The *Xenopus* homologue of Down syndrome critical region protein 6 drives dorsoanterior gene expression and embryonic axis formation by antagonising polycomb group proteins

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**ABSTRACT**
Mesoderm and embryonic axis formation in vertebrates is mediated by maternal and zygotic factors that activate the expression of target genes. Transcriptional derepression plays an important role in the regulation of expression in different contexts; however, its involvement and possible mechanism in mesoderm and embryonic axis formation are largely unknown. Here we demonstrate that XDSCR6, a *Xenopus* homologue of human Down syndrome critical region protein 6 (DSCR6, or RIPPLY3), regulates mesoderm and embryonic axis formation through derepression of polycomb group (PcG) proteins. *Xdsr6* maternal mRNA is enriched in the endoderm of the early gastrula and potently triggers the formation of dorsal mesoderm and neural tissues in ectoderm explants; it also dorsalises ventral mesoderm during gastrulation and induces a secondary embryonic axis. A WRPW motif, which is present in all DSCR6 homologues, is necessary and sufficient for the dorsal mesoderm- and axis-inducing activity. Knockdown of *Xdscr6* inhibits dorsal mesoderm gene expression and results in head deficiency. We further show that XDSCR6 physically interacts with PcG proteins through the WRPW motif, preventing the formation of PcG bodies and antagonising their repressor activity in embryonic axis formation. By chromatin immunoprecipitation, we demonstrate that XDSCR6 releases PcG proteins from chromatin and allows dorsal mesoderm gene transcription. Our studies suggest that XDSCR6 might function to sequester PcG proteins and identify a novel derepression mechanism implicated in embryonic induction and axis formation.

**KEY WORDS:** Transcriptional derepression, Embryonic axis formation, Mesoderm induction, Down syndrome, Polycomb group proteins, *Xenopus*

**INTRODUCTION**
During amphibian early development, mesoderm is formed through inductive signals emanating from endoderm cells of the vegetal hemisphere. The molecular basis of this inductive event has been extensively studied and now partly elucidated. Several conserved signalling pathways, including nodal, BMP, Wnt and FGF, are involved in mesoderm induction and patterning (Heasman, 2006; Kimelman, 2006). They act as morphogens to exert long-range effects on responding cells by activating the expression of various target genes (Smith, 2009). Subsequently, the dorsoventral patterning of mesoderm is largely mediated by factors expressed in the Spemann’s organizer, which is a source of secreted antagonists for zygotic BMP and Wnt signals (De Robertis et al., 2000; De Robertis and Kuroda, 2004; Niehrs, 2004).

The polycomb group (PcG) proteins are conserved chromatin-interacting factors that are essential for development. They are transcriptional repressors required for maintaining the correct spatial and temporal expression of key developmental genes (Kerppola, 2009; Sawarkar and Paro, 2010). The PcG proteins form multiprotein repressive complexes (polycomb repressive complexes, PRCs), which repress transcription by a mechanism that involves the modification of chromatin (Bracken and Helin, 2009). It has been shown that PcG proteins are concentrated in nuclear foci called PcG bodies, which contribute to epigenetic silencing of Hox genes in *Drosophila* (Bantignies et al., 2011; Pirotta and Li, 2012). Removal of PcG proteins in different tissues of vertebrates leads to the transcriptional activation of genes that are normally repressed (Pierson et al., 2008; Ezhkova et al., 2011; Juan et al., 2011). In the *Xenopus* embryo, misexpression of PcG proteins alters the expression of anteroposterior neural genes and produces anterior deficiency (Yoshitake et al., 1999; Barnett et al., 2001). Thus, the PcG system allows context-dependent gene expression and an appreciation of its control of the balance between repression and derepression is crucial in understanding developmental gene regulation; however, the mechanism of derepression by PcG proteins is hitherto unclear.

Down syndrome is one of the most frequent human birth defects, occurring in 1 out of 600 to 1000 births. Phenotypic and molecular analyses of patients have identified a region of chromosome 21, called the Down syndrome critical region (DSCR), which is responsible for many of the characteristic features of Down syndrome when present in three copies (Delabar et al., 1993). However, the function of most of the genes within this region in key early developmental events remains largely unexplored.

Here we report the characterisation of the *Xenopus* DSCR6 homologue (XDSCR6), which is member of the RIPPLY family proteins (*Dscr6* is also known as RIPPLY3). We found that *Xdsr6* is a maternal mRNA that is enriched in the endoderm of the early gastrula and functions as a novel and distinct mesoderm and axis inducer. It potently triggers the formation of dorsal mesoderm and neural tissues in ectoderm explants. XDSCR6 also possesses dorsalising activity and can induce a secondary axis. Knockdown of *Xdsr6* suggests that it is required for anterior development. We also show that a WRPW motif, which is present in all RIPPLY family proteins, is necessary and sufficient for these activities. Furthermore, we find that XDSCR6 physically and functionally interacts with PcG proteins through the WRPW motif, preventing their accumulation in PcG bodies, releasing them from chromatin, and counteracting their repressive activity. Our results thus identify a novel derepression mechanism and demonstrate that XDSCR6 is an...
endogenous inducer for dorsal mesoderm and the embryonic axis. These findings should also help in understanding the biological function and activity of the DSCR6 gene in early development and in the pathogenesis of Down syndrome.

