjected with 400 pg ActR-IIB mRNA either alone or together with 1 ng xGalntl-1 mRNA. The mesodermal marker genes *xbra* and *wnt8* indicated ActR-IIB activity, whereas *otx2* and *xag* indicated xGalntl-1 activity, and *xvent2* served as a marker for ventral mesoderm and *odc* as a loading control. (C) RT-PCR analysis of stage 20 animal caps microinjected with 40 pg ActR-IIB mRNA alone, together with xGalntl-1 morpholinos, and xGalntl-1 morpholinos and 100 pg xGalntl-1 mRNA. (D) Western blot analysis of Smad and phospho-Smad levels from HEK 293T cells transiently transfected with Smad1-FLAG, BMPR-II and xGalntl-1 (left) or Smad2-FLAG, ActR-IIB and xGalntl-1 (right). (E) Western blot analysis of phospho-Smad2 and Smad2 levels from HEK 293T cells transiently transfected with Smad2-FLAG, ActR-IIB and xGalntl-1 and incubated overnight in the presence or absence of 2 mM benzyl-GalNAc. (F) Whole-mount in situ hybridisation for *msx1* using stage 13 embryos microinjected with xGalntl-1 morpholinos, 100 pg xActR-IIB mRNA and 100 pg xGalntl-1 mRNA into dorsal-animal blastomeres at the 4- to 8-cell stage.
Microinjection of \( \text{ActR-IIB} \) mRNA interfered with \( \text{msx1} \) expression, comparable to the effects observed with the \( \text{xGalntl-1} \) morpholinos, and co-injection of \( \text{xGalntl-1} \) mRNA was able to restore \( \text{msx1} \) expression in ActR-IIB-overexpressing, as well as in \( \text{xGalntl-1} \)-depleted, cells (Fig. 5F). Taken together, these data suggest that mucin-type O-linked glycosylation interferes with ActR-IIB activity in the early \( \text{Xenopus} \) embryo as well as in human HEK 293T cells.

**Galntl-1 interferes with the formation of heteromeric TGF-\( \beta \) receptor complexes**

It has been reported that mucin-type O-linked glycosylation can affect protein stability, intracellular protein trafficking and protein-protein interactions (Hang and Bertozzi, 2005). Immunostaining of transiently transfected Cos-7 cells did not reveal changes in the membrane localisation of ActR-IIB, Alk-3, Alk-6 or Alk-4 protein that could explain the strong inhibitory effect of \( \text{xGalntl-1} \) co-expression on ActR-IIB activity (Fig. 6A and data not shown). In addition, western blot analysis of ActR-IIB, Alk-3, Alk-4 and Alk-6 proteins expressed in animal cap explants or HEK 293T cells in the presence or absence of \( \text{xGalntl-1} \), did not reveal significant changes in steady-state protein levels (Fig. 6B). However, as shown in Fig. 6B,C, co-expression of \( \text{xGalntl-1} \) in embryonic as well as 293T cells resulted in a migration shift of the ActR-IIB and Alk-3 proteins. Interestingly, the \( \text{xGalntl-1} \)-dependent shift of the ActR-IIB protein was not observed in HEK 293T cells treated with 2 mM benzyl-GalNAc (Fig. 6C).

Finally, we analysed the binding of ActR-IIB to the type I receptor Alk-4, which specifically mediates Activin/Nodal-related signals, and to the BMP-specific type I receptors Alk-3 and Alk-6. Expression constructs for HA-tagged type I receptors (Alk-3, Alk-4 and Alk-6), Myc-tagged ActR-IIB and FLAG-tagged \( \text{xGalntl-1} \) were transfected into HEK 293T cells, cell lysates immunoprecipitated with an anti-Myc antibody and co-immunoprecipitated type I receptors visualised using anti-HA antibodies. In these experiments, the binding of ActR-IIB to all three type I receptors was strongly reduced in the presence of \( \text{xGalntl-1} \) (Fig. 6D). Similar effects were observed using a Myc-tagged ActR-IIB (data not shown). By contrast, the binding of BMPR-II to Alk-

![Fig. 6. Galntl-1 inhibits the binding of ActR-IIB to type I receptors.](https://example.com/fig6.png)