RESULTS

**XDSCR6 triggers the formation of dorsal mesoderm and neural tissues**

XDSCR6 is a member of the RIPPLY family of proteins known to be involved in somite segmentation (Wardle and Papaioannou, 2008); however, whether it plays an early role in development is not clear. RT-PCR analyses indicated that Xdscr6 is a maternal mRNA that is expressed at a constant level from fertilisation until mid-gastrula stage (supplementary material Fig. S1A). In the early gastrula, Xdscr6 transcripts are enriched in the endoderm (supplementary material Fig. S1B,C), and its expression could be induced in the ectoderm by nodal signalling, but not by Wnt signalling (supplementary material Fig. S1D). This suggests that Xdscr6 might play an early role during development. We thus performed functional analyses by injecting Xdscr6 mRNA (0.2 ng) into the animal pole region and culturing ectoderm explants to different stages. At the early neurula stage equivalent, uninjected explants remained rounded (Fig. 1A), whereas Xdscr6-injected explants exhibited extensive elongation, which is characteristic of dorsal mesoderm formation (Fig. 1B). At the late neurula stage equivalent, in situ hybridisation analyses showed that Xdscr6-injected explants expressed the muscle-specific myosin light chain (MLC) gene, the cement gland marker XCG1 and the neuroectoderm gene Sox3 (Fig. 1C-H). This shows that overexpression of XDSCR6 triggered the formation of dorsal mesoderm and neural tissues.

We then injected different amounts (0.1, 0.4 and 1 ng) of Xdscr6 mRNA into the animal pole region and cultured ectoderm explants to the early gastrula and the late neurula stage equivalent to further analyse its inducing activity. At the early gastrula stage, Xdscr6 at both low and high doses potently induced the expression of dorsoventral mesoderm genes, but not the endoderm gene Sox17a or the Wnt/β-catenin target gene Siamois (Fig. 1I). However, Sox17a expression was induced in ectoderm explants treated with activin (1 ng). Injection of Xdscr6 also induced the expression of Xnr1, Xnr2 and Xnr6, but not Xnr4 or Xnr5 (supplementary material Fig. S2). Consistent with the induction of dorsal mesoderm genes at the early gastrula stage, ectoderm explants injected with high doses of Xdscr6 mRNA (0.4 or 1 ng) and cultured to the late neurula stage equivalent expressed somitic mesoderm genes including muscle actin and MyoD, as well as various anteroposterior neuroectoderm genes (Fig. 1J). Real-time RT-PCR analyses on early gastrula ectoderm explants injected with low (0.2 ng) and high (1 ng) amounts of Xdscr6 mRNA indicated a dose-dependent induction of mesoderm, but not endoderm, gene expression. In comparison, ectoderm explants treated with low (0.2 ng/ml) and high (1 ng/ml) doses of activin exhibited a dose-dependent induction for both mesoderm and endoderm genes (Fig. 1J). These analyses suggest that XDSCR6 induces only mesoderm gene expression at the early gastrula stage.

Since mesoderm induction and patterning depend on both maternal and zygotic factors (Kofron et al., 1999), we tested the activity of zygotic Xdscr6 in mesoderm and neural induction by injecting Xdscr6 DNA, which allows the transcription of Xdscr6 mRNA under the control of the CMV promoter to occur only after the mid-blastula transition (Rupp and Weintraub, 1991). As shown in Fig. 1I, injection of Xdscr6 DNA (0.4 ng) also induced, although...
weakly, the expression of dorsal mesoderm genes in ectoderm explants at the early gastrula stage and of neural genes at the late neurula stage. This result indicates that XDSCR6 is also able to induce dorsal mesoderm gene expression when expressed after zygotic transcription.

**XDSCR6 induces a secondary embryonic axis**

The activity of XDSCR6 in mesoderm and neural induction led us to examine whether it induces the formation of an embryonic axis. We first injected Xdscr6 mRNA (0.4 ng) in the ventro-vegetal region of the 4-cell stage embryo (Fig. 2A); this dose was sufficient to induce the expression of most dorsal mesoderm genes (see Fig. 1I). When injected embryos developed to the early gastrula stage, the formation of a precocious ventral blastopore was apparent (Fig. 2B, C). At the late neurula stage, an ectopic axis became evident (Fig. 2D, E). At the late tail-bud stage, Xdscr6-injected embryos formed a secondary axis with essentially trunk and posterior regions (Fig. 2F, G), although anterior structure including cement gland and eyes was observed in 9% of the injected embryos (supplementary material Table S3). Injection of a high dose of Xdscr6 mRNA (1 ng) did not further change the percentage or character of the secondary axis (not shown). Ventro-animal injection of Xdscr6 mRNA also induced a secondary axis, although less frequently. In addition, ventro-vegetal injection of Xdscr6 DNA (0.4 ng) induced a secondary axis with high frequency (Fig. 2H; supplementary material Table S3). We then co-injected lacZ mRNA (0.5 ng) into one ventral blastomere at the 4-cell stage to analyse how Xdscr6 induces a secondary axis from three independent experiments. When lacZ mRNA was injected alone, β-galactosidase-labelled cells were localised to the posterior and ventral region of the embryo at the late tail-bud stage (Fig. 2I; 100%, n = 48). However, in embryos co-injected with Xdscr6 and lacZ mRNAs, β-galactosidase-labelled cells were mostly localised to the anterior region of the secondary axis (Fig. 2J; 97%, n = 64) and formed a mass of muscle (Fig. 2K), indicating that Xdscr6 had transformed ventral and posterior cells into dorsal cells. In situ hybridisation analyses indicated that the secondary axis expressed the muscle marker MLC (Fig. 2L; 100%, n = 47), the forebrain marker Otx2 (Fig. 2M; 67%, n = 49) and the spinal cord marker HoxB9 (Fig. 2N; 100%, n = 58) but not the notochord marker keratin 8 (Fig. 2O; 98%, n = 55). Taken together, these results show that XDSCR6 can induce a secondary axis with somites and neural tissues.