**Fig. 6. Galntl-1 inhibits the binding of ActR-IIB to type I receptors.** (A) Immunohistochemical staining of transiently transfected Cos-7 cells for ActR-IIB containing a C-terminal HA-TAG and \( \text{xGalntl-1} \) containing a C-terminal FLAG-TAG. (B) Western blot analyses of stage 11 Xenopus animal cap cells expressing HA-tagged ActR-IIB, Alk-3, Alk-4 and Alk-6 and \( \text{xGalntl-1} \). (C) Western blot analyses of transiently transfected HEK 293T cells expressing HA-tagged XActR-IIB and \( \text{xGalntl-1} \) and incubated overnight in the presence or absence of 2 mM benzyl-GalNAc. (D) Co-immunoprecipitation of HA-tagged Alk-3, Alk-4 and Alk-6 with MYC-tagged ActR-IIB in the presence or absence of FLAG-tagged \( \text{xGalntl-1} \) from lysates of transiently transfected HEK 293T cells. (E) Co-immunoprecipitation of Alk-3 with Myc-tagged ActR-IIB or FLAG-tagged BMPR-II in the presence or absence of untagged \( \text{xGalntl-1} \). The binding of BMPR-II to Alk-3 does not seem to be inhibited by \( \text{xGalntl-1} \). (F) Co-immunoprecipitation of Alk-4-HA with ActR-IIB-Myc in the presence of \( \text{xGalntl-1} \), \( \text{sGalntl-1} \) or \( \text{xGalntl-6} \). Only \( \text{xGalntl-1} \) interfered with the formation of the heteromeric complex. In all experiments, 10% of the lysates used for co-immunoprecipitation was subjected to direct immunoprecipitation and the quantity of protein analysed by western blotting for the different epitopes.
3 was unaffected by xGalntl-1 co-expression (Fig. 6E). Thus, xGalntl-1 specifically interferes with the formation of heteromeric TGF-β receptor complexes containing ActR-IIA and ActR-IIB. The xGalntl-1 protein, which lacks the N-terminal transmembrane domain and did not display any activity in early Xenopus embryos (Fig. 3), also did not interfere with the binding of ActR-IIB to type I receptor proteins (Fig. 6F). In addition, other proteins of the N-acetylgalactosaminyltransferase family that are expressed in the early Xenopus embryo (Fig. 1), including xGalnt-6, had no effect on the binding of ActR-IIB to Alk-4 and did not interfere with mesoderm formation during gastrulation (Fig. 6F and data not shown). In summary, xGalntl-1 does not affect the stability or intracellular trafficking of TGF-β receptor proteins, but specifically interferes with the binding of ActR-IIA and ActR-IIB to type I TGF-β receptor proteins that can mediate BMP as well as Nodal signalling.

**DISCUSSION**

**Negative regulation of TGF-β signalling by Galntl-1**

The TGF-β signal transduction pathway is regulated on various levels of the cascade (Lutz and Knaus, 2002). A common level of control takes place in the extracellular space, where secreted antagonists prevent TGF-β ligands from binding to their cognate receptors (Miyazono, 2000; Balemans and Van Hul, 2002). In particular, recent studies have unravelled a complex system of interacting proteins that regulate the interaction of BMP ligands with their cognate receptors (De Robertis, 2006). In addition, regulation of TGF-β signals occurs at the level of the cell membrane, the cytoplasm and the nucleus (Lutz and Knaus, 2002; Shi and Massague, 2003) This study demonstrates an important role for the N-acetylgalactosaminyltransferase Galntl-1 in the regulation of TGF-β signalling. In the early Xenopus embryo, Galntl-1 interfered with Nodal/Activin-dependent mesoderm formation, inhibited BMP signalling and stimulated neural differentiation (Figs 2 and 3). In addition, Galntl-1 effectively interfered with the phosphorylation of BMP as well as of Activin/Nodal-specific R-Smads in the Xenopus embryo and in human HEK 293T cells, whereas specific xGalnt-1 morpholinos stimulated Activin and ActR-IIB activity in embryonic explants (Figs 2 and 5). Thus, endogenous xGalntl-1 negatively regulates TGF-β signalling in the early Xenopus embryo. In HEK 293T cells, Xenopus Galntl-1 interfered with the stimulation of R-Smad phosphorylation by Activin and BMP signals, demonstrating that Xenopus Galntl-1 can also act on human components of these signalling pathways. In addition, frog and mammalian Galntl-1 displayed similar activities on TGF-β signalling in the early frog embryo, HEK 293T cells and in our biochemical assays, despite the significant differences in the C-terminal Ricin domain (Fig. 3 and data not shown). Thus, the regulation of TGF-β signalling by Galntl-1 seems to be an evolutionarily conserved mechanism that could also be active during mammalian embryogenesis and adult tissue homeostasis.

**Requirements for xGalntl-1 in the early Xenopus embryo**

Although mucin-type O-linked glycans are very abundant, little is known about the function of this type of O-glycosylation in embryonic development. Targeted deletion of single N-acetylgalactosaminyltransferases has not revealed obvious functional deficits, arguing that some functional redundancy might exist (Haltiwanger and Lowe, 2004). We detected the expression of several N-acetylgalactosaminyltransferases throughout early Xenopus embryogenesis (Fig. 1). Nevertheless, our loss-of-function studies revealed a specific requirement of xGalntl-1 for the formation of the neural crest and the neural tube. The formation of these tissues is well known to be dependent on a tight regulation of TGF-β signalling (De Robertis and Kuroda, 2004; Barembaum and Bronner-Fraser, 2005; Stern, 2005). The msx1 and msx2 genes are required for the formation of neural crest cells and stimulate the expression of additional early neural crest marker genes, including snail, slug and foxd3 (Tribulo et al., 2003; Khadka et al., 2006). The msx1 gene is a direct target of the BMP signal transduction pathway (Takahashi et al., 1997; Alvarez-Martinez et al., 2002), but the transcriptional activation of msx1 is dose-dependent and requires intermediate levels of BMP signalling activity. Therefore, the territory expressing msx1 is expanded in Xenopus and zebrafish embryos with reduced BMP signalling activity, and locally applied Noggin protein can induce ectopic msx1 expression in neighbouring tissues (Nguyen et al., 2000; Tribulo et al., 2003). The requirement of endogenous xGalntl-1 for the determination of the narrow msx1-positive territory implicates an essential role for xGalntl-1 in the formation of the BMP activity gradient at neurula stages.