Although human RIPPLY proteins show relatively low overall identity (35-40%) with XDSCR6, they similarly induced mesoderm gene expression and a secondary axis (supplementary material Fig. S3). Furthermore, an amphioxus DSCR6 homologue (Li et al., 2006b) could also induce a secondary axis (data not shown). These observations suggest that RIPPLY family proteins from different species exhibit similar activity when overexpressed in the Xenopus embryo.

**XDSCR6 possesses dorsalising and neuralising activity**

We further characterised the activity of XDSCR6 in mesoderm patterning. Ventral injection of Xdscr6 mRNA (0.4 ng) at the 4-cell stage induced ectopic expression of Otx2 and chordin (Fig. 3A-D) and inhibited the expression of Wnt8 and Xvent1 (Fig. 3E-H) at the early gastrula stage, indicating that it could dorsalise ventral mesoderm. To directly test its dorsalising activity, Xdscr6 mRNA or DNA (0.4 ng) was injected into the two ventral blastomeres at the 4-cell stage and dissected ventral marginal zone (VMZ) was cultured to the late neurula stage equivalent in order to examine phenotypic and molecular changes (Fig. 3I). Dorsal marginal zone (DMZ) explants from uninjected embryos underwent elongation (Fig. 3J), which mimics gastrulation movements. However, VMZ explants from uninjected embryos remained rounded (Fig. 3K). By contrast, VMZ explants injected with Xdscr6 mRNA underwent extensive elongation as DMZ explants (Fig. 3L). VMZ explants injected with Xdscr6 DNA also elongate, albeit to a lesser extent (Fig. 3M). RT-PCR analyses indicated that injection of Xdscr6 mRNA or DNA induced ventral mesoderm to express dorsal mesoderm genes including muscle actin, Myf5 and MyoD, as well as the anterior neuroectoderm gene Otx2 and cement gland marker XCG1 (Fig. 3N). Furthermore, radial injection of Xdscr6 mRNA in the equatorial region at the 8-cell stage produced embryos with trunk
and posterior deficiency (supplementary material Fig. S4). These observations clearly indicate that XDSCR6 could dorsalise ventral mesoderm during gastrulation.

To examine whether XDSCR6 could initiate neural induction in the absence of dorsal mesoderm, we injected a low amount of Xdscr6 mRNA (0.1 ng) into the animal pole region along with VegT-EnR mRNA (0.2 ng) and antivin mRNA (0.4 ng) into one ventro-vegetal blastomere. Although RT-PCR analyses on dissected early gastrula ectoderm explants indicated that neither VegT-EnR nor antivin blocked the mesoderm-inducing activity of XDSCR6 (supplementary material Fig. S5), muscle was absent at the late neurula stage. However, expression of the cement gland marker XCG1, which correlates with neural induction, was detected (Fig. 3O; see also Fig. 1I). This suggests that XDSCR6 could trigger the formation of neural tissue in the absence of muscle, although this activity might be achieved by the early expression of chordin.

**Xdscr6 knockdown affects anterior development**

Since XDSCR6 possesses the activity to induce dorsal mesoderm and a secondary embryonic axis, we then examined whether it is required for these processes. We dorsally injected antisense MOs against Xdscr6 into 4-cell stage embryos and analysed the expression of mesoderm and neuroectoderm genes. We assayed three MOs (MO1, MO2 and MO3) targeting the 5’ untranslated region through the first 25 bases of coding sequence. Since MO1 was more efficient than MO2 and MO3 (data not shown), we used MO1, hereafter designated Xdscr6MO, in the following experiments. At the early gastrula stage, in situ hybridisation analyses revealed that injection of Xdscr6MO (40 ng) downregulated the expression of chordin, noggin and goosecoid (Fig. 4A-F), indicating that Xdscr6 knockdown affects dorsal mesoderm development. At the end of neurulation, Xdscr6 morphants exhibited reduced expression of Otx2, XCG1 and Sox3 (Fig. 4G-L). As development proceeds, injected embryos showed anterior deficiency characterised by the absence of head structure (Fig. 4M,N). These effects could be rescued by co-injection of myc-Xdscr6 mRNA (0.1 ng), which is not targeted by Xdscr6MO (Fig. 4O,P). Western blot analyses of early gastrula embryos previously co-injected at the 4-cell stage with Xdscr6MO, Xdscr6-Flag mRNA (0.2 ng) and myc-Xdscr6 mRNA (0.1 ng), showed that Xdscr6MO strongly inhibited protein synthesis from Xdscr6-Flag mRNA, which possesses the MO targeting sequence, whereas it had no effect on protein synthesis from myc-Xdscr6 mRNA (Fig. 4Q), indicating that the targeting effect of Xdscr6MO should be specific. We conclude that XDSCR6 is required for dorsal mesoderm formation and anterior development.

**The WRPW motif confers XDSCR6 activity**

RIPPLY family proteins have no clearly defined functional domain, except for a conserved WRPW tetrapeptide in the N-terminal region (supplementary material Fig. S3A). We overexpressed various Xdscr6 mutants (Fig. 5A) by injecting the corresponding mRNA (0.4 ng) into one ventro-vegetal blastomere at the 4-cell stage to investigate the structure-function relationship of XDSCR6 protein in axis induction. The results unambiguously showed that, similar to the wild-type protein, the N-terminal half of XDSCR6 (XDSCR6N-Flag) induced the formation of a secondary axis, whereas the C-terminal half of XDSCR6 (XDSCR6C-Flag) and a point mutant (XDSCR6m-Flag), which transforms WRPW into WRSG, had no axis-inducing activity (Fig. 5B). RT-PCR analyses indicated that wild-type XDSCR6 and XDSCR6N induced Otx2, chordin and Xbra expression in ectoderm explants, whereas XDSCR6C and XDSCR6m had no effect (supplementary material Fig. S6). This indicates that the N-terminal half, in particular the WRPW tetrapeptide, is required for XDSCR6 function.

Since XDSCR6-related proteins were shown to function as a repressor during somitogenesis through physical interaction with Groucho-related proteins (Kawamura et al., 2005; Kondow et al., 2006), we ventrally injected Xdscr6-EnR or Xdscr6-VP16 mRNA (0.2 ng) to examine whether the activity of XDSCR6 in mesoderm induction and axis formation is related to a repressor function. Phenotypic analyses showed that both Xdscr6-EnR and

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**Fig. 3. Dorsalisation of ventral mesoderm by XDSCR6.** (A-H) In situ hybridisation analyses of Otx2, chordin, Wnt8 and Xvent1 expression in uninjected (A,C,E,G) and Xdscr6-injected (B,D,F,H) embryos at the early gastrula stage. Ectopic uninjected and injected DMZ or VMZ explants were dissected at the early gastrula stage and cultured to late neurula stage equivalent. DMZ, dorsal marginal zone; VMZ, ventral marginal zone.

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**Fig. 4. Inhibition of protein synthesis by Xdscr6MO.** (A-P) Western blot analyses of early gastrula embryos previously co-injected at the 4-cell stage with Xdscr6MO, Xdscr6-Flag mRNA (0.2 ng) and myc-Xdscr6 mRNA (0.1 ng), showed that Xdscr6MO strongly inhibited protein synthesis from Xdscr6-Flag mRNA, which possesses the MO targeting sequence, whereas it had no effect on protein synthesis from myc-Xdscr6 mRNA (Fig. 4Q), indicating that the targeting effect of Xdscr6MO should be specific. We conclude that XDSCR6 is required for dorsal mesoderm formation and anterior development.

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XDSCR6-VP16 induced the formation of a secondary axis (Fig. 5B). Furthermore, injection of myc-WRPW mRNA (0.5 ng) was also sufficient to induce the formation of a secondary axis (Fig. 5B). To exclude the possibility that myc tags might behave as an activation domain for transcription (Ferreiro et al., 1998), we injected GFP-WRPW mRNA (0.5 ng) and found that it similarly induced a secondary axis (Fig. 5B,C). However, injection of myc-WRSG or GFP-WRSG mRNA (0.5 ng) had no effect (Fig. 5B,D). Western blot analyses indicated that wild-type and mutated proteins were expressed at a similar level (data not shown). In situ hybridisation analyses showed that XDSCR6-EnR, XDSCR6-VP16 and myc-WRPW similarly induced ectopic chordin expression (Fig. 5E). These analyses highlight the importance of the WRPW motif in dorsal mesoderm and axis induction and suggest that the axis-inducing activity of XDSCR6 is independent of a repressor or activator function.

**XDSCR6 physically interacts with PcG proteins**

Although the WRPW motif is generally involved in interaction with Groucho-related proteins (Fisher et al., 1996), we found that the mesoderm- and axis-inducing activity of XDSCR6 was not influenced by co-expression of the Groucho homologue XGrg4 [Xenopus groucho-related gene 4; also known as transducin-like enhancer of split 4 (Tle4)], or by inhibition of XGrg4 using an MO or the naturally truncated XGrg5, a dominant inhibitory Groucho protein (data not shown). Thus, interaction between XDSCR6 and Groucho-related proteins cannot be the mechanism underlying its mesoderm- and axis-inducing activity. We therefore performed a yeast two-hybrid screen in order to identify novel partners of XDSCR6 and attempt to provide a mechanism of XDSCR6 in mesoderm induction. By this approach, we identified enhancer of zeste homologue 2 (Ezh2). GST pull-down was used to confirm the physical interaction. Xenopus embryos at the 4-cell stage were injected with synthetic mRNA (0.5 ng) encoding XEzh2-myc, or XGrg4-myc as a positive control, and embryo extracts were prepared at the late gastrula stage. The results indicated that both XEzh2-myc and XGrg4-myc bound to GST-XDSCR6 (Fig. 6A). Co-immunoprecipitation further confirmed the interaction between XDSCR6 and XEzh2 (Fig. 6B).