The dorsoventral patterning of the neural tube is dependent on the establishment of a BMP signalling gradient (Placzek and Briscoe, 2005; Mizutani et al., 2006). In addition, it has been proposed that distinct types of BMP receptors regulate the switch between proliferation and differentiation of neural precursor cells in the neural tube (Chizhikov and Millen, 2005). Xenopus Galntl-1 is specifically expressed in the mantle (differentiating) territory of the spinal cord and is excluded from the central area containing proliferating neuronal precursors (Fig. 1E). Thus, the modulation of BMP receptor complex formation by xGalntl-1 might participate in the regulation of neural differentiation in the spinal cord. The effects of the xGalntl-1 morpholinos on the formation of the spinal cord were rather mild, but the lack of anterior neural crest and the reduction of neural tissue suggest an important role of xGalntl-1 in the regulation of proliferation and differentiation of these tissues. Interestingly, the morpholino knock-down of ActR-IIA and ActR-IIB in zebrafish revealed a requirement for both receptor subtypes in the formation of cranial neural crest and neural tissue (Albertson et al., 2005). In addition, overexpression of xActR-IIB in the early Xenopus embryo inhibited msx1 expression at neurula stage (Fig. 5F), similar to the reduction observed with the xGalntl-1 morpholino. Thus, at least some of the loss-of-function phenotypes observed for xGalntl-1 could be mediated by modulation of ActR-IIB activity.

**Regulation of heteromeric TGF-β receptor complexes by xGalntl-1**

The BMP and Activin/Nodal branches of the TGF-β superfamily signal through distinct type I receptors, but share type II receptors, including ActR-IIB (Attisano et al., 1992). The TGF-β superfamily member BMP3 antagonises BMP and Activin-like signals, generating phenotypes comparable to those we observed with xGalntl-1 (Hino et al., 2003; Gamer et al., 2005). However, the molecular mechanisms underlying the inhibitory activities of BMP3 are not completely understood. BMP3 binds to ActR-IIIB and inhibits ActR-IIIB activity, without interfering with ligand binding or receptor complex formation (Gamer et al., 2005). Similar to the effect of BMP3, xGalntl-1 did not inhibit BMP signalling completely. The remaining BMP activity might be mediated by BMP-PR-III. The binding of BMP-PR-II to Alk-3 was unaffected and BMP-PR-II activity was not inhibited by xGalntl-1 (Fig. 5D and Fig. 6E). Interestingly, a secreted form of Galntl-1 (sGalntl1) and an additional member of the
family of N-acetylgalactosaminyltransferases (xGalnt-1) expressed during early Xenopus embryogenesis did not interfere with the formation of heteromer receptor complexes in vitro and did not inhibit TGF-β signalling in vivo (Fig. 3 and Fig. 6E). Thus, the biological activity of Galnt-1 is specific and correlated well with our biochemical results.

Galnt-1 did not affect the steady-state protein levels or cellular localisation of type I or type II receptor proteins in Xenopus embryonic extracts or transfected cell lines (Fig. 6A,B). However, xGalnt-1 did induce a migration shift of Alk-3 and ActR-IIB proteins under denaturing gel conditions. A specific inhibitor of xGalnt-1 with Alk-3, Alk-4, Alk-6 or ActR-IIB and did not observe Galnt-1 protein in the cell membrane (Fig. 6A and data not shown). Thus, it is unlikely that Galnt-1 interferes with the formation of heteromeric complexes only through a direct interaction with TGF-β receptor subunits. However, we cannot exclude the possibility that Galnt-1 might interact with additional unknown factors in the Golgi compartment that are required for the efficient formation of ActR-II/B-containing receptor complexes.

In summary, we have shown that the activity of Activin and BMP signalling is regulated by a N-acetylgalactosaminyltransferase with a highly specific expression pattern, adding an additional component into the complex regulatory network modulating TGF-β signalling. Importantly, this novel regulatory mechanism is evolutionarily conserved between different vertebrate species. In the future, it will be important to understand whether different members of the N-acetylgalactosaminyltransferase family target different components of TGF-β signalling, providing a new paradigm for regulation in this important pathway.

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Supplementary material

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