Ezh2 is a component of PcG protein complex PRC2. We tested whether XDSCR6 also interacts with the core PRC1 component BMI1, which was not isolated in the yeast two-hybrid screen. Both GST pull-down and co-immunoprecipitation showed that XDSCR6, but not XDSCR6m, interacts with BMI1 (Fig. 6C,D). GST pull-down analysis shows the blockade of protein synthesis from Xdscr6-Flag mRNA, but not from myc-Xdscr6 mRNA, by Xdscr6 MO. Tubulin is a loading control. CoMO, mismatch control MO.
XDSCR6 disrupts the formation of PcG bodies and releases PcG proteins from chromatin

PcG proteins accumulate at pericentric heterochromatin as discrete loci called PcG bodies, which contribute to epigenetic gene silencing (Hernández-Muñoz et al., 2005; Bantignies et al., 2011). To explore the functional significance of the biochemical interaction with Ezh2 and BMI1, we examined whether XDSCR6 can influence the subcellular localisation of endogenous PcG proteins. Nuclear localisation and fluorescence intensity of endogenous Ezh2 and BMI1 in HeLa cells transfected with Xdscr6-RFP (250 ng), Xdscr6m-RFP (250 ng) or myc-WRPW (250 ng) were analysed by confocal microscopy. In untransfected cells (arrows in Fig. 7), Ezh2 and BMI1 exhibited a punctate nuclear distribution as previously described in this cell line (Saurin et al., 1998; Voncken et al., 1999). However, the nuclear fluorescence intensity for both Ezh2 and BMI1 was significantly reduced in Xdscr6-RFP- or myc-WRPW-transfected cells (arrowheads, Fig. 7A-C,1-K,M-O,U-W). By contrast, transfection of Xdscr6m-RFP did not affect the nuclear staining of Ezh2 and BMI1 (arrowheads, Fig. 7E-G,Q-S), highlighting the importance of the WRPW motif in interaction with Ezh2 and BMI1. Quantification of nuclear fluorescence intensity confirmed these results (Fig. 7D,H,L,P,T,X). Because PcG bodies represent the site of accumulation of PcG complexes and hence the site of transcriptional repression (Bantignies et al., 2011; Hodgson and Brock, 2011), this observation suggests that XDSCR6 might prevent the targeting of PcG proteins to chromatin and thus avert their transcriptional repression activity.

We then performed ChIP experiments to test this issue directly. We chose to examine the goosecoid promoter, which was activated by XDSCR6 in ectoderm explants. One-cell stage embryos were injected in the animal pole region with Xdscr6 mRNA (0.5 ng) and ectoderm explants were cultured until stage 11. Chromatin from un.injected and injected ectoderm explants was immunoprecipitated and analysed by semi-quantitative and quantitative PCR. The results showed that, in the absence of XDSCR6, a −390/−167 (relative to transcription start site at +1) goosecoid promoter region was amplified from chromatin precipitated by anti-Ezh2 and anti-BMI1 antibodies. However, in Xdscr6-injected explants, this region was less efficiently amplified (Fig. 8A-C). As a specificity control, no goosecoid promoter region was amplified from chromatin immunoprecipitated by an anti-GFP antibody, either in the absence or presence of XDSCR6 (Fig. 8A,D). This indicates that XDSCR6 specifically released Ezh2 and BMI1 from the goosecoid promoter, a region that also exhibits an increased acetylated histone H4 mark, reflecting an opened state of chromatin in this location (Shogren-Knaak et al., 2006). As a result, the goosecoid promoter could be amplified from chromatin immunoprecipitated by an anti-acetyl-histone H4 antibody, either in the absence or presence of XDSCR6 (Fig. 8A,D). These observations strongly suggest that the activation of mesoderm gene expression by XDSCR6 results from a derepression mechanism, which is achieved through the dissociation of PcG proteins from chromatin.

XDSCR6 and PcG proteins mutually antagonise in mesoderm and axis formation

To understand the functional significance of this antagonism during development, we co-expressed BMI1 with XDSCR6 in Xenopus embryos. Dorsal injection of Xdscr6 mRNA (0.2 ng) alone had no significant effect and most embryos (87%) developed normally (Fig. 9A,B; supplementary material Table S4). By contrast, dorsal injection of BMI1 mRNA (0.5 ng) alone produced anterior deficiency in 70% of injected embryos (Fig. 9C; supplementary material Table S4). In BMI1 and Xdscr6 co-injected embryos, this region was less efficiently amplified (Fig. 8A-C). As a specificity control, no goosecoid promoter region was amplified from chromatin immunoprecipitated by an anti-GFP antibody, either in the absence or presence of XDSCR6 (Fig. 8A,D). This indicates that XDSCR6 specifically released Ezh2 and BMI1 from the goosecoid promoter, a region that also exhibits an increased acetylated histone H4 mark, reflecting an opened state of chromatin in this location (Shogren-Knaak et al., 2006). As a result, the goosecoid promoter could be amplified from chromatin immunoprecipitated by an anti-acetyl-histone H4 antibody, either in the absence or presence of XDSCR6 (Fig. 8A,D). These observations strongly suggest that the activation of mesoderm gene expression by XDSCR6 results from a derepression mechanism, which is achieved through the dissociation of PcG proteins from chromatin.
embryos, 76% of embryos were morphologically normal (Fig. 9D; supplementary material Table S4), indicating that XDSCR6 counteracts the activity of BMI1 during anterior development. Conversely, ventral injection of Xdscr6 mRNA (0.1 ng) induced a secondary axis in 66% of injected embryos (Fig. 9E,F; supplementary material Table S5), whereas ventral injection of BMI1 mRNA (0.5 ng) had no effect (Fig. 9G; supplementary material Table S5). Co-injection of BMI1 mRNA with Xdscr6 mRNA reduced the prevalence of a secondary axis to 36% (Fig. 9H; supplementary material Table S5), which correlated with a blockade of ectopic chordin expression induced by XDSCR6 (supplementary material Table S6, Fig. S7). In accordance with these in vivo observations, in vitro analyses showed that XDSCR6 strongly activated a −1500/+3 goosecoid and a −907/−5 Xnr1 promoter-luciferase reporter gene, whereas these were repressed by co-expression of BMI1 (Fig. 9I,J). Altogether, these results suggest that XDSCR6 releases PcG proteins from chromatin (Fig. 9K) and thus contributes to transcriptional derepression.

DISCUSSION

This study has uncovered a novel derepression mechanism underlying dorsal mesoderm and embryonic axis formation during Xenopus early development. We show that XDSCR6, through interaction with PcG proteins, removes them from chromatin and triggers the expression of dorsoanterior genes, which then repress the expression of ventral genes.

**XDSCR6 is a novel dorsal mesoderm and axis inducer**

The activity of XDSCR6 in mesoderm and secondary axis induction presents several characteristic features. First, both maternal and zygotic XDSCR6 potently induces the expression of dorsal mesoderm genes, including several Xnr members. However, the activity of XDSCR6 to initiate the formation of mesoderm and neural tissue is unaffected in the presence of activin, implying that it might function independently of nodal signalling. Second, XDSCR6 does not have endoderm-inducing activity even at high concentration, whereas other mesoderm-inducing factors, including activin and XNR proteins, can induce both endoderm and mesoderm (Green et al., 1992; Osada and Wright, 1999) (this study). Third, in the absence of muscle, XDSCR6 potently triggers the formation of both anterior and posterior neural tissues. Finally, XDSCR6-expressing cells participate in the formation of the secondary axis, which lacks a notochord.

The ability of XDSCR6 to induce a secondary axis may be attributed to its dorsal mesoderm-inducing and/or dorsalising activity during gastrulation. Although ventral gene expression could be detected in Xdscr6-injected ectoderm explants, whether this is direct or a consequence of mesoderm formation remains to be determined. In situ hybridisation analyses clearly showed that ventral overexpression of XDSCR6 dorsalises ventral mesoderm through ectopic induction of dorsoanterior genes and downregulation of ventral genes including Wnt8 and Xvent1. Thus, the induction of dorsal genes and the repression of ventral genes trigger the formation of a secondary axis. Our findings suggest that XDSCR6 represents a novel mesoderm and embryonic axis inducer with both distinct and shared activity with respect to previously identified mesoderm-inducing factors.

**A WRPW motif confers the mesoderm- and axis-inducing activity of XDSCR6**

XDSCR6 belongs to the RIPPLY family, which have no conserved functional domain except for a WRPW motif in the N-terminal half. This motif was shown to interact with Groucho-related proteins during somitogenesis (Kawamura et al., 2005; Kondow et al., 2006); however, we find that neither overexpression nor knockdown of XGr4 affects the activity of XDSCR6 in mesoderm induction and axis formation. This indicates that XDSCR6 and XGr4 have distinct activity in mesoderm induction. Most strikingly, we found that the WRPW motif is strictly required and sufficient for the dorsal mesoderm- and axis-inducing activity of XDSCR6, whereas the C-terminal half is totally dispensable. By contrast, the C-terminal region is required for interaction with Tbx24 during somitogenesis (Kawamura et al., 2008). Furthermore, we find that the axis-inducing activity of XDSCR6 is independent of an activator or repressor function. Together, these differences imply that RIPPLY family proteins regulate mesoderm induction and somitogenesis through distinct mechanisms.

The observation that a WRPW motif alone could function as an axis inducer raises the question of whether other proteins with this motif have similar activity. It is likely that such proteins, to some extent, might function as active trimeric complexes.
extent, exhibit similar function to XDSCR6. For example, Hairy-related proteins may function as dual transcriptional regulators (activator and repressor) in embryonic axis formation (Murato et al., 2006). In the ascidian embryo, Posterior end mark (PEM), which has a WRPW motif and lacks a DNA-binding domain, plays a role in anteroposterior patterning (Yoshida et al., 1996). Thus, WRPW-containing proteins might regulate embryonic patterning with both shared and distinct specificity.

**XDSCR6 counteracts the repressor activity of PcG proteins**

We also demonstrated that the WRPW motif is indispensable for physical and functional interactions with protein components within both PRC1 and PRC2, which are chromatin-associated multiprotein complexes involved in the maintenance of stable gene repression, especially during early development and stem cell fate decision (Schwartz and Pirrotta, 2008; Kerppola, 2009; Sawarkar and Paro, 2010; Bantignies et al., 2011; Ezhkova et al., 2011). In
functional assays, XDSCR6 prevents the proper nuclear localisation of both Ezh2 and BMI1 in PcG bodies, which contribute to epigenetic silencing. Thus, XDSCR6 may regulate embryonic induction and patterning through interference with endogenous PcG protein function and derepression of dorsal mesoderm genes.

At present, it is unclear how PcG protein-mediated transcriptional silencing established during development is reversed to allow gene expression. In Drosophila, it has been demonstrated that testis-specific TATA box-binding protein (TBP)-associated factors bind to target promoters, reduce binding of PcG proteins and promote gene expression (Chen et al., 2005). XDSCR6 might counteract PcG proteins through both a similar and a distinct mechanism. Based on the interference of XDSCR6 in the formation of PcG bodies and on the binding of PcG proteins to chromatin, we propose that a physical interaction between XDSCR6 and PcG proteins may remove the latter from target promoters, open the chromatin and thus promote gene expression. In support of this hypothesis, it has been shown that several key genes involved in mesoderm induction, including nodal and brachyury, are targets of PcG proteins. In the absence of PcG function, these genes are expressed independently of the inductive signals (Dahle et al., 2010). Furthermore, there are several lines of evidence showing that conditional knockout of Ezh2 in different mouse tissues is accompanied by transcriptional activation of genes that are normally repressed in these cells (Chen et al., 2009; Juan et al., 2011). BMI1 has been shown to bind to a specific locus in pancreatic β-cells and decreased binding leads to transcriptional derepression (Dhawan et al., 2009). Thus, it is likely that XDSCR6 triggers mesoderm induction by releasing PcG proteins from chromatin to allow transcriptional activation of mesoderm genes.

In conclusion, the DSCR on chromosome 21 is associated with specific features of Down syndrome that are likely to be caused by an increased gene dosage (Pritchard et al., 2008). Functional analyses of these genes should help to understand how they regulate key aspects of embryogenesis and how their duplication leads to the pathogenesis of Down syndrome. The finding that XDSCR6 antagonises the repressor activity of PcG proteins in embryonic axis formation suggests a novel mechanism of derepression during development.

**MATERIALS AND METHODS**

**Embryo manipulations**

*Xenopus* eggs were obtained from females injected with 500 IU of human chorionic gonadotropin (Sigma) and artificially fertilised. For activin treatment, ectoderm explants at the blastula stage were incubated in 0.2 to 1 ng/ml activin solution for 1 hour and then cultured to appropriate stages.

**Plasmid constructs**

The full-length *Xdscr6* (AB073615) and human *RIPPLY2* (NM_001009994) sequences were PCR amplified and cloned into the pCS2 vector (Rupp and Weintraub, 1991). *Xdscr6*-Flag, *Xdscr6N*-Flag and *Xdscr6C*-Flag were cloned in the pCS2 vector, upstream of the sequence coding for the Flag epitope.

Fig. 9. XDSCR6 and BMI1 are mutually antagonistic during embryonic axis formation. (A-D) XDSCR6 rescues anterior deficiency produced by BMI1 overexpression. Embryos at the 4-cell stage were either left uninjected (A) or dorsally injected with *Xdscr6* mRNA (B), BMI1 mRNA (C) or co-injected with BMI1 and *Xdscr6* mRNAs (D), and cultured to the stage 32. (E-H) BMI1 inhibits the axis-inducing activity of XDSCR6. Embryos at the 4-cell stage were either left uninjected (E) or ventrally injected with *Xdscr6* mRNA (F), BMI1 mRNA (G) or co-injected with BMI1 and *Xdscr6* mRNAs (H), and cultured to the stage 35. (I,J) BMI1 represses the *goosecoid* promoter (*gsc-luc*) and *Xnr1* promoter (*Xnr1-luc*) activity that had been stimulated by overexpression of XDSCR6 in ectoderm explants. Values were expressed relative to the value obtained from uninjected control explants. Mean ± s.e.m.; *P*<0.05 (Student’s *t*-test). RLU, relative luciferase units. (K) Model of XDSCR6 function in transcriptional derepression. (Top) In the absence of XDSCR6, PcG proteins bind to chromatin and repress the transcription of mesoderm genes. (Bottom) The interaction between XDSCR6 and PcG proteins removes PRC1 and PRC2 from chromatin and contributes to transcriptional derepression.
Xdscr6m-Flag, bearing mutations in the WRPW coding sequence, was created by site-directed mutagenesis according to the manufacturer’s recommendations (Stratagene). A myc-tagged version of Xdscr6 (myc-Xdscr6) was made by cloning the coding sequence, without the initiation codon, after the six myc epitopes in the pCS2 vector. Xdscr6b-EnR and Xdscr6b-VP16 were constructed by cloning the Xdscr6 coding sequence in frame with the engrailed repressor domain or the VP16 activator domain, respectively. The myc-tagged or GFP-tagged WRPW and WRSW constructs were obtained by cloning the WRPW or WRSW coding sequence after the six myc epitopes or the GFP in the pCS2 vector. BMII-Flag, BMII-GFP, Xdscr6-myc, Xdscr6m-myc, Xdscr6b-RFP and Xdscr6b-RFP were also obtained by PCR. All constructs were sequenced before use. XEzh2-myc, VegT-EnR and antivin constructs were described previously (Thissie and Thissie, 1999; Barnett et al., 2001; Li et al., 2006a). Capped mRNAs were synthesized by in vitro transcriptions using appropriate RNA polymerases as previously described (Djiane et al., 2000).

**Morpholinos (MOs)**

The MOs for Xdscr6 (MO1, 5′-AACTCCGTGTAGTCCGCTCCAF-3′; MO2, 5′-CGCAACTCCGTTGATTCGCTGT-3′; MO3, 5′-AGA-ACAGTCCGCGTCTGTCAG-3′) and the control MO (CoMO) incorporating five mismatches (indicated in lowercase) in the MO1 sequence (5′-AAGTCCCeCTGTCATATCCTG-3′) were from Gene Tools, and suspended in sterile water.

**In situ hybridisation and cell lineage tracing**

Whole-mount in situ hybridisation was performed according to a standard protocol (Harland, 1991). Probes were labelled using digoxigenin-11-UTP and appropriate RNA polymers. Cell lineage tracing was performed by injection of lacZ mRNA followed by β-galactosidase staining using X-Gal as substrate.

**RT-PCR and real-time RT-PCR**

For RT-PCR, total RNA extraction and reverse transcription were as described (Li et al., 2010). PCR primers are listed in supplementary material Table S1 or were described previously (Shi et al., 2002). PCR products amplified in a reaction mixture containing 1 µCi [α-32P]dCTP were resolved on a 5% non-denaturing polyacrylamide gel and visualised using a phosphorimager (Bio-Rad).

For real-time RT-PCR, total RNA from 15 ectoderm explants was extracted using the RNaseasy Mini Kit (Qiagen) and reverse transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to the manufacturer’s instructions. Quantitative PCR was performed using SsoAdvanced SYBR Green Supermix (Bio-Rad) according to the manufacturer’s instructions. PCR primers are listed in supplementary material Table S2. The data were analysed using both ornithine decarboxylase (ODC) and EF1α as reference.

**Yeast two-hybrid screening**

The Xdscr6 coding sequence was cloned in the pGBK7 vector (Clontech) in frame with the Gal4 DNA-binding domain (Gal4-Xdscr6). The A1109 yeast strain was transformed with this construct and expression of the fusion protein was detected by western blot using anti-Gal4 or anti-myc antibody. The A1109-Gal4-Xdscr6 strain was mated with an 11-day mouse embryo Matchmaker cDNA library pre-transformed into yeast strain Y187 (Clontech) and ~2.4×10⁶ independent clones were screened at high stringency.

**GST pull-down and co-immunoprecipitation**

GST-Xdscr6 and GST-BMI1 constructs were obtained by cloning their coding sequences in pGEX4T1, and fusion proteins produced in E. coli cells were bound to glutathione-agarose (Sigma). Synthetic mRNAs encoding epitope-tagged proteins were injected into Xenopus embryos and lysates were incubated with glutathione-agarose-bound GST fusion proteins overnight at 4°C. Bound proteins were subjected to western blot analyses. The experiment was also performed with in vitro translated proteins using the TNT Coupled Reticulocyte Lysate System (Promega). Co-immunoprecipitation was performed as previously described (Carron et al., 2003).

**Cell transfection and confocal immunofluorescence microscopy**

Xdscr6-RFP, Xdscr6m-RFP or myc-WRPW plasmids were transfected into HEK293 cells using Fugene 6 reagent (Roche). Transfected cells were cultured for 24 hours and then fixed in PBS-buffered 4% paraformaldehyde. After permeabilisation in PBS containing 0.1% Tween 20 and blocking in PBS containing 5% BSA, immunodetection was performed with rabbit anti-Ezh2 antibody (1:800; Abcam, 1 µg/ml stock, code ab3748), mouse anti-BMI1 antibody (1:100; Abcam, 1 mg/ml stock, code ab14389) or 9E10 antibody (1:2000; Santa Cruz Biotechnology, 200 µg/ml, code sc-40), followed by Alexa Fluor 488- or Alexa Fluor 594-conjugated secondary antibody. The cells were then stained with 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI). Images were acquired with a Leica SP5 confocal microscope and quantification of nuclear fluorescence intensity was by fluorescent particle counting in an area of 20 µm² using ImageJ software (NIH). Box plots were generated using R software. Statistical significance was assessed by Student’s t-test in triplicate.

**Chromatin immunoprecipitation (ChiP)**

ChiP was based on a published method (Havis et al., 2006). One-cell stage embryos were injected in the animal pole region with Xdscr6 miRNA (0.5 ng) and ectoderm explants were dissected at stage 11. Sonicated chromatin from 100 ectoderm explants was incubated with 10 µg anti-Ezh2 antibody (Abcam), 5 µg anti-BMI1 antibody (Abcam), 8 µl anti-acetylated histone H4 antibody (Millipore, unpurified serum, code 06-866) or 10 µg anti-GFP antibody (Roche, 400 mg/ml stock, code 1181446001). The goosecoid promoter regions (~390–167 and ~390–290) were analysed by semi-quantitative PCR or by quantitative PCR in triplicate using the primers listed in supplementary material Tables S1 and S2.

**Luciferase assay**

The −1500/+3 goosecoid and −907/−5 Xnr1 promoter-luciferase reporter plasmid DNAs (Watabe et al., 1995; Cao et al., 2007; Cao et al., 2008) were injected at 100 pg with synthetic mRNAs in the animal pole region at the 2-cell stage, and the luciferase assay was performed using ten early gastrula ectoderm explants as described (Cao et al., 2012). The experiments were carried out in triplicate using different batches of embryos and the data were analysed using Student’s t-test.

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**Competing interests**

The authors declare no competing financial interests.

**Author contributions**

-H.Y.L. performed the experiments with assistance from R.G. and A.S.; C.C. and D.-L.S. designed and performed the experiments, analysed results and wrote the paper.

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**Supplementary material**

Supplementary material available online at http://dev.biologists.org/lookup/doi/10.1242/dev.098319/-/DC1

**References**


